

MALIGNANT LYMPHOMA

A Study Based on the Classification of the
Constituent Cells by Surface Marker Techniques

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Note to the Reader

This work is a survey of lymphoma which makes use of the greatly increased knowledge of the physiology of the lymphocyte and mononuclear phagocyte gained over the last two decades. The approach to the problem of lymphoma has undergone a quiet revolution in this period as techniques for the classification of lymphoid cells have become available. Today the physiology of lymphoid cells is well known, but this knowledge tends naturally to be restricted to a relatively few individuals closely concerned with the problems of experimental immunology. This was taken into account in writing this thesis, and a comparatively large section of the work is devoted to a summary of normal lymphoid cell physiology (Chapters 3-6). This forms the basis of the understanding of the lymphoma in terms of lymphocyte function which forms the experimental section of the work presented here. Chapters 7 to 10 contain a more detailed analysis of the surface of the lymphocyte and show how this contributes to our knowledge of B and T cell differentiation in the immune response. Chapter 12 is a complete survey of the available literature on surface markers in lymphoma and leukaemia published to date. The experimental work is presented in chapters 13-23, and the conclusions are discussed in Chapter 24. For ease of reading, the quoted references are included at the end of each chapter.

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INTRODUCTION

"Pathological physiology is the main fortress of Medicine, while pathological anatomy and the clinic are outlying bastions"

Rudolf Virchow 1847

"Über die Standpunkte in der wissenschaftlichen Medicin"

Virchows Archive 1, 3.

"Insofar as cellular pathology deals only with the changes of the form and mixture of the living body it remains merely pathological anatomy, i.e. not pathological physiology, and insofar as it seeks to raise itself to the latter we must, in accordance with all that has been said before, call it a decisive step backward to a past stage of development"

Gustav Spiess 1855

"Die Cellular-Pathologie in Gegensatz zur Humoral und Solidarpathologie"

Virchows Archive 8, 303.

"Until the cytologists can convincingly demonstrate just which cells are plasma cells and which are not, which are lymphoid but not lymphocytes, what the life cycle of the lymphocyte really is, and what is the relationship of these cells to one another and to macrophages, polymorphs and fixed reticuloendothelial cells we shall remain confused"

McMaster 1953

In The Nature and Significance of the Antibody Response. Editor Pappenheimer, Columbia University Press, New York.

CHAPTER 1

HISTORICAL REVIEW

INTRODUCTION

Lymphomas were probably first recognised by David Craigie, and an account given of them as follows:-

"Either after repeated attacks of inflammation, alternating with resolution, or with a slow and indistinct form of the disease, a gland or cluster of glands gradually enlarges, and resisting all forms of resolution becomes unusually hard." Craigie recognised the malignant potential of such growths, and realised that they were distinct from carcinoma by their unique anatomical distribution (Craigie 1828).

In 1832 came the classical paper of Thomas Hodgkin "On Some Morbid Appearance of the Absorbent Glands and Spleen" in which the clinical histories and post-mortem findings in six cases were described of the disease which now bears his name. Sections prepared from tissue from Hodgkin's original cases by Fox (1926), showed that cases 2 and 4 as originally described were compatible with the modern descriptions of that condition, and it has been subsequently argued (Kaplan, 1972) that probably 4 out of the original 6 cases would be accepted as cases of Hodgkin's Disease.

Rudolph Virchow (1856) in his book "Cellular Pathology" distinguished two forms of leukaemia, which he called the "ordinary splenic form" (i.e. Myeloid leukaemia) and the "lymphatic form" (i.e. lymphocytic leukaemia). He appreciated that the cells in these two forms of leukaemia were different. "In the well marked lymphatic form the cells are small, the nuclei large in proportion and single, usually sharply defined with dark outlines and somewhat granular, whilst the cell wall is frequently in such apposition/...

apposition to them that an interval can hardly be demonstrated". He goes on to say that these facts "can hardly be interpreted in any other manner than by supposing the spleen and lymphatic glands are really intimately concerned in the development of the blood".

Virchow appreciated the normal histological appearances of lymph nodes, and described the reactive changes of sinus histiocytosis and reactive fibrosis. Following his description of leukaemia or "white blood" (1845) Virchow subsequently classified the two forms of leukaemia into leukaemic and aleukaemic forms. The aleukaemic forms probably included most of what would today be regarded as lymphomas. Cochin (1865) proposed the term pseudoleukaemia for these conditions.

In 1893 Kundrat distinguished from the group of pseudoleukaemia a number of cases in which disease remained localised to the lymph nodes without apparent bone marrow involvement. These he called "lymphosarcoma", and noted that this group itself contained several variants which may have represented other disease.

In 1916 Sternberg described eight cases of leukosarcoma and "myeloblastic leukaemia" associated with enlargement of thymus and mediastinal lymph nodes. This type of lymphoma is now identified as "Sternberg Sarcoma".

Brill, Baehr and Rosenthal (1925) described "Generalised Giant Follicular Hyperplasia of Lymph Nodes and Spleen", as a separate entity and three years later Oberling (1928) described "Reticulo-endothelial sarcoma" distinguishing this entity from the multiple myeloma of bone marrow. In 1930 Roulet further distinguished/...

distinguished the primary Reticulo-endothelial sarcomas of lymph nodes from those in bone as "retothel. sarkom".

As pointed out by Robb-Smith (1938) numerous accounts of lymphoma reached the literature under the description of reticulosis or reticulo-endotheliosis. According to Sternberg (1936) lesions so described were so ill classified as to contribute little more to the concept of lymphomatous disease than Conheim's description "pseudoleukaemia". Whilst retaining the title "reticulosis" to describe these conditions Robb-Smith divided them into three broad classes on the basis of cell type and anatomical arrangement into Follicular reticulosis, Sinus reticuloses and Medullary reticuloses. In this classification he attempted to relate both benign and malignant neoplastic conditions of lymph nodes in one histopathological classification, far too cumbersome to use diagnostically.

In 1942, Gall and Mallory adopted a straightforward approach to pathological classification of these diseases, and made no attempt to explore the histogenesis of the lesions, which in previous years had led to great confusion. Their criteria were primarily cytological and they required their classification to show a reasonably close correlation with the clinical course of the disease. They discerned seven categories of lymphoma using these criteria.

The most recent addition to the classes of lymphoma now distinguished was the report of Burkitt (1958) on Multicentric Sarcoma of the jaw in African children, now named Burkitt's lymphoma.

The most extensively used modern classification of lymphoma is probably that of Rappaport Winter and Hicks (1956) brought up to/...

to date by Rappaport (1966).

With the emphasis laid on the cytological appearances of the cell types in lymphoma and the appreciation of their importance in prognosis, further attempts to classify lymphoma by these means have been made. The category of Reticulum Cell Sarcoma was severely criticised by Gall (1958) who found no cytological grounds for its diagnosis. Lukes and Collins (1974, 1975) and Lukes (1968) have proposed several variants of a classification scheme based on the cytology of the normal lymphocyte as it develops, from the small resting lymphocyte to the large reactive or transformed lymphocyte such as occurs in the germinal centres.

The most recent view of lymphomata is based on the identification of the classes of lymphoid cells which compose the lymphoma. This follows the discovery of at least two classes of lymphocytes, and identification of the receptors thought to be specific for the class of lymphocyte involved.

TABLE IHistorical and early classification of lymphoma

Craigie 1828	Distinction of Vascular Sarcomas from carcinoma.
Hodgkin 1832	Description of Hodgkin's Disease.
Virchow 1845	Description of Leukaemia.
Conheim 1865	Leukaemia & Pseudoleukaemia.
Kundrat 1893	Pseudoleukaemia. Subclass "Lymphosarcoma".
Sternberg 1916	Leukosarcoma with myeloblastic leukaemia and mediastinal involvement - "Sternberg Sarcoma".
Brill Baehr Rosenthal 1925	Giant Follicular Hyperplasia.
Oberling (1928)	Reticulo Endothelial Sarcoma - distinguished from Myelomatosis.
Roulet (1930)	Retothela Sarcome - "Reticulum cell Sarcoma".
Robb Smith (1938)	Concept of Reticulosis. Follicular. Sinus. Medullary.
Gall & Mallory (1942)	First Modern Classification.
Rappaport Winter & Hicks (1956)	Second Modern Classification.
Burkitt (1958)	Describes "Multicentric Sarcoma of the jaw". "Burkitt's lymphoma".
Gall (1958)	Dismisses the Reticulum Cell Sarcoma.
Lukes	Cytological Classification (Follicular Centre Cell Concept).

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CHAPTER 2

DEFINITION OF LYMPHOMA

SECTION IIDefinition of the Lymphoma

A lymphoma is a malignant neoplasm of lymphoid tissue arising in either central (thymus bone marrow) or peripheral (lymph nodes, spleen, gut) lymphoid tissue. Lymphomas are characterised by a progressive increase in cell mass of one more of the cell types normally present in such tissue.

Most lymphomas are tumours of lymphocytes. Lymphocytes are non cohesive cells which under normal conditions give rise to the cellular and humoral immune functions of the body. Lymphocytes under normal circumstances exhibit some features which with other tissue cells would be regarded as malignant behaviour. They have considerable mitotic potential, they are invasive of other normal tissues, and they exhibit a wide range of cellular changes (transformation) including changes in nuclear morphology, and none of these features necessarily indicates a malignant state. Lymphocytes recirculate from lymph to blood, and back to the lymph through pathways in the lymph node cortex and the splenic white pulp. They are able to rapidly accumulate within a reactive lymph node - and hence produce lymphadenopathy - without active division. Under antigenic stimulation, emigration from the blood and mitotic division within the node can soon produce an enlargement of the node, with some disturbance of normal architecture, and in extreme cases invasion of local fat, whilst retaining the characteristics of a benign lesion. Upon cessation of the stimulus the reactive features abate and the architecture regains its normal appearance. This pattern of reaction is clearly/...

clearly seen in some drug induced lymphadenopathies, and in toxoplasmosis, conditions which in the past were, and occasionally still are confused with lymphomatous neoplasms.

A second constant cell type encountered in lymphoid tissue is the macrophage, present in the spleen and in the sinusoids, compact cortex and paracortex of lymph nodes. The ubiquity of this cell, and its protean features led in the past to considerable controversy over its origins. Happily these problems have recently been resolved. The macrophage originates from a stem cell precursor in bone marrow, and enters the blood as the monocyte. Circulating monocytes settle in tissues, where they modulate to macrophages. In some tissues, lymph nodes, spleen and lung in particular, a resident macrophage population is built up which alters both in morphology and in cell chemistry to adjust to the local environment. Such populations frequently retain a limited capacity for in situ division. Specialised macrophages are frequently distinguished by terms descriptive of their site (e.g. sinusoidal, alveolar macrophage, histiocyte) or by other eponymous terms (e.g. Kupffer Cell, Tingible body macrophage). As with the lymphocyte, the normal monocyte shows the capacity to infiltrate normal tissues, to accumulate from the circulating pool at sites of injury or antigenic stimulation, and to alter its morphology. None of these features are indicative of malignant behaviour. An additional feature leading to some confusion lies in the resemblance of early cells of the monocyte series to the lymphocyte, when assessed by light microscopy. Some non-neoplastic lesions characterised by large accumulations of macrophages (sinus histiocytosis, Whipples disease) have in the past/...

past been classified as malignant neoplasms, but in general reactive conditions involving macrophages are easily recognised.

A third component of lymphoid tissue - in this thesis called the reticular cell system - has been recently defined. In the past the cells of this system were called "reticulum cells" and they were regarded as progenitors of either lymphocytes or macrophages or both. It is now doubtful that this is so. The reticular cell is a stable differentiated derivative of early mesenchyme, particularly of the coelomic epithelium. It is readily isolated from spleen, lymph node, and peritoneal sac in man and shows features in vitro which distinguish it from the fibroblast and the macrophage. In lymphoid tissues it is intimately associated with reticulin fibre, but does not necessarily secrete it. Such cells can be found in and around the periphery of germinal follicles where they may play a role in antigen trapping. Tumours of this cell probably do occur, and there may yet be a role for the term reticular cell sarcoma in the classification of tumours of reticular cells.

Finally mention must be made of two other classes of cell which, because of the influence they exert on lymphocyte differentiation and recirculation, may yet prove important in the study of lymphomas. The epithelial cell of the thymus gland, present in the thymic medulla, is essential to the differentiation of the class of T lymphocytes. This effect is produced both locally (induction) and through the effects of a trophic hormone acting in peripheral lymphoid tissue. The endothelial cell of the post capillary venule in the lymph node, and similar structures in the spleen control the entry of lymphocytes into lymph/...

lymph nodes from the blood. This structure is undoubtedly influenced by trophic factors from the thymus gland, and perhaps by the lymphocytes themselves, but the mechanisms by which the blood/lymph node filter operates are obscure, and little is known about lymphocyte recirculation patterns in man. It is conceivable that lesions of these two tissues could exert a significant influence on the types of cell contributing to the neoplastic mass in an affected lymph node.

One of the features of the normal biology of these cell types, lymphocyte, macrophage and reticular cell, is their capacity to co-operate or interact in producing an immune response to antigen. Macrophages for example will interact with both B and T lymphocytes in the inductive and effector phases of the humoral or cellular immune response. T lymphocytes will co-operate with B lymphocytes in the humoral immune response to many antigens. The reticular cell may play an essential role in the transformation of B lymphocytes in a germinal centre. In the normal physiological reactions of these cells a pure response by only one cell type to one antigen is never seen. Changes in cell populations other than the one which eventually produces the antibody, or the cell mediated immune response are always detectable. Moreover the presence of two reactive cell populations does not always produce the same result. T lymphocytes may help B lymphocytes to proliferate and secrete antibody, or may inhibit the B lymphocyte response. The response of T lymphocytes to an antigen may be abrogated by the antibody secreted by B lymphocytes (blocking or enhancement effect), while the response of macrophages to the same antigen/...

antigen may be facilitated by either antibody (a B lymphocyte product) or by lymphokines (T lymphocyte product). It is therefore difficult to account for the proliferation of B lymphocytes in a lymphocytic lymphoma when such a proliferation may be the consequence of T cell stimulation, lack of T cell suppression, increased macrophage stimulation of B cells, or the presence of a mitogenic substance for B lymphocytes to mention but a few of the extrinsic factors known to result in B cell proliferation experimentally.

In the lymphoma the histological features can vary from forms confusingly like the patterns seen in the extreme forms of reactive hyperplasia, to monomorphic proliferations of one cell type. In the past, attempts to classify these lesions have resulted in the attachment of labels to each histological type with scant regard paid to the functional significance of the lesions. Recently, with improvements in our knowledge of the lymphocyte and its habits, several important advances have been made.

Firstly tumours of individual classes of lymphocytes have been identified because of the secretion by the cells of a single unique class of immunoglobulin. By these means Waldenstroms disease, and multiple myelomatosis may be diagnosed without recourse to the microscope. The extreme abnormality or perversion of normal physiological behaviour, which lies behind the progressive growth of secretory B lymphocytes (plasma cells) stands in marked contrast to the normal controlled secretion by the same cell types of a restricted but polyclonal antibody as a consequence/...

consequence of encounters with antigen. In fact a more or less monoclonal immune response can be produced experimentally, but it does not result in progressive expansion of the antibody secreting cells as is the case in Waldenstroms disease or myeloma.

Secondly, the accumulation of a monomorphic class of lymphocytes, frequently seen in lymphomas, is seldom if ever seen in lymph node reactions. The explanation of this event, interpreted as a "maturation block" in the normal progress of lymphocytes through phases of immaturity and reactivity to inactivity and final oblivion, has much to recommend it. Even though there remain difficulties in defining the stage at which this "block of maturation" occurs, the concept is a dynamic one, in which lymphocytes entering a proliferative phase for some reason fail to mature and remain fixed in an attitude of proliferation, resulting in an enlarging population of non-functioning cells at the same stage of development and hence of uniform appearance.

Here again such gross perturbations of normal physiology can scarcely fail to be recognised, when compared with the reactive lymph node, particularly because of the presence of cells in all stages of development in the reactive node, and in the tendency of normal reactions to pass through the reactive/hyperplastic phase to a phase of resolution accompanied by functional maturation of the participating cells.

Thirdly, with a better appreciation of the co-operation of lymphocytes with other cell types the pathologist has to some extent discarded the reluctance with which he classified lesions of mixed cellularity. Once the concept that lymphoma must be a tumour of/...

of lymphocytes alone was shown to have no factual basis since normal responses invariably involve other cell types the lesion containing mature and immature lymphocytes and macrophages can clearly be called "mixed". Such an interpretation is more than descriptive, and leads to the consequence of expecting co-operative neoplasms in which the proliferation of the neoplastic cells is dependent upon the mutual interaction of diverse cell types within a single lesion (Horror Pathologicus).

By far the most important advance has recently been the discovery and application of methods which allow identification of living cells within lymphoid tumours. The lymphoma is a proliferation of the cells composing lymphoid tissues. This proliferation differs from the physiological proliferative state - defined as an immune response - in that no inductive agent or antigen can be demonstrated, and the proliferation fails to terminate in the production of a state of immunity.

The immune response is unique in that an encounter with an antigen leads physiologically to two effects:- division of cells specifically reactive to that antigen, and the secretion of molecules which either react with the antigen specifically or which influence the local reaction of other cell types with that antigen.

It can thus be seen as a reaction in two phases, one the inductive phase in which antigen is detected and encountered by cells, and the other the effective phase in which the consequences of the inductive process are expressed, by ultimately the elimination of the antigen or neutralisation of its effects.

The cells which either react with antigen or induce or control/...

control the reactions of other cells as a consequence of the presence of antigen have been substantially and accurately defined. The means of their definition have been the experimental manipulation of cell populations in whole animals, and the recognition of cell surface antigens constantly associated with one cell class. In addition other techniques used involve the reaction of these surface components with detectable particulates, with other cells, or with chemical agents which produce or inhibit proliferation or otherwise cause changes in the population under study. These reactions in or on the cell surface are intimately involved in the immune response. The reaction of a cell with any particle or molecule in its vicinity takes place at the cell surface. Since the surface and membrane of the cell is so complex that physical interpretations of particle-cell interactions become for practical purposes meaningless, the concept of the "surface receptor" has been widely used to explain the kinetics of such reactions. According to this hypothesis, the surface of each and every cell possesses families of molecules which can react by weak bonding to complementary regions on particles or other cells; that is possess "receptors" which "bind" the "ligand" or particle. These receptors are determined by genes expressed during the differentiation of the cell. For some of the molecular families on the cell membrane, no "ligand" is identified, but the molecules themselves can be identified by their immunogenicity - these are the cell surface antigens - and they have proved of great significance in understanding the physiology of the immune response. The ability to identify the cell surface antigens has led to the mapping or description of the genes/...

genes responsible for their expression.

The importance of the cell surface is two-fold. Firstly it is the "eyes and ears" of the cell, and dictates the response of that cell to its environment. Secondly it is accessible and can be used to define both the cell class and its functional state.

The essence of this thesis is to relate the abnormal proliferation of lymphoid cells in lymphoma to the normal known reactions of lymphoid tissue to antigens. This involves a comparison of the known surface structures such as surface receptors for immunologically reactive intermediates, on the surface of the neoplastic lymphoid cell with the same structure on the normal or reactive lymphoid cell.

In this thesis the data obtained by examining the surface of lymphoma cells for the presence of surface markers is presented. The markers employed detect differences in lymphoid cell populations, and by this means quantitation of different subclasses of lymphoid cells in a lesion can be achieved.

The examination of receptors on the cell surface goes beyond this point. There is evidence that lymphocytes, during their complex differentiation and interaction in immune responses, express receptors which they would otherwise not in the "resting" or inactive phase. There are morphological equivalents of these alterations in receptor expression, notably in those cell interactions which lead to the formation of reactive nodules or germinal centres in the normal lymph node, or perhaps in the nodular variants of the lymphomas. From the receptor patterns observed in the lymphomas examined it is suggested that in the majority/...

majority of cases the lymphoma can be interpreted in terms of lymphoid differentiation reflecting the normal differentiation seen in an immune response. It is not possible to determine whether this is an abnormal response by normal lymphoid cells due to some peculiarity of the antigen responsible for its initiation (e.g. a virus) or whether abnormal lymphoid cells react in a manner reminiscent of their normal counterparts but fail to bring an immune response to a useful conclusion.

The lymphoma is thus interpreted as:-

AN ABNORMAL IMMUNE RESPONSE IN WHICH THE PARTICIPATING CELLS BECOME FIXED IN AN ATTITUDE OF PROLIFERATION BY FAILING TO TERMINATE THE RESPONSE IN A NORMAL WAY.

This interpretation may not be factually correct, but it is the contention of the author that since virtually all that is known about lymphoid cell proliferation is determined from experimental manipulation of the immune response any responsible viewpoint must correspond to this interpretation to make most use of the available facts. Since all data on lymphocyte proliferation concern the response to antigens, so the neoplasm can be usefully regarded as an abnormal immune response.

Lymphomas occur principally and primarily in lymph nodes. These are not merely aggregates of lymphocytes, but contain important phagocytic, structural and endothelial elements. In an enlarged lymph node of reactive type there is no difficulty in grasping the concept of co-operation - that is the concerted activity of phagocytic, endothelial, structural and lymphoid cells in producing the resultant immune response. The difficulty in grasping/...

grasping this concept in respect of lymphomas is greater. In a neoplastic lymph node, a minority of the cells present may be objectively "neoplastic" (that is have chromosomal or antigenic variations which can be demonstrated as a property of neoplastic cells - for example monoclonal immunoglobulin secretion). The remaining cells, according to at least one modern viewpoint, are not part of the neoplasm. However, it could be argued that the non-neoplastic lymphocytes are as essential to the continued proliferation of the neoplastic lymphocytes in a lymphoma, as are the macrophages and the T lymphocytes to the few B lymphocytes proliferating and secreting antibody in a reactive lymph node. As will be shown the study of surface receptors in lymphomas does give some factual support to these arguments.

CHAPTER 3

PHYLOGENY OF THE LYMPHORETICULAR TISSUES

An account of the phylogeny of this system demonstrates some important features of immune responses are older than the phyla in which they are now studied. Of the three most important classes of immune reaction, non-specific cellular immunity, specific cell mediated immunity and humoral immunity, two were present before vertebrate phyla evolved.

SECTION III

The phylogeny of the Lymphoreticular Tissues

Phylogeny is the tracing of a common cell, or cellular function back, as it were, in time by examining that function in coexistent phyla which have undergone diverse evolutions. By this means the macrophage or the lymphocyte, and their functions can be examined in invertebrates, in fishes, in cold blooded animals, marsupials and mammals. As a general rule it is found that physiological functions which are important to a species, are never lost once gained during the course of evolution. On the other hand they may undergo changes in which the ancestral characteristic becomes barely recognisable when expressed in more recent phyla.

The immune response exhibits such characteristics. In invertebrates there are two types of response described. One - the response of phagocytosis of invading bacteria or particulate matter-evolved from the primitive feeding mechanism of protozoa, and is expressed in all triploblastic invertebrate phyla so far studied (see review by Stuart, 1970). An additional type of immunity, that is the capacity to interact with competing equivalent organisms, is well developed in the colonial hydroids Gorgonians (Theodore, 1970). These simple animals do not coalesce with "foreign" species - rejecting them by inducing cell necrosis at points of contact - a mechanism developed by the ectoderm. Another notable feature of invertebrate immunity is the dependence of the phagocytic reaction upon "opsonins" in the body/...

body fluids (Stuart, 1968; McKay et al., 1969; Hostetter and Cooper, 1972) and of the latter reaction on cell contact (Theodore, 1970). Thus at such a primitive level of animal evolution the main features of the mammalian immune reaction are observed:-

- 1) Phagocytosis dependent upon opsonins in body fluids
- 2) The ability of ectodermally derived cells to cause contact necrosis in foreign tissues.

In the terminology of mammalian immunity it could be said that 1) is an example of phagocytic-immunity initiated by opsonins and 2) is cell mediated immunity, in the vertebrate dependent upon the thymus gland.

In the absence of lymphocytes, invertebrate organisms exhibit a kind of humoral immunity in which the phagocyte is the effector cell and a kind of cellular immunity in which the effector cell is ectodermal. The further development of the immune system is characterised by modifications of the phagocyte (with the development of blood forming tissue) of the "opsonins" (with the development of circulating antibodies) and of the Cell contact/necrosis reaction involving ectoderm (development of the thymus and thymic dependent lymphocyte system).

The phylogeny of the immune system in cold blooded vertebrates has been extensively reviewed (Du Pasquier, 1973). There is a continued development and modification of the phagocytic tissues (the primitive reticulo-endothelial system, Stuart, 1968) of the invertebrate with the formation of an organ the "Lymphomyeloid complex" which contributes lymphocytes, nucleated red cells, and granulocytes to the circulation. Such tissue/...

tissue has now been identified in many primitive vertebrates, such as agnathes, save for a few transitional forms (e.g. *Amphioxus*). The appearance of the lymphomyeloid complex in primitive vertebrates is associated with a very marked increase in the susceptibility of the organism to irradiation and cytotoxic drugs (Finstadt, et al., 1969) in comparison with the invertebrate (Sparks, 1972). In addition to the haematopoietic organ of the gut wall the agnathes show a primitive thymus which develops on the paryngeal aspect of the gill bars (Fichtelius, 1969).

In the jawed fishes, the thymus, although primitive, has already acquired some of the physiological characteristics associated with mammalian thymus, notably it shows involution with age. In the larval tadpole the thymus is located behind the eye, which is also the site of the monocyto-poetic organ of the octopus - the "corps blanc" (Stuart, 1968). In fishes, and larval amphibians, lympho- and haematopoiesis occurs in the spleen, kidneys, gut, liver and bone marrow, a situation which differs from the agnathes where it is found only in gut wall. Antibodies of IgM type are produced in fish (Marchalonis and Edelman, 1965).

In amphibians, both thymic and extrathymic lymphopoiesis can be identified, the extrathymic tissues contributing lymphocytes with identifiable surface immunoglobulin of IgM type. Antibodies are formed and secreted by cells morphologically like the IgG producing plasma cells of mammals, and two classes of antibody are produced. Both however are susceptible to cleavage with mercaptoethanol and resemble IgM rather than IgG (Yamaguchi et al., 1973). Antibody production in toads and in fish is dependent on environmental temperature (Lin and Rowlands, 1973). Thymectomy in/...

in the amphibian has little or no effect on antibody production but will prevent graft rejection. The phenomena of second set graft rejection and immunological enhancement (both Thymic dependent phenomena) have been shown in the leopard frog (Vulpe and Gebhardt, 1965).

The reptilia have no lymph nodes - these are found only in marsupials and mammals, but they are notable for two features - the presence of pharyngeal tonsillar tissue is noted in the alligator, and in some species the presence of a cloacal bursa. Evolution at this stage present a divergence between the immune system of the bird, with the cloacal bursa of Fabricius, and the mammal which subsequently develops true lymph nodes.

In terms of function, the cells which characterise the lymphocyte system (Thymus dependent, T, and thymus independent, B, lymphocytes) and the phagocytic and structural cells are all present in the free swimming jawed fishes. Subsequent vertebrate evolution has added the pharyngeal lymphoid tissue, bursal lymphoid tissue, and lymph nodes, and with them the capacity to secrete low molecular weight antibodies. In the mammal five classes of antibody are identified compared with two in the chicken (IgM and IgY) and only one in amphibians (IgM, both pentameric 19s and monomeric 7s) and one in fish. It is obvious that increased capacity to secrete immunoglobulin is the most marked characteristic feature of the later evolution of the immune system. There is also the implied genetic instability of antibody formation which the rapid evolutionary modifications of this system demonstrate.

This brief summary serves to illustrate five major points

1) The/...

- 1) The extraordinarily primitive character of the mechanism of phagocytosis and ectoderm-mediated cell necrosis reactions.
- 2) A positive correlation between the evolution of haemopoetic and lymphopoetic tissue and increased susceptibility to irradiation and cytotoxic drugs.
- 3) The capacity to reject grafts in vertebrate phyla invariably depends upon the presence of an epithelial thymus of ectodermal derivation with its associated lymphocyte population.
- 4) That phylogenetically, normal lympho- and haematopoesis can occur in spleen, liver, kidney, gill and gut wall.
- 5) The development of the major lymphoreticular organs of mammals, pharyngeal lymphoid tissue, lymph nodes, and the gut-associated lymphoid tissue, is a recent event in evolutionary terms and is accompanied by an increase in quantity and in number of subclasses of immunoglobulin secreted.

Biology and Physiology of the Lymphocyte System

The lymphocyte is derived from a stem cell population in bone marrow. Ford and Micklem (1963) showed that suspensions of bone marrow cells will repopulate the irradiation depleted thymus with lymphocytes. The bone marrow cells in these experiments bore a marker chromosome, which, following proliferation in the thymus of the irradiated host, could subsequently be identified in secondary lymphoid organs, thoracic duct and blood. Cells from the thymus, lymph nodes, thoracic duct and blood were much less potent than bone marrow in repopulating thymus in irradiated hosts (Ford et al., 1956; Micklem et al., 1968). These facts suggested that lymphocytes originated from a stem cell precursor in bone marrow - and not from lymphoid tissue primarily.

The marrow itself contains a population of lymphocytes diffusely admixed with myeloid and erythroid cells (Yoffey and Courtice, 1970) but in man follicular or nodular aggregates of lymphocytes can normally be found (Rywlin, 1974; 1975). In the guinea-pig the bone marrow lymphoid population is manufactured at the rate of 1×10^9 lymphocytes a day; the half life of the marrow lymphocyte is estimated at 24 hours. (Everett and Tyler, 1967). The lymphocyte population of marrow itself cannot be regarded as a true "stem cell pool" although it is the progenitor of a class of antibody forming lymphocytes. Miller and Mitchell (1969) using purified populations of thymic lymphocytes, and bone marrow lymphocytes carrying marker chromosomes transferred to irradiated animals, showed that the progeny of bone marrow lymphocytes/...

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CHAPTER 4

PHYSIOLOGY OF THE LYMPHOCYTE

More is known about the lymphocyte than about any other cell type (with the possible exception of the neurone). The overwhelming nature of lymphocyte associated literature tends to depress rather than elate those not intimately acquainted with this cell. Nonetheless it is important, particularly in a study of this kind, to indicate a few of the basic facts about its origins and functions. Without this knowledge attempts to investigate tumours of lymphocytes become confusing.

lymphocytes - but not thymocytes - became antibody forming cells.

The stem cell of the lymphoid series has been studied by Tyler and Everett (1966) and Edwards, Miller and Phillips (1970). The former authors used a system in which one of a pair of parabiotic animals was lethally irradiated, and cells in the other labelled with H^3 Thymidine while the connection between the two was occluded. When circulation was re-established, the repopulation of marrow, lymph nodes, and spleen by H^3 thymidine labelled cells could be studied. The cell responsible for this repopulation was "Monocytoid" rather than lymphoid. Edwards, Miller and Phillips (1970) using a chromosome aberration induced by irradiation in myeloid cells, found that bone marrow "marked" by this method could be used to reconstitute x-irradiated congenitally anaemic mice. Myeloid colonies bearing the marker chromosome were recovered from the spleens of recipient mice. On transfer of these colonies into recipients concurrently injected with sheep erythrocytes, B and T antigen-reactive cells of lymphoid origin were found to possess marker chromosomes.

The bone marrow stem cell will form colonies of granulocytes or monocytes upon cultures in soft agar. These "colony forming units" (CFU) are regarded as the putative stem cell, but no evidence of the direct generation of lymphoid colonies from bone marrow stem cells exists. The proliferation of the CFU isolated from marrow requires the presence of additional factors, colony stimulating factors, for colony growth. In the mouse, one source of colony stimulating factor appears to be the activated T lymphocyte, and administration of antigens to mice changes the frequency and distribution/...

distribution of haematopoietic cells in blood and in spleen due to release of this factor (Cerny, 1974). It is apparent that the lymphocyte progeny of the bone marrow stem cell cannot be directly produced by culture of bone marrow, only monocytes and granulocytes result from this procedure. However, lymphoid nodules will develop in vitro from fragments of lymph node and spleen in irradiated mice reconstituted with bone marrow (Metcalf, et al., 1975). In vivo experiments also clearly show the ability of bone marrow cells to restore the depleted lymphoid population of the irradiated thymus or thymus graft. Treatment of bone marrow cell suspensions with antisera designed to remove the thymic dependent lymphocyte produces an increase in the numbers of in vitro colonies formed by stem cells (Mookerjee and Poulter, 1974). The number of colonies is also increased during early infection of mice with ectromelia virus (McNeill and Killen, 1971a) and there is evidence of secretion of a suppressor substance probably from the lymphoid system later in the disease (McNeill and Killen, 1971b).

The stem cell is normally confined to the bone marrow, but there is evidence in CBA mice, reconstituted with blood from CBA/T6 marked animals that a poorly proliferating peripheral blood lymphoid stem cell exists. (Micklem, Anderson and Ross, 1975). This has been regarded as a stem cell "eliminated" from the bone marrow pool.

In man cells isolated from the peripheral blood have also been shown to possess colony forming potential. The various subpopulations of human peripheral blood mononuclears can be separated and substantially purified by "rosetting" with appropriate test particles and differential centrifugation of the resulting rosetted/...

rosetted and non-rosetted cells. When all T (thymic dependent) lymphocytes were removed, a monocyte enriched fraction could be prepared from which colony forming units making macrophages, granulocytes, megakaryocytes, and even occasional monoblasts were clearly present. From this fraction, further removal of cells expressing Complement receptors, left a population of "stem cells", which were neither T lymphocytes, B lymphocytes, or monocytes (Barr, Whang-Peng and Perry, 1975). B and T lymphocytes form 8.6% (T) and 15.4% (B) of the mononuclear cells in human marrow. 74% of cells have no B or T lymphocyte characteristics (Fauci, 1975).

In summary, the lymphoid stem cell is present in bone marrow, but is not a bone marrow lymphocyte. Lymphocytes can only develop from this stem cell in the appropriate inductive environment of thymus, bone marrow itself, or peripheral lymphoid tissues. It is this exposure which determines the subsequent life history of the lymphocyte, as it becomes committed at this stage to develop as either an antibody forming cell (B cell) or as one of several further categories of thymic dependent (T) lymphocyte. The experimental evidence which has led to the interpretation of the lymphocyte population in terms of their bone marrow (or Bursal) dependence, and their thymic dependence, i.e. as "B" or "T" lymphocytes forms the bulk of the cellular immunology of the last decade. This evidence has led to slightly different viewpoints on the nature of the B and T lymphocyte development. Strictly speaking, in mammals, only the thymic dependent class of lymphocytes is present, as no "Bursal equivalent" (that is tissue performing the inductive role of the Bursa of Fabricius) has been identified/...

identified in the mammal. However, in birds, the Bursa produces lymphocytes which become antibody forming cells on antigen stimulation, and the presence of these cells is independent of the thymus gland - i.e. are thymic independent. The terms thymic dependent and thymic independent lymphocytes are possibly more academically acceptable than the terms more popularly used T lymphocyte (or T cell) and B lymphocyte (or B cell).

The lymphoid stem cell enters peripheral blood, and may seed to the thymus gland, lymph node, gut associated lymphoid tissue or spleen. There is some evidence that the fate of such lymphocytes depends upon the inductive influence of the "primary" lymphoid organs - the thymus, bone marrow, gut associated lymphoid tissue and spleen. It has been shown in the rabbit that if lymphoid suspensions are prepared from thymus, gut associated lymphoid tissue, bone marrow or spleen, significant antigenic differences between the populations can be shown, following immunisation of horses with the lymphoid suspensions (Richter, Colas de la Noue and Hardy, 1975). This implies that, from the stem cell in marrow, lymphocytes differentiate and acquire different functional capacities dependent upon the peripheral site in which they develop.

This finding is of some relevance to the substance of this thesis. The finding that lymphocytes of the spleen contain a population which carries spleen specific antigens indicates that

(a) the bone marrow and thymus may not be the only primary lymphoid organs, gut associated lymphoid tissue and spleen must also be so classified.

(b) that all lymphoid organs except lymph node and blood are capable of inducing differentiation of stem cells resulting/...

resulting in a unique organ associated lymphocyte population, distinguishable by surface antigenic characteristics.

The Thymus and thymic dependent lymphoid population

Removal of the thymus in the neonatal period is associated with four effects.

- 1) Wasting of the thymectomised animal, due to failure of cell mediated immune reactions resulting in infection. This can be corrected with broad spectrum antibiotics.
- 2) Decrease in lymphocyte population of blood, lymph nodes and spleen.
- 3) Diminished capacity to produce antibody following injection of certain antigens.
- 4) A permanent failure to reject skin grafts from incompatible donors.

Thymectomy in adult animals is not associated with significant immune defects. If adult thymectomy is followed by irradiation and reconstitution with bone marrow there is failure to develop immunity against grafts, or to fully restore the circulating lymphocyte population. If the animal is merely irradiated, and protected with bone marrow from a syngeneic donor without thymectomy, full immunocompetence can be restored (Miller and Osoba, 1963).

The thymic dependent lymphocyte is the major population of thoracic duct lymph. Gowans (1962) and Gowans and Knight (1964) showed that by cannulation of the thoracic duct, and removal of the lymphocytes/...

lymphocytes with thoracic duct lymph the lymphocyte population of peripheral blood was greatly reduced. Similar observations were made on the depletion of lymphocytes in spleen, lymph nodes, bone marrow and thymic cortex in rats subjected to prolonged thoracic duct drainage (Yoffey et al., 1964). Such depleted animals can still mount normal primary and secondary humoral immune responses (Gowans and McGregor, 1965). The small lymphocyte population drained from the thoracic duct lymph could terminate the state of tolerance in neonatally grafted animals, and was thus shown to contain immunologically competent cells (Gowans et al., 1963). Since this circulating TDL (Thoracic Duct Lymphocyte) population was the one most severely depleted by thymectomy it was established that the small lymphocyte of thoracic duct lymph was "thymic dependent". The cells of the thoracic duct lymph will migrate to the periarteriolar lymphocyte sheaths in the spleen and to the paracortical areas of lymph nodes, but not into bone marrow (except for a small subpopulation) or into germinal centres of lymph nodes or spleen (Ford, 1969; Ford and Gowans, 1969). The recirculating lymphocyte pool, most of which consists of long-lived, slowly dividing lymphocytes, is largely, but not completely, derived from thymic dependent lymphocytes. Thymectomy depletes the circulating pool, as well as reducing the rate of entry of bone marrow derived lymphocytes into this recirculating pool (Ford and Marchesi, 1971). Thymectomy reduces the lymphocyte population in the paracortical areas of lymph nodes, and also the periarteriolar lymphocytic sheath of the spleen (Parrott, de Souza and East, 1966). The T lymphocyte population is not exclusively dependent upon the presence of/...

of an intact thymus, since in congenitally thymusless (NU/NU) mice a small number of theta antigen positive T lymphocytes can be detected (Loor et al., 1975).

The thymus gland

As previously mentioned the thymus gland develops as an epithelial tissue derived from foregut structures. During the second in-utero months, the epithelium of the primordium becomes lobulated and a cortex develops by rapid accumulation of lymphocytes so that the organ is largely lymphoid by the 9th week of intrauterine life. Lymphocytes were originally thought to develop from the epithelium of the thymus itself (Auerbach, 1961) but it is now certain that they migrate from yolk sac or bone marrow into the thymic rudiment and proliferate in the specialised environment of the thymic epithelial cell (Moore and Owen, 1967; Micklem et al., 1968). Repopulation studies have shown that thymus grafts of epithelium become repopulated with cells originally derived from bone marrow (Ford et al., 1965). The rate of proliferation of the lymphocyte population is determined by the thymic epithelial component (Miller and Osoba, 1967; Metcalf et al., 1961; El-Arini and Osoba, 1973). The bulk of the lymphocytes produced in the thymus gland probably die in situ. The lymphocyte rich cortex is particularly vulnerable to so called "stress involution". In some species, notably the opossum, very marked thymic involution occurs at birth (Block, 1964). Thymic lymphocytes leave the cortex to enter the spleen, and after following a rather complicated circulatory pathway within the periarteriolar lymphocyte/...

lymphocyte sheath (Ford, 1969) re-enter the blood and lymphatic circulation as part of the recirculating lymphocyte pool. As previously indicated the great majority of thymic lymphocytes are probably destroyed within the thymus, and the cortical lymphocytes are particularly susceptible to irradiation and lysis by steroids. A residual medullary population of thymic lymphocytes is steroid and radiation resistant, and it has recently been shown that these cells can persist in thymus grafts (Elliot, 1973) and are able to transfer G.V.H. reactivity (i.e. are Immunocompetant) in contrast to the labile cortical lymphoid component which is incompetent. The concept of heterogeneity among thymic, and thymus-derived lymphocyte populations is recent. It is further complicated by the known ability of thymic epithelium, but not thymic lymphocytes or thymus-derived lymphocytes alone to restore the ability of a thymectomised bone marrow reconstituted animal to mount a humoral immune response to thymic dependent antigens (Dukor et al., 1965). Not only is the thymus a major "factory" for the reproduction of circulating lymphocytes (at least up to early adult life) it also exerts a humoral influence on lymphocyte differentiation in the periphery (Yoffey and Courtice, 1970).

The thymic lymphocyte (thymocyte) and the thymic dependent lymphocyte (T lymphocyte) do not secrete immunoglobulin, although they can both bind certain immunoglobulin classes passively to their surface (e.g. IgM)(Webb and Cooper, 1973) and have surface receptors which are immunoglobulin-like (Cone et al., 1974). The thymic dependent lymphocyte is the primary reactive cell in graft versus host disease (although it influences the activity of the macrophage/...

macrophage in the effector phase of graft versus host disease). The specifically reactive T lymphocyte populations can be depleted of graft versus host reactive lymphocytes by adherence to cells bearing the sensitising antigens. This implies that in GVH a population of T lymphocytes carry surface receptors for histocompatibility antigens from "foreign" tissues, and that this population alone is responsible for the production of graft versus host disease on transfer to irradiated recipients (Bonavida and Kedar, 1974). The nature of the receptor for histocompatibility, and other antigens, on the surface of the thymus dependent lymphocyte is dealt with in a later section.

Among the population of thymus derived lymphocytes are found several subpopulations with different functions. In graft versus host disease, there is a population of T lymphocytes which are cytotoxic to cells carrying the sensitising antigens. This population can be inhibited by pretreatment with anti-thymocyte serum and complement, indicating that the cytotoxic T cells carry surface antigens similar to those expressed on thymic lymphocytes (Sanderson and Franks, 1974). The cytotoxic T lymphocyte can also be inhibited by antibody prepared to its surface antigen receptors (Kimura, 1974) showing that such receptors are required for both sensitisation and subsequent T cell-target cell interaction leading to cytotoxicity.

A second important class of thymic dependent lymphocyte is the carrier specific helper cell, defined by its ability to react with the carrier protein of a haptene carrier complex, and subsequently to stimulate B lymphocytes to divide and form antibodies/...

antibodies to the haptene. Such cells can be induced in vitro by appropriate exposure to carrier determinants (Kontianen and Feldman, 1973).

The third class of T lymphocytes defined by their function in immune responses is the suppressor T cell. These cells act to suppress or prevent antibody formation by B lymphocytes in vitro and in vivo. If a transferred reactive cell population, in an adoptive humoral immune response is treated by antisera against thymocytes greater humoral response on subsequent immunisation occurs. This is reminiscent of the enhancement by anti-T-cell serum of the proliferation of bone marrow CFU in haematopoietic colony assay. In a recent survey of subpopulations of T lymphocytes it was shown that the separate functional groups of T cells showed different surface antigenicity involving the Ly surface antigen system (Kisielow et al., 1975).

In addition to helping B cells in the immune response, T cell helper populations have also been defined in T cell cytotoxicity towards incompatible cells. Both helper functions, and GVH cytotoxicity are specific in their induction and expression. T cell mediated suppression of antibody formation is a non specific function of thymic dependent lymphocytes (Janeway et al., 1975).

In addition to cytotoxic T lymphocytes induced by contact with target cell antigens, cytotoxic T cell populations can be generated by IgM antibody to target cells, using thymocytes not previously exposed to target cell antigens. These are cells normally immunologically incompetent but they are rendered specifically cytotoxic for target cells coated with IgM (Lamon et al., 1975).

The/...

The B lymphocyte system

The term B lymphocyte implies a differentiation from the mammalian equivalent of the Bursa of Fabricius. This is probably the bone marrow, and it has been shown that the bone marrow lymphocyte population contains the precursors of antibody forming cells (Miller and Mitchell, 1969; Benner et al., 1974). In addition to bone marrow derived lymphocytes there is also evidence, at least for the rabbit, that B cells are supplemented by gut-derived (appendix and sacculus rotundus) and possibly spleen-derived antibody synthesising populations (Richter et al., 1975). The term B cells, embraces the entire population of antibody synthesising cells and their precursors whether strictly of bone marrow derivation or not. The stem cell of the B lymphocyte system matures in both bone marrow and in gut associated lymphoid tissue in some species (Nieuwenhuis, 1974a). The development of antibody synthesising cells is probably controlled by the "antigen drive", i.e. the rate of entry into the body of bacterial and other antigens. The presence of an association between the gut and the differentiation of large numbers of antibody forming cell precursors could be accounted for by the antigen load in the gut - and hence the antigen drive to cells associated with it, without the need to invoke a special role for the gut in the differentiation of B lymphocytes (Nieuwenhuis et al., 1974a,b). The end cell of the B lymphocyte system is the plasma cell, which secretes antibodies (Fagreus, 1948). Removal of B lymphocytes from bone marrow or spleen of immunised animals, by techniques which eliminate cells expressing surface immunoglobulin, abolish the ability of irradiated/...

irradiated animals reconstituted with this depleted population to make antibodies, but leaves cellular immune responses more or less intact (Campbell and Grey, 1972). The ability to inhibit the uptake of antigen by B lymphocytes with the use of anti-light chain antisera (Basten et al., 1971) in antigen binding reactions (Ada, 1970) shows that the antigen receptor on this class of cell is surface immunoglobulin (Wigzell et al., 1969, 1970, 1971). This constant feature of the B lymphocyte, the presence of surface immunoglobulin, can be used to define the B cell population. Mouse bone marrow lymphocytes are capable of synthesising antibody in vitro after challenge with antigen in vivo. Subsequent detection of antibody by the plaque forming cell (PFC) assay demonstrates this capability (Benner et al., 1974). Bone marrow B lymphocytes may selectively migrate to the germinal centre (Nieuwenhuis, 1971) where they differentiate into "memory" B cells.

B lymphocytes of the mouse, in addition to carrying immunoglobulin also express sub-class specific receptors for immunoglobulin IgG (the Fc receptor), receptors for complement components C3b, C3d, and receptors (in man) for Epstein Barr virus particles. Using these criteria B lymphocytes are readily identified. B lymphocytes, after production from bone marrow stem cell lie in peripheral lymphoid tissues where they occupy two anatomical zones, the diffuse lymph node cortex and the cortical follicles. In the mammal, the gut associated lymphoid tissue is well endowed with prominent secondary follicles, which show a "cap" on the luminal surface, composed of small B lymphocytes. Flash labelling with H^3 thymidine in tonsil demonstrates very rapid labelling of this lymphocyte/...

lymphocyte cap, the labelling rate exceeding that even of the thymus (Koburg, 1967; Mitrou et al., 1969). This speed of labelling was also shown for the rabbit appendix (Nieuwenhuis, 1974a,b.).

In the spleen, B cells are localised to the peripheral areas of the Malpighian body, around the marginal sinus. This area, the peripheral white pulp (PWP), is not depleted by thymectomy (Parrott and de Soussa, 1967), shows presence of specific antibody forming cells after immunisation, and accumulates labelled B cells from appendix or bone marrow (Nieuwenhuis, 1974b).

Nossal and Pike, (1973), demonstrated that B cells originate from a non-B stem cell in the haemopoetic organs of the foetal mouse about 3 days before birth. B cells do not arise in lymph nodes but reach and populate them 5 days after birth. Recovery of B cell populations after irradiation is rather slow, and the B cell is more susceptible to irradiation than the T lymphocyte.

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CHAPTER 5

THE MONONUCLEAR PHAGOCYTE SYSTEM

The macrophage can lay claim to the longest experimental history of any cell type. Its vigorous phagocytic response was noted by the early biologists. In the last two decades it has experienced an experimental renaissance. It is now no longer regarded as a ubiquitous dustbin, but as a subtle and important member of an intricately organised cellular community.

The Macrophage System (Mononuclear phagocyte system)

The cells which compose this system have certain defined characteristics. These are:-

- (a) Avid phagocytosis.
- (b) Strong adhesion to glass or plastic surfaces.
- (c) Surface receptors for Fc portion of IgG molecule, fragments C3b, C3d or complement, IgM molecules, and altered fibrinogen.
- (d) Receptors for cytophilic homocytotropic antibodies.
- (e) Response to factors, such as the chemotactic factors released from complement activation, and M.I.F. (migration inhibition factor).
- (f) Cytotoxicity towards cultured cells if "armed" with antibody, or M.A.F. ("macrophage arming factor").

In addition, there are certain cytological characteristics seen by electron microscopy. These include the presence of phagolysosomes, presence of leashes of microfilaments in the cytoplasm, and evidence of pinocytosis and macropinocytosis. They have no endoplasmic sacs, and no rough endoplasmic reticulum. The plasma membrane is highly convoluted with broad cytoplasmic veils. Few mitochondria are present in unstimulated cells. The nucleus is unremarkable, and a small single nucleolus is usually present in human cells.

The macrophage was one of the earliest tissue cells for which a function was clearly demonstrated. Metchnikoff showed the role of the phagocyte in defence against bacteria (1905) as a scavenging cell in necrobiosis (1899) and in inflammation (1892) and as a constituent/...

constituent of the granuloma of tuberculosis (1888). The source of macrophages in mammalian tissues remained a mystery. Various authorities argued an origin from the local tissue fibroblasts and reticulum cells (Aschoff, 1924) and from the blood monocyte (Bloom, 1928), or, more controversially, from the lymphocyte. This view, now considered erroneous, stemmed from the initial observations of Metchnikoff (1888) on the formation of the cells of tuberculous granulomas. He observed blood cells (i.e. lymphocytes) metamorphosing to first macrophages, then to epithelioid cells, and finally to giant cells. The ultimate disciples of this origin of the macrophage from the peripheral blood lymphocyte were Rebeck (1947) and Downey (1955).

The origin of the macrophage, the blood monocyte and promonocyte, from the bone marrow stem cell was revived only recently, although this correct view of its origins was shown many years ago.

Murray, Webb and Swann (1926) studying *Bacterium Monocytogenes* infection in rabbits, found only a few cells not readily distinguishable from lymphocytes among the monocyte population of peripheral blood. As they pointed out although the output of small lymphocytes from the thoracic duct was increased by infection, no increase in the macrophage population was seen. If lymphocytes turned into macrophages or monocytes in lymph nodes and reached the blood by way of the thoracic duct then an increased thoracic duct monocyte population should have been detected. The increased monocyte population of peripheral blood could only therefore have occurred by release of monocytes from spleen/...

spleen or bone marrow. Witts and Webb (1927) confirmed these findings, and emphasised the importance of supravital staining in recognising forms of monocytes difficult to distinguish from lymphocytes morphologically.

Spector, Walters and Willoughby (1965) showed by a dual phagocytic and radioactive labelling technique that mononuclear phagocytes in an inflammatory exudate were almost entirely derived from the monocyte population of the peripheral blood. They concluded that lymphocytes and "lymphoid monocytes" could be excluded as a major population contributing macrophages to an inflammatory exudate. In the same year Volkmann and Gowans (1965) demonstrated that following labelling of thoracic duct lymphocytes, few or no labelled cells accumulated on coverslips in a "skin window" technique. The only labelled tissue which consistently gave rise to monocytes was bone marrow, although the spleen did contain some monocyte precursors.

Van Furth and Cohn (1968) showed conclusively that the mononuclear phagocyte took origin from a rapidly dividing precursor cell, the promonocyte, in bone marrow. There was no evidence of the origin of macrophages from lymphocytes. Local macrophages, e.g. those in lung, show a limited capacity for proliferation under normal conditions (Golde, Byers and Finley, 1974).

Spector and Willoughby (1968) investigating the histogenesis of the macrophage in chronic inflammation and tuberculin reactions in the rat showed that lymph node or thymus cells alone could not reproduce the features of chronic specific inflammation. They clearly/...

clearly showed the monocyte or macrophage to be the "effector cell" in such inflammation and established its ability to proliferate locally under the influence of the sensitised small lymphocyte.

The kinetics of the bone marrow phagocyte (the monocyte and its precursor the promonocyte) were established by Van Furth and Dieseloff-Den-Dulk (1970) and by Van Furth (1970). They showed that promonocytes are proliferating cells, and 70% enter division every 24 hours. The S (synthetic) phase of the cell cycle in monocytes occupies 13.6 hours, and the mean generation time was 19.5 hours.

Bell and Shand (1972) re-examined the problem of the origin of macrophages in graft versus host disease by using transferred thoracic duct cells labelled by chromosome markers, and detected by an immunofluorescence test based on antigen differences between the donor and xenogeneic recipient. They could find no evidence that macrophages of donor origin were present, or had originated from the transferred thoracic duct population. A further study (Shand and Bell, 1972) demonstrated in their xenogeneic (rat versus mouse) G.V.H. disease model, that bone marrow cells gave rise to macrophages of donor karyotype and antigenicity in recipients. These macrophages were found in all tissues and included the Kupffer cell population of the liver. The conclusion was that bone marrow contains a stem cell for the free and fixed macrophage population, which is non-phagocytic, and non-glass adherent.

Despite clear evidence of the bone marrow origin, and the demonstration that the monocyte population was not derived from the lymphocyte population, occasional reports of the origin of macrophages/...

macrophages from lymphocytes in man (Berman and Pollack, 1967) and in the rat (Elves, 1970) still continue to appear. The reasons for this conflict of fact are worthy of examination.

The first reason is that of misinterpretation of morphology. On looking at human peripheral blood, cells which are cytologically "lymphoid" can be seen to alter their function and become macrophages. The literature contains many such accounts (see review by Rebuck, 1947; Maximov, 1924). De Bruyn (1945) showed by study of locomotion of peripheral blood cells that cells with the cytology and characteristic locomotion of lymphocytes changed into cells with the characteristics of macrophages. In man, the peripheral blood contains cells with lymphoid morphology which are either stem cells (Barr, Whang-Peng and Perry, 1975) or which can functionally modulate to monocytes (i.e. are promonocytes).

Finally what defines a macrophage or monocyte? If the phagocytosis of particles is taken as proof of the monocyte or macrophage then the mononuclear phagocyte system contains many cells of different lineages corresponding to the Reticulo-endothelial system of Aschoff. An extreme view of this mode of classifying cells is taken by Chevremont (1942) in which he defined the phagocytic cell as a functional state (the histiocytary state) which was completely independent of the histogenetic commitment of the cell.

At the beginning of this section, the functional criteria by which the macrophage can be defined are set out, and these are now discussed. Reviews of the mononuclear phagocyte and macrophage system are to be found in Nelson (1969), Carr (1973) and Spector (1974). The basis for the older views on the origins of/...

of macrophages can be found in Maximov (1924) and Aschoff (1924).

Macrophages are glass adherent. Their facility in adhering to glass, plastic, and even siliconised surfaces is a feature of cells of this type. Lamvik (1966) showed that the majority of macrophages derived from cultures of peripheral blood, were adherent to glass and not present in suspension. Gough et al (1965), Elves et al (1966) and Jones (1966) had reported that macrophages were formed from blood lymphocytes in suspension cultures, especially when polymorphonuclear leukocytes were present. The basis of proof was the presence of large amounts of acid phosphatase in the suspension culture, and not on the basis of functional tests (Gough and Elves, 1967). It is notable that on morphological grounds alone, pure lymphocytes stimulated with P.H.A. are not glass adherent, and are non-phagocytic (Robbins, 1964). The requirement for polymorphs is now felt to be a requirement for, in fact, monocytes which contaminate polymorphonuclear suspensions (Lamvik, 1967). The necessity of determining which cells stick to glass is an essential point in the study of macrophage surface receptors, and of phagocytic function, as the requirements for opsonins, complement and antibody, and the ability to phagocytose itself is dependent upon the type of culture and the environment of the phagocytic cell (Habeshaw, 1970). There is a strong tendency to regard all glass adherent cells from peripheral blood, lymph node, or spleen suspensions as potential macrophages, even though not all glass adherent cells exhibit the features of macrophages derived from the mononuclear phagocyte system (e.g. the Reticular Cell/...

Cell of Stuart and Davidson, (1971).

Nonetheless investigation of macrophage surface receptors and other functions is still, strictly speaking, the investigation of surface receptors and functions of glass adherent cells. The difficulties of preparing such populations free of contaminants such as fibroblasts or "serosal" (endothelial) cells has been demonstrated by Ross and Lilly white (1965) who showed that the act of cardiac puncture can lead to significant contamination of cultures by fibroblasts.

The identification of macrophages by their ability to phagocytose iron powder (Sanderson et al., 1975) carbon particles or natural red dye (De Halleux Taper and Deckers, 1973) or polystyrene or latex beads is used in studies of receptors on mononuclear cells in man or animals. Carbonyl iron powder can be used as an effective method of removing phagocytically active cells from peripheral blood, since the iron powder and the offending cells can be removed with a magnet.

The removal of phagocytes with iron powder is a complex phenomenon. If uncoated iron particles are added to the cell suspension, a greater proportion of cells are removed than if iron pre-incubated in serum is added. In the latter case functional phagocytes are removed, in the former case adherent cells whether phagocytic or not are removed (Sanderson et al, 1975).

Mononuclear phagocytes have receptors for IgG, which can be detected by the use of IgG coated test red cells. Adherence or phagocytosis of these particles can be inhibited competitively by/...

by free IgG, aggregated IgG, or by the Fc fragment of the IgG molecule (Lo Buglio Contran and Jandl, 1967; Gelfand et al., 1971; Schmidt and Douglas, 1972; Mantovani, Rabinovitch and Nussenzweig, 1972; Nelson, 1969).

Uhr showed that complexes of salmonella and antibody would attach to the surface of the mouse macrophage when either 7S IgG or 19S IgM antibody was used to complex with the antigen. Neither cytophilic antibody nor complement was required for this attachment reaction (Uhr, 1965).

The receptor for Fc IgG is demonstrated by treating the washed macrophage with aggregated IgG (MacLennan, 1972; Huber and Fudenberg, 1968) or with red cells coated with immune IgG. Mouse macrophages will bind such particles, (Berken, Benacerraf, 1966) and will also bind human red cells sensitised with iso-haemagglutinins (Stuart and Cumming, 1967). The receptor for IgG is lost during phagocytosis, but reappears 6-8 hours after the phagocytic event (Schmidt and Douglas, 1972). The classes of antibody bound by human monocytes are restricted in that no binding of IgG₄ occurs, although IgG₁ and IgG₃ subclasses of immunoglobulin are bound (Huber and Holm, 1975). In the mouse, macrophages will bind antibodies of 7S 2 immunoglobulin class without prior interaction with antigen. They will also bind cells sensitised with heterologous IgG or IgM, but homologous α_2 globulin with cytophilic properties has also been described (Nelson, 1969). There is no evidence that macrophage receptors for cytophilic antibody or for the Fc portion of IgG differ in any important respect (Tizard, 1971).

The/...

The sensitivity of the IgG receptor on the human macrophage can be measured. Less than 1,000 molecules of antibody on a target red cell can be easily detected by a rosette test (Arend and Mannik, 1975). The Fc receptor appears to be specific for IgG, since cells coated with IgM, IgA, IgE or IgD were not bound by human monocytes (Huber et al., 1971a). The subclass specificity of the macrophage receptor for IgG was limited to IgG1 and IgG3, whereas that on lymphocytes is active towards IgG1, 2 and 3, although this may be related to the method by which the receptors are evaluated, different results being obtained with aggregated IgG (MacLennan, 1972) than with particles coated nonspecifically with IgG subclasses (Huber and Holm, 1975; Hay, Torrigiani and Roitt, 1972; Huber et al., 1971b).

The actual number of receptor sites on the rabbit alveolar macrophage has been estimated at 2 - 2.35 million molecules per cell (Phillips-Quagliata et al., 1971). There is an interesting report of the expression of an Fc/IgG receptor by heterokaryons formed between mouse macrophages and melanoma cells in culture. When the heterokaryon was first formed the Fc receptor was present, but was lost by two days in culture. It could readily be detected again by gentle trypsinisation of the cells. The loss of Fc receptor was due to production of a masking protein by the fused cell, which could be inhibited by cycloheximide (Gordon, 1975). If fused with a chick fibroblast the Fc receptor decayed more slowly (five days) but was not recoverable following trypsinisation. These experiments show that the receptor for IgG antibody on the macrophage is a secretion product/...

product of that cell, and is not merely acquired from the serum or culture medium.

The IgM receptor

The presence of IgM receptors on macrophages is controversial and the evidence for their existence conflicting. Huber and Holm (1975) could find no IgM receptor on human monocytes, using RBC coated with cold agglutinins of IgM class. In mouse systems, cultured peritoneal exudate cells react with mouse 19S IgM coated red cells in the presence of Ca^{++} ion (Lay and Nussenzweig, 1969). Stuart and Cummings (1969) found that the mouse peritoneal macrophage would bind human red cells coated with isohaemagglutinins (which are IgM antibodies). Rhodes (1973) showed that guinea pig spleen macrophages possessed a receptor for 7S IgM, but not for 19S IgM, using the sheep RBC as the test particle. Free IgM antibody in the medium is ineffective in inhibiting binding of IgG coated red cells to macrophages (Arend and Mannik, 1975). There are no reports extant of IgM antibody binding receptors on the monocytes of man.

Anti-Macrophage Serum

Antisera which react specifically with macrophages have been prepared (Loweliet al., 1969; Feldman et al., 1972). These antisera will inhibit phagocytosis, macrophage processing of antigen, and macrophage mediated cytotoxicity. Anti-mouse macrophage serum reacts with the mouse peritoneal macrophage but not with bone marrow or peripheral blood monocytes (Feldmann et al., 1972). Maintenance of bone marrow or peripheral blood monocytes/...

monocytes in vitro for some days allows the development of macrophage specific antigens (Virolainen et al., 1971). Macrophage antisera are specifically cytotoxic when incubated with complement, or can "arm" macrophages untreated with the antiserum for cytotoxic reactions with antiserum sensitised macrophages. Sensitised macrophages release chemotactic factors (Leibovich and Ross, 1975).

TABLE 2MONOCYTES AND THEIR SUBCLASSES

<u>CLASS</u>	<u>FEATURES</u>
Stem Cell	No E receptor (man) No C or Fc receptor ThyI negative Surface Ig negative
Promonocyte	"Lymphoid" morphology Circulates (in man) Glass adherent Matures to functional Phagocyte Has Fc receptor Surface Ig negative C receptor ?
Monocyte	Fc, C ₄ , C3d receptors Binds IgG ₁ , IgG ₂ , IgG ₃ but not IgG ₄ . Has IgM receptors (Disputed)
Macrophage	Fc and C3 receptors Phagocytic Glass adherent Surface Ig negative Makes C ₂ and C ₄ Has IgM receptors (for isohaemagglutinin) Expresses SD and LD Alloantigens (mouse) Expresses Ia antigens Expresses H-2 antigen (mouse)

Note only "species specific" antigens detected. Other differentiation antigens not known. All classes are cytotoxic for antibody coated cells.

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CHAPTER 6

THE RETICULAR TISSUES

Not all the cells in lymph nodes are as well known or as well characterised as the lymphocyte and macrophage. In this chapter the misfits of the lympho reticular system will be considered. The reticular, dendritic and endothelial cells are poorly understood despite the importance attributed to them in the historical fantasies of an earlier era.

The Reticular Tissues

The term reticular tissues is here used to include the structural components of primary and secondary lymphoid tissues. In normal terminology the reticular tissues are descriptive of several classes of cell, including the reticular cell, and the dendritic cell of the germinal centre.

The dendritic cell (Steinmann and Cohn, 1973) was isolated from peripheral lymphoid tissues of mice. Spleen was teased into a single cell suspension and an adherent population of cells was obtained. Typical phagocytic forms were rare, and the major cell type was morphologically dendritic, forming long thin cell processes. Motion was not typically that of the macrophage, and the cell showed features which differed from conventional macrophages. These cells lacked phagolysosomes, surface ruffles and pinocytotic vesicles. The dendritic cells lack membrane associated ATP'ase, and contain little acid phosphatase, while macrophages possess both these enzymes. When spleen was trypsinised (Steinmann and Cohn, 1973) more macrophages than dendritic cells were recovered. Dendritic cells were unresponsive to IgG coated red cells, lacking Fc receptors.

Some of the qualities Steinmann and Cohn ascribed to the dendritic cell of mouse spleen were reported previously by Stuart and Davidson (1971a,b,c) for the "reticular cell" present in culture of peritoneal exudate, tonsil, lymph nodes and human spleen. This cell also lacked receptors for IgG, and showed slightly different cytochemistry from the macrophage, but was phagocytic for yeast and for injured nucleated lymphoid cells.

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The cells contained leashes of microfilaments, as do the cells of endothelial or macrophage lineage. In prolonged culture the human peritoneal "reticular cell" produced an extracellular "coat" or secretion which Henry (1975) described as a "feltwork" or "felting" which identifies the reticulin fibre forming cell.

The dendritic cell of the germinal centre supposedly differs from the "dendritic cell" of Steinmann and Cohn, the "Reticular cell" of Stuart and Davidson or the Reticulin fibre forming "Reticular cell" of Kirsten Henry. The difference between the "dendritic" cell of Steinmann and Cohn, and the dendritic cell of the germinal centre lies in the capacity of the latter to take up immune complexes in vivo whereas there is no evidence of this occurring with isolated dendritic cells in vitro (Steinmann and Cohn, 1975).

The "reticular cell" of Stuart and Davidson has been isolated from human spleen and from tonsillar germinal centres. It lacks Fc receptors but is phagocytic towards injured lymphocytes. (Compare "clustering" of lymphocytes around dendritic cells described by Steinmann and Cohn, 1975) In cytochemistry it resembles the macrophage, but differs from the fibroblast, even though bearing a superficial morphologic resemblance to fibroblasts in early cultures.

The controversy about the terminology of these cell types, and of the structural matrix of the fibre network in lymph node and spleen has been reviewed by Stuart (1975), who distinguishes two types of cell within this group. One, the fibrocytic "reticulum" cell forms the structural fibres, and the other, the reticular/...

reticular cell forms an anastomosing framework of living protoplasmic fibres, which are separate from the structural component. These cells are present in germinal centres, and in the marginal zone of the Malpighian corpuscle (Streefkerk and Veerman, 1971) and differ from the conventional mononuclear phagocyte.

The dendritic cell of the germinal centre was identified by studies on the uptake of 125 I or fluorescent labelled antigen by germinal centres (Hanna and Szakal, 1968)(Nossal et al, 1968) (McDevitt et al., 1966)(Korngold and Mellors, 1963)(Ortega and Mellors, 1957). In germinal centres antibody was found in cells which had "elongated and seemingly continuous cell processes which appeared as though attached to reticulin fibres". (Mellors and Korngold, 1963). These experiments which purported to show antibody formation, accidentally demonstrated the dendritic antigen retaining cell. McDevitt et al (1966) in the first of a series of papers on the distribution of 125 I labelled T-G-A-L (a synthetic polypeptide) showed that all the follicular labelling was due to antigen complexed with antibody, and that the intensity of such labelling was dependent upon the amount of antibody present. No antigen was retained by lymphocytes, but was retained in a lacy pattern relating to the cell processes between the lymphocytes rather than to the lymphocytes themselves.

Nossal Abbot and Mitchell (1968) showed that antigen localised to the follicles faster in immunised than in virgin rats. It remained extracellular attached to dendritic processes of "follicular reticular" cells. A second type of reticular cell associated with the follicle is illustrated (Fig. 6. Nossal Abbot and Mitchell, 1968) which corresponds to the cell described in/...

in the periphery of the rat Malpighian body by Streefkerk and Veerman (1971).

The "reticular dendritic" cell of the germinal centre was shown by Maruyama and Masuda (1964) to show "desmosomes" or tight junctions between processes at points of contact. Schwartzendruber and Hanna (1965) described these cells with H3 thymidine autoradiography, and showed the "dendritic cell" of the germinal centre to be a relatively stable slowly dividing component. By comparison with the epithelial component of the thymic medullary cell, the epithelial cell of the bursa of Fabricius, and of the thymus of the embryonic chick, all of which show the presence of desmosomal junctions, Schwartzendruber maintained that the dendritic cell of the germinal centre was epithelial in nature. (Schwartzendruber, 1965). The syncytium formed by these cells would therefore represent an epithelial component similar to the thymic medullary epithelium, exerting a trophic function on the bone marrow derived lymphocytes in their passage through this area.

There are two problems in attempting to understand the nature of the "structural" cells of lymphoid tissue. The first is historical, the second is technical.

Historically the structural component of lymphoid tissue was regarded as the stem cell source for lymphocytes and monocytes and was called the "Totipotential, or stem cell reticulum". The lymphocyte, produced in lymph nodes entered the bone marrow to differentiate into haematopoietic cells (Jordan and Speidel, 1923; Alden and Dawson, 1927). In an investigation of agyrophillic cells in the spleen and lymph nodes, Marshall described four types of metalophil and non-metalophil cell, which he also subdivided into those/...



those with phagocytic capacities, and those which had not. All the cells he described were more or less constantly associated with the structural reticulin (reticulum)(Marshall, 1956). The four cell types described (macrophage, sinus lining cell, irregular and coarse branching cell, and white pulp metalophil cell) were further investigated by Marshall and White (1950) during an antibody response. Their findings were interpreted as differentiation of the non-metalophil "stem-cell" of the reticulum into lymphoblast forms. It is only relatively recently, with the advent of electron microscopy and autoradiography, that study of the microscopic and functional differentiation of the structural cells has been possible. Can such information be obtained from tissue culture of "reticular" and "dendritic" cells (Stuart and Davidson, 1971a, b,c) (Steinmann and Cohn, 1974, 1975)? As pointed out by Bloom (1937) it is extremely difficult in tissue culture to distinguish genetically programmed cell differentiation from cellular "modulation" caused by changes in environmental conditions. Richter (1958) after extensive cultural investigations of the mesenchymal cell could come to no definite conclusions about "genetically programmed" differentiation of the mesenchyme, but showed that the coelomic epithelium was a separate, definitive structure which yielded dendritic cells in culture, but which did not differentiate into either macrophages or lymphoid cells. It is quite possible that the different functions and morphologies defined in different systems for the "dendritic cell" and the "reticular" cell can be the effect of different approaches to isolation and culture of such cells.

In/...

In the author's opinion the structural component of lymph node consists, from the available evidence, of possibly four cell types, these are:-

- (1) endothelial cell of capillary and minor blood vessels.
- (2) reticulin fibre forming cell.
- (3) reticular cell - non fibre forming and selectively phagocytic.
- (4) dendritic cell of the germinal centre.

The most simple approach would be to consider that types 3 and 4 are derived from mononuclear phagocytes - there is some evidence that the "resident" macrophage in lymph nodes can divide and is somewhat different from the classical mononuclear phagocyte (Carr, 1975). The dendritic cell of the germinal centre has receptors on its surface for IgG immune complexes which distinguishes it from the cell isolated by Stuart and Davidson or the cell of Steinmann and Cohn. The presence of desmosomes and/or tight junctions is an interesting observation but not really helpful, as similar structures are found in the endothelium of spleen, between epithelioid cells of granulomata, and in the synovial membrane. The dendritic and reticular cells described by Cohn and by Stuart are now accepted as being the same cell type (Steinmann, Adams and Cohn, 1975).

It should be pointed out that the isolation of the same cell type from a variety of tissues in which it is constantly associated with reticulin fibre (e.g. peritoneum, lymph node, tonsil, spleen) and which shows consistent features (phagocytosis of yeast, lymphocytes, unique cytochemistry and morphology, lack of IgG receptors/...

receptors) is as far as our understanding goes of the structural components of lymphoid tissue.

TABLE 3Other Classes of "Lymphoid" Cell

<u>CLASS</u>	<u>FEATURES</u>
<u>Null Receptor Cell</u> (Receptor silent cell)	Capable of antibody mediated cytotoxicity. Many in the spleen (20+%)
<u>Cytotoxic "K" Cell</u>	Fc receptor positive Complement receptor positive Surface Ig negative Non-glass adherent Non-phagocytic
<u>Reticular Cell</u>	Stuart and Davidson Phagocytic for yeast and nucleated cells Non-fibroblast Non-fibre forming ? Surface receptors No Fc receptor Extracellular coat
<u>Dendritic Cell</u>	Steinmann and Cohn No features really different from above.
<u>Reticular Dendritic Cell</u>	Maruyama and Matsuda Has desmosomes, is present in germinal centres. Has Fc but not C3 receptors.
<u>Metalophil Cells</u>	Marshall and White (1956) Irregular and coarse branching cell. Sinus lining cell Non metalophil "reticulum cell".

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CHAPTER 7

SURFACE CHARACTERISTICS OF LYMPHOID CELLS

SURFACE ANTIGENS

From the study of the normal physiology and the characteristics of the lymphocytes, macrophages and structural cells of the lymph node, the theme now transfers to the more detailed analysis of these cells, in particular the lymphocyte.

In this chapter the surface antigens of the lymphocyte are considered. These are known to be functionally important in three instances, firstly in histoincompatibility reactions which are determined by the HLA gene complexes, secondly in genetically determined immune responses related to the expression of the Ir genes, and thirdly in the relationship between the expressed surface antigens and infection with vertically transmitted viruses.

Surface Antigens

These are substances which can be identified either serologically (SD antigens) by the preparation of a specific antiserum or immunologically by the demonstration of graft rejection, graft versus host disease, or stimulation of lymphocytes in a mixed lymphocyte reaction (LD antigens). The two classes of antigens are therefore either serologically defined or lymphocyte defined. The former are SD, and latter LD antigens. The LD antigens are unigenic, and determined by the non H-2 M Locus.

In the mouse, many surface antigen characteristics are determined by the H-2 gene complex, a linked series of genes present on a small segment of the 15th chromosome. The H prefix stands for histocompatibility and these genes control the ability of tissues to exist in mutual harmony, providing identity at major histocompatibility loci is present.

Within the H-2 complex is a cluster of genes which determine (1) the antibody response to a variety of antigens, (2) the susceptibility of lymphoid cells to lymphoma induction by tumour virus, (3) the ability of cells to react to differences in antigenicity in the mixed leukocyte culture and graft versus host disease reactions, and (4) determine a number of lymphocyte alloantigens and transplantation antigens of minor type. The region controlling this multiplicity of immune functions is called the I or Ir gene (Ir standing for immune response).

Closely related to the H-2 gene complex is a further gene TL, which although not part of the complex is probably linked to the H-2 gene. The mechanisms for defining these antigens are highly involved/...

involved, necessitating the use of congenic mouse strains (i.e. strains genetically identical to a standard inbred strain of mice: e.g. B10). Different "haplotypes" (i.e. different sequential arrangements of alleles within the gene) can be introduced into this standard highly inbred strain, and their effects measured by sophisticated techniques for demonstrating the resulting antigens or immune reactivity (Shreffler and David, 1975).

In the mouse the H-2 and TL complex genes have the regions: K, I, S, D and TL, each region being responsible for a subset of phenomena traceable to the H-2 TL complex.

The major histocompatibility antigens are determined by alleles in the K and D regions of the H-2 complex, minor alleles determining histocompatibility differences are also found in the TL region (HTLa) and the I region (H-2I). The S region determines the quantity (Ss) and the alloantigen on $\alpha 2$ globulin molecules (SsLp) which is also male sex limited (SL). The I region controls some surface lymphocyte antigens (Ia) certain aspects of the humoral immune response (Ir-1A, Ir-1B), and the mixed leukocyte and GVH reaction with sections of K and D regions (Lymphocyte activating determinants - L.A.D.) The TL region controls the expression of the TL antigens (thymus leukaemia cellular alloantigens). The K and I regions also control two important functions: the susceptibility of mice to tumour virus (MULV) and the interaction of T lymphocytes and B lymphocytes in the immune response.

Stimulation in mixed lymphocyte cultures can occur with blast transformation and proliferation of co-cultured cells, even when histocompatibility antigens of the major histocompatibility system (H-2) do not differ. Apart from minor histocompatibility differences/...

differences traceable to other regions of the H-2 complex, another locus called the M locus in mice appears to be a major factor determining reactivity in mixed leukocyte reactions. Histocompatibility antigens expressed by genes of the H-2 major histocompatibility system are present on both B and T lymphocytes and are called lymphocyte activating determinants (LAD's). LADs of the minor histocompatibility system (M locus LADs) are expressed only by B lymphocytes, and in the M locus assay system in vitro B lymphocytes are required as stimulator cells, and T lymphocytes as responder cells. (Van Boehmer and Sprent, 1974). Recently it has been shown that both H-2 determined lymphocyte activating determinants and M locus determined LADs are present on macrophages (Schirmacher, Pena-Martinez and Festenstein, 1975), which may account for the ability of macrophages to stimulate T lymphocytes, and B lymphocytes, particularly since an integrated immune response by lymphocytes to macrophage processed antigen is determined by genetic compatibility at the I region of the H-2 complex. M locus antigens may also be responsible for stimulation in xenogeneic (e.g. rat vs mouse) systems. There is evidence that human lymphocytes can recognise mouse H-2 SD antigens (Lindhal and Bach, 1975).

The I region genes are the most important from the point of view of lymphocyte activation, co-operation, and the immune response. These genes form a part of the H-2 complex in mice. They determine immune responses to individual synthetic polypeptide antigens (McDevitt et al., 1972). In this group are genes which control synthesis of IgG (Ir-IgG) and IgA (Ir-IgA) antibodies/...

antibodies (Lieberman et al., 1972). The ability of cells to respond in mixed lymphocyte reactions are also determined by this gene group (Bach et al., 1972), which controls the expression of lymphocyte defined (LD) alloantigens. These genes also control the presence on lymphoid cells of serologically defined antigens, known as the Ia antigens (Hauptfeld, Klein and Klein, 1973). Indeed a whole new category of lymphocyte associated antigens has been uncovered in studies of the I region of the H-2 complex. These include the so called new lymphocyte antigen system (Ina) (David, Schreffler and Frelinger, 1973) and a B lymphocyte antigen, specific for peripheral B lymphocytes called Beta antigen (Sachs and Cone, 1973). Similar genes control interactions between macrophages and T cells, and T cell and B cells, in both guinea pig (Shevach and Rosenthal, 1973) and in mice (Katz et al., 1973). In the rhesus monkey, Ir genes have been described, and are shown to be related to the major histocompatibility genes RhL-A (Dorf, Balmer and Benacerraf, 1975). Ia like antigens have been described on the Daudi cell line, and on normal human B lymphocytes from which HLA antigens have been "stripped" using anti-B₂-microglobulin antiserum (Jones et al., 1975).

The Ia subregion of the H-2 complex also controls the expression of an antigen specific factor secreted by T cells, which co-operates with B cells to produce an humoral immune response (Taussig et al., 1975). Ia antigens themselves are preferentially expressed on B cells, but have been isolated from thymocytes (Goding, White and Marchalonis, 1975). There is a close association between the receptor for immunoglobulin G. (IgG/Fc receptor/...

receptor discussed in the next section) and the expression of Ia surface antigen (Dickler and Sachs, 1974).a However, isolated Fc receptors do not appear identical to the Ia antigen (Rask et al., 1975) and more recent evidence suggests that the Fc receptor is associated with a further class of antigens which are not part of the H-2 complex (Dickler et al., 1975). H-2 linked genes, probably of the Ia region determine the specific B lymphocyte antigens in mice (Sachs and Cone, 1973) and also appear to control the rate of expression of the complement receptor (CRL-1 gene) in mice (Gelfand et al., 1974a). The relationship between the Ia antigen, detected serologically, and the Fc receptor which is inhibited by anti-Ia alloantisera, has recently been shown to be spurious, since any antisera (e.g. anti MBLA, anti Ly4.2) which are directed against B cells can inhibit the Fc receptor (Schirmacher, Halloran and David, 1975).

There are, in addition to antigens determined by the H-2 gene complex in the mouse, a variety of other alloantigens not determined by the H-2 or TL complex. These are the theta antigen, the Ly antigens Ly1, Ly2, Ly3, Ly4 and G_{1X} antigen, and the antigen PC.

Species specific antigens are also present, MSLA (Mouse specific lymphocyte antigen), MBLA (Mouse B lymphocyte antigen), MSPCA (Mouse specific plasma cell antigen) and the Hy antigens. Some viral antigens are expressed by murine lymphocytes infected with C type virus particles, a good example being the G_{1X} antigen expressed on virus infected lymphocytes in G_{1X} negative strains but also on normal thymocytes in some strains. (Old and Boyse, 1973).
The/...

The group of MSIA, MBLA and MCPCA antigens are all defined by the use of hetero-antisera (i.e. from a rabbit) and are therefore called "species" antigens. Human surface antigens (other than HL-A) correspond to the species antigens rather more closely than to the alloantigens in the mouse.

The theta antigen, now called thy1 was first associated with the thymocyte, but was later shown to occur on all peripheral T lymphocytes, skin and brain (Reiff and Allen, 1963). Recent analysis of this antigen shows that it is glycoprotein in nature, and is superficially located in the membrane. It is not related genetically or structurally to the H-2 antigen group, and there is clear evidence that it is not an antigen receptor for the T cell since anti-thy1 antisera do not prevent antigen recognition by T cells (Vitetta et al., 1973; Vitetta et al., 1974; Roelants et al., 1973). Theta CH3 antigen is now termed thy1.1, and theta AKR antigen thy1.2.

The originally proposed glycolipid nature of the thy1 antigen has recently been challenged, as a cell surface glycoprotein (T2S) has been isolated from T cells and reacts strongly with anti-thy1 sera (Trowbridge, Weissman and Bevan, 1975).

The antigens Ly-A, Ly-B were first described on thymocytes and peripheral lymphocytes by the use of cytotoxic antisera. These alloantigens are controlled by two genetic loci, Ly-A, Ly-B and there were initially shown to be two alleles of these loci, giving four antigens in all, LyA-1, LyA-2, LyB-1 and LyB-2. A third locus LyC has also been described (Boyse et al., 1968; Boyse et al., 1971).

The Ly antigen loci have now been renamed Ly1 and Ly2 and Ly3.
Ly/...

Ly antigens have not been detected on cells other than lymphoid cells, and do not occur to any great extent in thymectomised mice. They appear to be specific for lymphocytes undergoing thymus dependent differentiation. Each Ly antigen system comprises a genetic locus, Ly-1 on chromosome 19, Ly-2 and Ly-3 on chromosome 6. Each locus determined 2 alleles, which specify one of two alloantigens 1 or 2, thus the Ly antigens are described as Ly1.1, Ly1.2, Ly2.1, Ly2.2, Ly3.1 and Ly3.2. All inbred mice specify one allele from each genetic locus on their thymic dependent lymphocytes (e.g. Ly1.2, Ly2.2, Ly3.1) as their phenotype. In contrast thy antigen expression is limited to only thy1.1 or thy1.2 positive cells (theta positive cells).

The expression of Ly antigen correlates with the differentiation and maturation of thymic dependent lymphoid cells. In the neonate nearly all Ly + cells were Ly1,2,3 positive, but during ageing the pattern changed with Ly1+ and Ly23+ cells forming a substantial spleen population (Cantor and Boyse, 1975a). Ly1+ cells appear to co-operate with B cells in antibody formation. Ly2+ or Ly3+ cells appear to generate cytotoxicity. The cytotoxic T cell has the phenotype Ly2 or Ly3, and this is stable and distinguishes the class of cytotoxic as opposed to helper T cell which has the phenotype Ly1. It also shows that helper and cytotoxic T cells are committed to their roles before antigen stimulation. Both categories, Ly1+ cells and Ly23+ cells can recognise alloantigens and respond by proliferation (Cantor and Boyse, 1975b). Takahashi (1972) showed that in mouse T lymphoma/leukaemia K36, the T cells were negative for theta and LyA antigens (Ly1), but expressed Ly2 (LyB) while another leukaemia E G2 expressed theta (thy1) and Ly1 (LyA) antigens, but not Ly2 (LyB). This finding suggests that leukaemia/...

leukaemias of functional groups of differentiated T cells (T helper and T cytotoxic) can occur, whilst retaining their differentiation.

A further Ly group antigen, Ly₄ has been described on B lymphocytes, for which it appears specific (McKenzie and Plate, 1974).

In man the lymphocyte surface antigens are less well defined, particularly since alloantisera are available only through foetal-maternal immunisation by foetal cells in pregnancies (Winchester et al., 1975). Nonetheless, the histocompatibility antigens, in this case denoted by the letters HL, have been well characterised by a mixture of serological and mixed leukocyte culture tests. In addition Ia-like antigens which, in the mouse, are B cell associated and histocompatibility linked, have been defined on B cells in man (Jones et al., 1975), but little is known of them (Winchester et al., 1975).

The HL-A alloantigens have been isolated from human lymphocyte membranes (Mann et al., 1969). This group of antigens is now serologically defined, as pure HL-A antigen does not stimulate in mixed lymphocyte reactions (Ling et al., 1974). It is said that HL-A antigens alter rapidly with the establishment of cell lines in culture. This event was proposed to account for the observed ability of isologous cultured lymphocytes to stimulate in mixed lymphocyte reactions (MLR). Indeed, lymphoid cell lines are among the strongest stimulators in mixed lymphocyte reactions, possibly because most cell lines are B cell, and thus express "M locus" determined IADs.

Apart/...

Apart from the histocompatibility antigens on human lymphocytes, it has proved possible to raise antisera against human T lymphocytes (Wortis et al., 1973) and against human B cells (Greaves and Brown, 1973). Antigenicity of cells tends to vary with the cell cycle, and recently antisera raised against human thymocytes, and Burkitt lymphoma cells, reacted with thymocytes, B lymphoblasts, T lymphoblasts, and lymphoid cell lines. These antigens, one of which resembles the i antigen on red cells, are expressed only on neoplastic cells, (both lymphoid and non lymphoid) or on dividing cells (Thomas and Phillips, 1973; Thomas, 1974).

As in mouse systems, specific antigens have been defined on leukaemic cells (Monahanakumar et al., 1974; Baker, 1975), these are probably analogous with the G_{1X} antigen system in mice but have not been as well defined.

Most studies of surface antigenicity have involved murine T lymphocytes, but there are B cell specific antigens in the mouse on both B cells and plasma cells. In man B cell specific antigens have been described (Greaves and Brown, 1973).

The mouse B lymphocyte antigen (MBLA) was described on circulating B cells and lymph node cells using a heteroantiserum rendered specific by adsorption (Raff et al., 1971). An antigen on B cells, PC1, was described by Takahasi et al. (1970). This antigen is an alloantigen, determined by a gene, PCa, which segregates as a Mendelian dominant, and is not histocompatibility linked. This alloantigen, specific to mature mouse B cells, is not present on thymocytes, leukaemia cells, or T cells but is found on liver, kidney, brain, lymph node and myeloma cells, and is present/...

present on immune haemolytic plaque forming cells. A second B cell/plasma cell antigen has also been described (Yutoku et al., 1974) which, unlike PC1, is also represented on about half of the thymocytes, and is not present on liver, kidney or brain cells.

The G_{1X} antigen system in the mouse is of interest in that it is related to the Murine lymphoma virus (MuLV) (Gross virus). Infection of mice with this virus is ubiquitous, spontaneous leukaemia strains (AKR, C58) are overt carriers. Other strains show no overt infection but they develop MuLV virus and MuLV induced antigens on induced tumours, or in tissue cultures. MuLV antigen is detectable in embryo mice of virtually all tested strains during gestation. Normal mice (i.e. those showing no lymphomas) show a virus related antigen, called G (for Gross) and 1X (chromosome linkage group 1X). This antigen differs from the directly virus induced cell surface antigens GCSA, and GSA (Gross cell surface antigen, and Gross soluble antigen). Because of its ubiquity, antisera against G_{1X} were first raised in rats to which Gross Virus leukaemia was transplanted. The resulting antiserum showed strong cytotoxicity for the thymocytes of some mouse strains (G_{1X} positive) but little or none for others (G_{1X} negative). The same antigen G_{1X} can be induced on rat thymocytes temporarily by the MuLV virus showing that its expression is virus linked (Stockert, Old and Boyse, 1971).

TL antigens in TL+ mouse strains are demonstrated only on thymocytes and leukaemia cells. Leukaemia induced in a TL negative strain, results in the expression of TL antigens on circulating leukaemic cells. Even though the antigen is not demonstrable on peripheral/...

peripheral lymphoid cells (spleen and lymph node lymphocytes) in TL positive mice, injection of these cells into a TL negative mouse strain will produce the appropriate anti-TL alloantiserum (Komuro, Boyse and Old, 1973). The thymus is necessary for the maturation of T1- to T1+ cells in TL-recipients of spleen or lymph node cells. Further experiments reveal that thymic hormone induces in vitro differentiation of spleen and bone marrow lymphoid cells from adult animals. These cells also acquire both thy1 (theta) antigen and the Ly antigen. Similar antigen induction occurs with cells from congenitally thymusless mice and cells from embryo mouse liver (Komuro and Boyse, 1973). These findings emphasise the role played by the thymic epithelium in inducing maturation of the surface phenotype of the T lymphocyte. If thymus lymphoid cells are co-cultured in vitro with thymic epithelium, there is an increase in H-2 antigen expression and a gradual decrease in thy1 antigen expression, features which characterise the peripheral T lymphocyte. This process of "maturation" was preceded by cell division as a necessary step (Mosier and Pierce, 1972).

The presence of the subclass and differentiation specific surface markers on the murine T lymphocyte has proved valuable in defining subpopulations of mouse thymocytes and their functional characteristics. For example the TL negative thymocyte population in TL positive mice is effective in causing GVH disease, is steroid resistant, and has helper cell activity. These cells also have increased amounts of H-2 antigen on their surface, and can recirculate to spleen and lymph nodes. Indeed the steroid resistant thymocyte population is virtually identical, both antigenically/...

antigenically and functionally with the splenic T lymphocyte population (Stobo, Rosenthal and Paul, 1973; Konda, Stockert and Smith, 1973; Schlesinger, Korzash and Israel, 1973).

The study of cell surface antigens of similar type in human lymphoid populations is by no means as advanced technically as in the murine system. Some antigens have been defined in man, as for example the membrane antigens expressed by thymocytes, and infectious mononucleosis cells (Thomas, 1972). One antigen on human lymphoid cells which reacts with anti-brain sera, and is therefore analogous to thy1, has been described (Brown and Greaves, 1974) as have antigens specific for CLL cells and normal B lymphocytes (Greaves and Brown, 1973). Antisera prepared against human thymocytes in rabbits will react with a subpopulation of human peripheral lymphocytes, and will inhibit rosette formation with sheep erythrocytes (T rosettes) but not with complement coated red cells (B rosettes) (Wortis, Cooper and Brown, 1973). Treatment of peripheral blood by such antisera in the presence of complement removes all surface Ig negative cells (Smith et al., 1973).

Attempts have been made, using specific human thymus lymphocyte antisera in conjunction with "lectins" such as concanavalin A, phytohaemagglutinin, or pokeweed mitogen, to define subclasses among human thymocytes, similar to those described for the mouse. Anti-human thymic lymphocyte sera can inhibit mixed lymphocyte reactions by preventing proliferation of responder cells, but quite failed to prevent a proliferative response to either phytohaemagglutinin or to concanavalin A. It did interfere with the proliferative response to P.P.D. of tuberculin/...

tuberculin, and had no effect on Lymphokine release. The interpretation offered is that the cells responding to stimulation by lectins are a subpopulation of human T lymphocytes (Woody et al., 1975). In fact from the evidence presented it appears that B cell proliferative responses in man are inhibited as strongly as T cell responses by anti-T cell sera. For example, the mitogenic effect of PPD is primarily on B rather than T lymphocytes (Sultzer and Nilsson, 1972) yet it is inhibited, whilst response to phytohaemagglutinin is not inhibited, although it is primarily a T cell response (Greaves, Janossy and Doenhoff, 1974).

The use of heteroantisera to define human lymphocyte subpopulations is in its earliest stages, but providing that antisera of sufficient specificity to define differentiation, and subclass specific antigens can be prepared, free of anti-HLA and other activities, these techniques offer a quick and reliable guide to cellular differentiation. Antisera to B₂-microglobulin seem relatively B cell specific in human systems (Ringden and Moller, 1975).

TABLE 4 : Antigen Expressed on Lymphoid Cells in the Mouse:
Histocompatibility, Differentiation, Species and
Subclass Specific Antigens.

<u>Antigen</u>	<u>Cells on which Expressed</u>	<u>Antigen Class</u>
H-2	SS Thymocytes weak + all cells except Red cells SR Thymocytes +	Histocompatibility D
Thy 1.1, 1.2 (Theta CH3) (Theta AKR)	Thymocytes & thymus dependent Lymphocytes Skin and brain cells	Alloantigen Class Specific
TL (Thymic Leukaemia)	SS Thymocytes (in TL+ strains) Leukaemia Cells (TL-Strains)	D Alloantigen Subclass Specific
G _{1Y} (p30) glycoprotein	SS Thymocytes (in TL+ strains)	D Alloantigen Subclass Specific
(gp69/71)	Normal Thymocytes	MuLV Associated
Ly A, B, C. (Ly 1, 2, 3, 4)	Strongly Expressed on Thymocytes Weakly on Peripheral T cells Ly 4 on B Lymphocytes	Class & Subclass Specific D Alloantigen
MSLA	Expressed on Peripheral T cells Not on Thymus	Species and Subclass Specific
MBLA	Expressed on B cells	Species Specific
MSPCA	Plasma Cells B Lymphocytes	Species and Subclass Specific
PC ₁	B Cells Liver, Kidney	Species and Subclass Specific
PC ₂	Plasma Cells Thymocytes (50%)	Species Specific

Hy/...

<u>Antigen</u>	<u>Cells on which Expressed</u>	<u>Antigen Class</u>
Hy	Lymphoid & Tissue Cells	Species
SK	Lymphoid and Skin Cells	Species
Ia	Histocompatibility Linked	B cell/macrophage Specific determine cell interactions
SS steroid sensitive	SR steroid resistant	D differentiation

References:

(Old and Boyse, 1973; Stockert et al., 1971; Schlesinger et al., 1973; Konda et al., 1973; Kisielow et al., 1975; Boyse et al., 1968; Takahashi et al., 1970; Raff et al., 1971; Vitteta et al., 1973; Vitetta et al., 1974; Schreffler and David, 1975).

TABLE 5T Cells and their Subclasses

<u>CLASS</u>	<u>FEATURES</u>
<u>Thymocyte</u> (Steroid Sensitive Population)	Theta (thy 1) antigen TL positive Ly positive H-2 negative HTLA positive (man) E Receptor (man)
<u>Thymocyte</u> (Steroid Resistant Population)	TL negative Theta (thy 1) weak H-2 positive
<u>Peripheral T cells</u>	Theta (thy 1) weak TL negative H-2 positive E Rosette positive (man) HTLA positive (man) PHA responsive
<u>"Activated T cells</u>	Fc receptor positive
T cell Helper (antibody formation)	Ly1 positive Ly2, Ly3 negative Theta (thy 1) positive
T cell Helper (cytotoxicity)	IgM receptor positive (chicken mouse and man)
Cytotoxic T cell	Steroid Resistant Ly2, Ly3 positive
Supressor T cell	Theta (thy 1) positive

All T lymphocytes express Sd alloantigens, none express LD alloantigens. They express Ia antigens weakly. A few T cells react with isologous RBC. Note that differentiation of T cell subclasses is independent of antigen.

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CHAPTER 8

SURFACE CHARACTERISTICS OF LYMPHOID CELLS

ANTIGEN RECOGNITION UNITS AND SURFACE RECEPTORS

The prime function of the lymphocyte, is to interact with and respond to antigens. This account is concerned with the surface receptors by which lymphocytes trap antigen, and with surface receptors concerned with the subsequent cellular response to antigen.

Surface Receptors for combination with Antigen

Lymphoid cells react to the presence of antigen in their environment by binding that antigen to the cell surface. Such binding can be recognised by labelling the antigen radioactively, and the physiology of the antigen binding cells studied by their elimination (so called antigen suicide technique) or by separation (antigen binding cell depletion or enrichment procedures). The receptor for antigen has two characteristics, firstly it is specific for the antigen, and secondly it is present on only a small population of lymphoid cells.

Direct proof that antigen binding lymphocytes were responsible for the immune reaction to the bound antigen stemmed from the experiments of Ada and Byrt (reviewed by Ada, 1970) using the antigen suicide technique. In this technique an antigen is heavily labelled with I^{125} with very high specific activity. The labelled antigen is mixed with lymphoid cells, which are then washed, and injected into an irradiated syngeneic recipient reconstituted with bone marrow or foetal liver cells. Both B and T cells binding more than say 10,000 molecules of antigen are killed by the radiation dose they receive and on subsequent immunisation no antibody is formed (Basten et al., 1971). Techniques using red cells coated with specific antigen have also been employed (Bach, 1971). In this test, antigen coated erythrocytes are reacted with lymphocytes, and antigen binding cells give rise to rosettes or clusters of bound RBC surrounding the binding lymphocyte. These clusters can be removed by centrifugation and the residual cells tested for their capacity to respond/...

respond to the test antigen. Lymphoid populations so treated fail to respond to subsequent antigen challenge as in the antigen suicide technique.

The binding site for antigen on the B lymphocytes is surface immunoglobulin. This can be shown by the capacity of anti-Ig, anti-u chain or anti-L chain sera to inhibit antigen binding (Roelants et al., 1973) or antigen suicide (Ada, 1970). The number of specific antigen reactive B cells is characteristically small, but rises quite steeply following immunisation with that antigen but not with unrelated ones (Wilson, Munro and Coombs, 1969). There is abundant evidence that the antigen receptor on the B lymphocyte is immunoglobulin, and that its combining site is of equal affinity to the antibody the progeny of that B cell will eventually produce (Makela, 1970).

T lymphocytes also respond to immunisation by proliferation, showing immunological memory and being susceptible to tolerance induction (Greaves, Owen and Raff, 1973). The receptor for antigen on T cells is not usually demonstrable by mechanisms which succeed with B lymphocytes. Conflicting views of the presence of immunoglobulin as the receptor on T cells has been long a subject of controversy. One problem is demonstrating antigen binding by T lymphocytes. It has been shown that substantial numbers of antigen binding cells can be eliminated by treatment with theta (thy 1) anti-serum and complement (Greaves and Moller, 1970; Roelants, 1972). Cells in the mouse which are theta antigen positive will bind SRBC to their surface, and can be shown to "cap" this antigen in the same manner as B cells. This does not prove that the T cell receptor is immunoglobulin (Ashman and Raff, 1973).

Binding/...

Binding of antigen can be inhibited by anti-immunoglobulin, but not with anti-theta sera, and the "capping" of antigen did not alter the distribution of theta antigen, showing that the alloantigen is not the antigen receptor on T cells. Antigen receptors on both B and T lymphocytes, 1-2 months after antigen priming were found to be "capped" by anti-immunoglobulin antisera. Binding of antigen to both B and T cells was inhibited by anti-u and anti-L chain antisera, but not by anti thy 1 antisera, suggesting that IgM is the T cell receptor (Roelants, Forni and Pernis, 1973). Other evidence is less conclusive.

The genes controlling the immune responsiveness of T lymphocytes, and the co-operation of these cells with B lymphocytes are linked to the major histocompatibility gene complex. In the mouse these immune response (Ir) genes map to the I region of the H-2 complex. The Ir genes determine antigenic molecules (Ia antigens) which are expressed largely on the B lymphocyte. There is definite evidence that Ia antigens are expressed also on murine T lymphocytes (Goding, White and Marchalonis, 1975). Recent trends have thus been directed towards the identification of T cell antigen receptors by using closely related strains in which, by appropriate immunisation, antibodies can be prepared to the antigen combining sites (Ir gene products) on both B and T lymphocytes (Mitsuda, 1974). In one model, alloantibodies directed against the SD (serologically determined) alloantigens are raised by injecting spleen cells from one parent (P 1) into the other parent (P 2). The alloantibodies so produced are isolated and then used to immunise the P1 x P2 hybrid recipient. Such antialloantisera/...

anti-alloantisera produced by the F1 hybrid will react preferentially with the antibody combining site of the parental P2 alloantiserum, since the F1 hybrid is congenitally tolerant both of parental alloantigens (expressed on cells) and of parental allotypic determinants on the antibody molecules. The only "new" antigenic structure presented to the F1 hybrid is the antigen combining site of the alloantibody formed in the P2 parent. Such anti-alloantisera will react with B cells only, because the antigens are SD antigens, and T cells are unreactive to these antigens and lack receptors for them (Binz, Lindenmann and Wigzell, 1974).

This model has been further refined by the above authors in an attempt to identify the antigen combining site on the T lymphocyte for the LD (lymphocyte determined) alloantigens. These LD antigens cannot be serologically detected but can be reacted against by T lymphocytes, and can also be used to separate, by immune adsorption, reactive T lymphocytes from a lymphocyte population (Wekerle, Eshhar and Feldman, 1974). T lymphocytes express SD alloantigens but not LD alloantigens, while B cells express both SD and LD alloantigens. B cells will respond to SD alloantigens and will stimulate T lymphocytes by the LD alloantigen (Simpson, 1975). Preparations containing B cells cannot be used for immunisation of the F1 (P1 x P2) hybrid since anti-allotype antibodies occur against SD alloantigen binding sites on the B cell surface and could confuse the issue. If, however, pure T cell populations from parent P2, immunised with spleen cells from parent P1, are injected into the F1 hybrid, the T cells reactive/...

reactive against P1, LD alloantigens, which are not present in P2 T lymphocyte populations, will express antigen binding sites which are antigenic in the P1/P2 F1 hybrid. The anti-T cell receptor antibody so produced exhibits a capacity to react with a proportion of P2 T lymphocytes - these T cells can be removed on anti-idiotypic-antibody coated bead column which leads to a specific depletion of P2 T lymphocytes capable of reacting against P1 LD alloantigens. This work shows that the B and T lymphocyte share the same set of genes which determine the specificity of the antigen receptor site, since anti-idiotypic antibody so produced will react both with purified IgG alloantibody combining site and with the normal parental T lymphocytes (Binz and Wigzell, 1975; Binz, 1975).

Evidence supporting the close linkage of T cell antigen-receptor sites and the H-2 gene complex has been obtained by studying the requirement for histocompatibility in the effector phase of T cell mediated cytotoxicity (Doherty and Zinkernagel, 1975a), between the stimulator cells and reactive T lymphocytes, and for histocompatibility between T cells and macrophages in the generation of helper cells (Erb and Feldman, 1975). It appears from studies of hybrid animals that the antigen receptor mechanism of the T cell is better at detecting "self" or "altered self" antigens than at reacting with foreign antigens. This accounts for relatively high numbers of self reactive T lymphocytes (Cohen and Werkerle, 1972), or T lymphocytes reactive with closely related strains (Ford, Simmonds and Atkins, 1975), the better immune surveillance of hybrid as against inbred animals with LCM infection/...

infection (Doherty and Zinkernagel, 1975b) and the high levels of spontaneous T lymphocyte/red cell reactions observed in mice (Micklem, Asfi and Anderson, 1970) and man (Baxley et al., 1973).

The receptors for antigen on T cells were originally demonstrated to be of IgM antibody (Dwyer, Warner and Mackay, 1972) but the recent demonstration of IgM receptors on T cells, allowing the binding of environmental antibody and hence conferring antibody-like specificity on T cell reactions, has clarified the picture considerably (Moietta et al., 1975; Cooper and West, 1974).

The demonstration that anti-idiotypic antibodies, passively administered can sensitise mice for a secondary response to the antigen recognised by the immunoglobulin idiotype used for preparation of the anti-idiotypic antibody. Such sensitisation can occur in both B and T cell systems (T helper, B precursor lymphocytes) and lends support to the theory of antigenic identity of the T cell receptor with B cell surface Ig idiotype. Reaction with anti-idiotypic antibody is, then, equivalent to interaction with antigen (Black et al., 1976). Further investigations show that the idiotypic markers of the T cell receptor (T helper cell) in this system are not connected with the H-2 haplotype (i.e. are not produced by the Ir gene) but are probably produced by the Ig-1 gene complex, the "heavy-chain linkage group" which determined immunoglobulin expression in the mouse (Hammerling et al., 1976). This is further evidence that the T cell receptor - even if not conventional Ig, is certainly controlled by those genes controlling Ig expression.

In summary it is not now believed that the T lymphocyte antigen/...

antigen receptor is determined by genes closely associated with the major histocompatibility gene complex as proposed by Shevach, Paul and Green (1972) (Hammerling et al., 1976). It is not a conventional immunoglobulin molecule, but it is a T lymphocyte specific antigenic structure (Wekerle, Eshhar and Feldman, 1974). The receptor for antigen on the B lymphocyte is antibody, but both B and T lymphocyte receptors for a single alloantigen show the same antigenic structure, as determined by the reaction of anti-alloantisera prepared against both antibody and T cell receptors.

Anti-idiotypic antibodies can act to promote sensitisation of B cells for Ig production, and T cells for the helper effect, and both classes of receptor appear to be determined by heavy chain linkage group (Ig1) genes rather than Ir genes in the mouse.

The cells secreting an antibody bearing a specific idiotypic marker can be suppressed by the administration of anti-idiotypic antibody. This was shown when rabbits were treated with anti-idiotypic antibody, and failed to secrete antibody expressing that idio type upon subsequent antigen challenge (Cosenza and Köhler, 1972).

Investigations of this kind point to the possibility of selective elimination of cells expressing single idio type determinants, a possible method for eliminating clones of neoplastic cells.

Surface Receptors for Immunologically active Intermediates

1)/...

1) The Fc receptor.

A subclass of B lymphocytes expresses a surface receptor for the Fc fragment of immunoglobulin IgG in both mouse and man (Dickler and Kunkel, 1972; Paraskevas et al., 1972; Basten et al., 1972a, b). This is conventionally detected by rosetting tests in which antibody coated red cells are incubated with lymphocyte suspensions. Lymphocytes binding with the complex of red cells and antibody express the Fc receptor. In the human system, the Fc rosetting lymphoid population can also be identified using heat-aggregated immunoglobulin (Dickler, 1974). Free immunoglobulin is not bound and the reaction appears to be specific for B lymphocytes (Dickler and Kunkel, 1972). The studies of Dickler (1974) also show that the receptor is specific for the Fc portion of IgG, F(ab) fragments which retain the antigen combining site do not sensitise red cells for adherence to lymphocytes in this test. The surface Ig of the B lymphocyte is not involved in this reaction, as mild trypsinisation which removes surface Ig does not prevent binding of an IgG antigen complex. The receptor for IgG Fc is important in that it mediates the cytotoxic reactions attributed to a subpopulation of lymphoid cells, K cells, which are able to kill tumour cells sensitised with IgG antibody. The evidence that IgG Fc receptors are expressed only on B lymphocytes (among the lymphocyte population - macrophages also express receptors for IgG Fc) lies in the conjunction of stainable surface immunoglobulin and Fc receptors on the same population (Dickler, 1974). In normal human blood, 16-21% of the lymphocyte population express IgG Fc receptors, and the/...

the bulk of these are surface Ig positive cells. About 3-4% of normal T lymphocytes (surface Ig negative) also express Fc receptors. The marker Fc receptor is better regarded as being expressed on a population of lymphocytes which overlaps extensively with that population expressing surface Ig, but is not a specific marker only of B lymphocytes. In the mouse, a population of cytotoxic (K) cells showing IgG Fc receptors, but lacking any conventional B cell (surface Ig) or T cell (thy 1.2) surface markers has been described (Greenberg et al., 1975). In man lymphocytes, monocytes and neutrophil polymorphs all express receptors for Fc IgG. There is however some subclass specificity of the Fc receptor on these cells. Lymphocytes will bind unaggregated IgG of IgG₁ or IgG₃ subclasses, but not IgG₂, IgG₄, IgA, IgD or IgM. Monocytes will bind IgG₁ or IgG₃, but bind IgG₄ poorly if at all. Neutrophils bind IgG₁, IgG₃, IgA₁, IgA₂ secretory IgAS and also IgG₄. The Fc portion of the immunoglobulin molecule appears to confer cytophillia for certain cell surfaces (Lawrence et al., 1975). Not only lymphoid cells express receptors for Fc IgG, these have also been described on mouse placenta, and yolk sac cells of the mouse embryo (Elson et al., 1975).

Apart from the circulating lymphocyte of B lineage further studies have revealed similar receptors on lymph node cells (Siegal, 1972) and on cultured plasmacytoma cells (Cline et al., 1972). Ramasamy and Co-workers (1974) put forward the interesting proposition that expression of the Fc receptor was related to activation of B lymphocytes, in that it acted as a reservoir on the/...

the B cell membrane for immunoglobulin secreted by that cell. Thus in a pro-secretory B cell, the bulk of the Ig Fc receptor sites would be unoccupied, and the passive binding of antibody coated red cells would be strong. As secretion of immunoglobulin by the B cell increased, fewer Fc receptor sites would be available, and the increase in surface fluorescence would be paralleled by a decrease in antibody coated red cell binding. Finally, the plasma cell would fail to express Fc receptor at all, as it is totally committed to IgG production, and all its receptor sites are occupied by secreted IgG. There is evidence that lymphokine secretion by the B lymphocyte can be triggered through not only the Fc receptor but also the surface Ig itself and the receptor for complement (Wahl, Iverson and Oppenheim, 1974).

In the reports on Fc receptor distribution on subclasses of lymphoid cells it is frequently difficult to reconcile individual observations because of the widely divergent techniques of assay, cell separation and experimental manipulation. As an example cytotoxic activity towards antibody coated cells expressed by spleen cells from the mouse, reveals a null receptor cell (Greenberg et al., 1972) which is largely represented in the glass adherent cell population (Greenberg, Shen and Roitt, 1973) and is almost certainly a macrophage (Goldstein et al., 1973). "Null receptor" cells have had a variety of origins postulated for them, for example immature "stem" cells (Barr, Whang-Peng and Perry, 1975) antibody forming cell precursors (Basten, Warner and Mandel, 1972) or plasma cells (Ramasamy et al., 1974). However, other classes of non adherent mouse spleen cell are also cytotoxic to/...

to antibody coated cells (Greenberg et al., 1975), and there are null cells, characterised only by their receptor for IgG₂ subclass in the mouse. Thus at least two classes of spleen cell exhibit as a common characteristic the ability to kill antibody coated target cells. This property is also shared by neutrophil polymorphs and macrophages. The other property all share is the receptor for IgG. The "class" of antibody dependent cytotoxic cell (K cell) is therefore to a very large extent an artefact of experimental method rather than a "nosological entity". This work is noteworthy since it illuminates the difficulties in defining subclasses of lymphoid cell on the basis of their receptors, as distinct from their surface antigenic characteristics.

There is evidence to show that, under some circumstances, T lymphocytes in both mouse (Yoshida and Andersson, 1972; Andersson and Grey, 1974; Stout and Herzenberg, 1975) and man (Ferrarini et al., 1975) can express receptors for Fc IgG. T cells in the Guinea pig also appear to express Fc receptors (Van Boxel and Rosenstreich, 1974). In the papers of Yoshida and Andersson, and Stout and Herzenberg, IgG antibody complexed with erythrocytes or with IgG anti-IgG were used as the test particles. Resting, i.e. inactive, T lymphocytes did not express Fc receptors, but transformed reactive T lymphocytes did express this receptor (Yoshida and Andersson, 1972). It was further shown that Fc receptor positive T lymphocytes were not helper cells (Stout and Herzenberg), a finding which implies that expression of Fc receptors may be a property of transformed responding (i.e. "effector")

T/...

T lymphocytes. In the studies of Andersson and Grey (1974) aggregated IgG in a mouse system was used in an attempt to quantitate the Fc receptors on B and T lymphocytes. Fc receptors were found on 20-45% of thymus cells, and on 70-85% of spleen cells. A very sensitive radiolabelling assay technique was employed. Both "null cells" (thy-1 negative SIg negative) and thy-1 positive SIg negative thymus derived lymphocytes could bind substantial amounts of aggregated immunoglobulin.

Fc receptors have been described on human lymphocytes which form rosettes with sheep erythrocytes (Ferrarini et al., 1974) and which are presumptive T cells. The same group has also described a receptor for IgM antibody on human T cells, although this is distinctive and does not show inhibition with free IgG, arguing that the receptor for IgM antibody on T cells is not an Fc receptor (Moriotta et al., 1975). In the mouse, it has been shown that IgM receptors present on normal thymocytes can mediate T cell cytotoxicity against LCM virus infected cells (Lamon et al., 1975).

The biochemical nature of the Fc receptor, and the genes determining its expression have recently come under scrutiny. Since the Ir gene products are involved in many of the reactions which might also involve Fc receptors, the relationship of the Fc receptor to the H-2, and Ir gene products was an obvious starting point for such studies. The relationship of the Fc receptor to the H-2 complex, and the antigen binding sites on T and B lymphocytes (which are Ir gene products) was studied by Basten, Miller and Abraham (1975). These authors concluded that/...

that one group of H-2 determined alloantigens was in close proximity to the gene determining the Fc receptor. Anti-H2 antibody, administered to cell suspensions as the Fab fragment, could block the Fc receptor on B cells. This antiserum reacted predominantly with the H-2 alloantigens not coded for by H-2K or H-2D segments, thus lying in the region of the Ir gene complex. The Ir determinant for Fc receptor seems to be expressed only on the B cell, but appropriate anti H-2 antisera prevented T cells binding to antigen present on B cells - implying that one of the Ir gene products of the B cell may be an Fc receptor, while in the T cell it may be the antigen receptor site.

Schirmmayer, Halloran and David (1975) re-examined the data of Dickler and Sachs (1974) which claimed to show close association of I region determined alloantigens, and the Fc receptor on B cells, by the use of I antigen alloantisera prepared in H-2 compatible animals. They showed that not only would anti-Ia antisera inhibit the Fc receptor, but anti-Ly 4.2, rabbit anti-mouse B cell antiserum (MBLA) and rabbit anti-mouse immunoglobulin would also effectively inhibit Fc receptors. On the other hand, antisera against thy 1 or xenogeneic anti-thymocyte antisera failed to inhibit Fc receptors. Fab fragments of inhibitory antisera were also inhibitory, and appropriate inhibition of the K cell by Fc receptor was obtained by whole Ig molecules, but not by Fab fragments. It was therefore concluded that the Fc receptor was not, antigenically at least, part of the Ia molecule. Further attempts to relate the Fc receptor to Ia antigens biochemically also have failed to demonstrate identity of these two components (Rask et al., 1975).

The Complement Receptor

The phenomenon of "immune adherence" described by Nelson (1953) between normal human erythrocytes and *T pallidum* sensitised with antibody in the presence of complement, stimulated further research into the mechanisms of adherence, and of the cells with which immune adherence takes place. In his original description Nelson regarded such adherence as a method of increasing the efficiency of phagocytosis. It was therefore natural to examine the neutrophil and macrophage for immune adherence reactions. Receptors for complement were described on blood leukocytes, including lymphocytes by Lay and Nussenzweig (1968), and receptors for complement on macrophages by Huber et al (1968). It was shown that the human monocyte expressed a receptor for the third component of complement (activated C3), that this receptor required large amounts of bound complement to function, and was separate from the receptor for IgG (IgG Fc) on the same cell.

The first definitive account of complement receptors on human peripheral blood lymphocytes was by Michlmayr and Huber (1970). The test particle was the sheep erythrocyte, coated with IgM antibody and human complement. Both monocytes and lymphocytes bound these cells, but monocyte binding was reported to be inhibited by EDTA. A mean of 20%, with a range of 12% to 29% of lymphocytes had complement receptors by this test. These findings were confirmed by Bianco, Patrick and Nussenzweig (1970) who also used EDTA to inhibit binding to monocytes and polymorphonuclear leukocytes. Bianco and Nussenzweig (1970) later showed/...

showed, in the mouse, that lymphocytes which bound complement sensitised red cells lacked theta (thy 1) antigen, and that CRL (complement rosetting lymphocytes) were B cells. This was confirmed by demonstrating that complement sensitised red cells reacted preferentially with lymphocytes contained in the B lymphoid areas of spleen, and lymph node, but not in thymus (Dukor, Bianco and Nussenzweig, 1970; Silveira, Mendes and Tolnai, 1972). The latter authors showed preferential binding of complement sensitised erythrocytes to the germinal centre in lymph nodes in man, but failed to show binding to thymus using Frozen sections. Apart from the presence of complement receptors on normal human lymphocytes and monocytes, they were also demonstrated on leukaemic lymphocytes and on cells from lymphoid cell lines (Shevach et al., 1972a) in man and in the mouse (Shevach et al., 1972b).

The specificity of the binding in complement receptor studies involves two variables:- firstly the class of cell to which the complement sensitised particle is presented, and secondly the complement components expressed on the surface of the particle. Extensive studies have been undertaken to exactly determine which classes of cell bind to which complement component.

Briefly complement consists of eleven components, of which C_1 , C_2 , C_4 , C_3 are relevant. C_1 (composed of three subunits C_{1q} , C_{1r} , C_{1s}) is bound by IgM antibody (and IgG if present in sufficient concentration) and subsequently interacts with C_4 , C_2 and C_3 in that sequence. Each interaction involves the splitting/...

splitting of a fragment from the native free complement component, the major portion being bound, and the minor component entering the fluid phase. For example C₄ is split by C₁s into C_{4a} and C_{4b}. C_{4b} is bound to C₁ to give C_{14b}. This complex fixes C₂ to form C_{14b2a}, a complex called C₃ convertase which splits C₃ into C_{3a} and C_{3b}. C_{3b} is fixed to the cell surface, and there is acted on by C₃ inactivator which converts bound C_{3b} to C_{3d} by way of an intermediate C_{3c} component. Erythrocytes expressing C₁, C_{14b}, C_{14b2a}, C_{14,2,3}(b or d), C_{4,3b}, C_{4,3d}, can be prepared and these are used to test the specificity of the receptors on the cells studied; e.g. human red cells express receptors for C_{3b} component but not for the C_{3d} component. C_{3b} cells are "immune adherence positive" with human red cells, C_{3d} cells are "immune adherence negative". Using such techniques it has been shown that the C₃ component was the major one influencing the binding to lymphocytes, as complement coated red cell binding could be inhibited with split products of C₃ (Eden et al., 1973). Human lymphocytes were in fact shown to possess two receptors: for C_{3b}, or for the inactive product C_{3d} (C_{3b} inactivator cleaved C_{3b}). Human neutrophils expressed receptors for C_{3b} and C₄ but not for C_{3d}. (Ross et al., 1973; Ross, Polley and Grey, 1973; Eden, Miller and Nussenzweig, 1973).

Studies of CRL in human blood or tissue preparations also have to take into account the class of erythrocyte (e.g. sheep, ox, human) used, and the source of complement (mouse, rabbit, guinea pig, human) in preparing the test particle. Sheep erythrocytes/...

erythrocytes, sensitised with rabbit antibody and mouse complement, will react with human T cells as well as B cells, under the conditions cited by Ross et al. (1973). This was not the experience of the author of this thesis, using a similar system but with great attention paid to standardisation of the initial sensitisation by IgM antibody, and not of some other laboratories (Gatien et al., 1975a, 1975b), using essentially similar techniques.

More careful study paid to the lymphoid cells expressing the complement receptor, particularly when these were combined with tests for responsiveness to phytomitogens, and for the Fc receptor, revealed that not all cells possessed both Fc and C3 receptors together, although there was considerable overlap between these populations. 70% of Fc receptor cells had C3 receptors as well and no cells expressed the C3 receptor only. This work, in the mouse, revealed that rapidly dividing lymphoblastoid B cells responding to bacterial lipopolysaccharide (a B cell mitogen) retained their Fc receptor, but lost the receptor for C3 (Moller, 1974).

There have also been further reports showing that the receptor specificities attributed to C3b in previous reports, may well have been due to the expression on human lymphocytes at least, of a receptor for C4. This C4 receptor will cross react with C3b:- the second lymphocyte receptor, for inactivated complement (C3d) reacts only with C3d, and not with C4 or C3b (Ross and Polley, 1975). The C4 receptor (Bokisch and Sobel, 1974) but was first described, again on the human erythrocyte, by Cooper (1969).

The/...

The role of the receptors for complement and IgG Fc in the development of the immune response, and in the regulation of B lymphocyte proliferation and differentiation is currently being explored. It has been shown that immune complexes containing complement bind to the complement receptor in preference to binding to the receptor for Fc IgG (Theofilopoulos, Dixon and Bokisch, 1974). These authors also managed to show that receptor for Fc and complement on human lymphoid cell lines did not universally occur together, and did not always occur only on SIg positive cells.

The complement and Fc receptors seem to appear rather later than the surface immunoglobulin on the B lymphocyte surface. Although the numbers of SIg positive B lymphocytes in the neonatal mouse spleen achieve adult or near adult proportions by the end of the first two weeks of life, the numbers of Fc or complement receptor expressing lymphocytes do not reach adult levels until at least 6 weeks of age (Gelfand et al., 1974a). If, as seems probable, the major immunoglobulin expressed is IgM, then the Fc receptor will not be occupied by IgM molecules, and consequently its rate of expression is not simply due to masking by immunoglobulin molecules, but due to actual synthesis by the cell. It is reasonable to believe that the expression of complement receptors is under genetic control since the rate of appearance of a complement rosetting lymphoid population depends upon a gene located with the H-2 complex (Gelfand et al., 1974b).

The presence of the complement receptor is of great importance in the activation of B lymphocytes. Cobra venom factor (which destroys C3) prevented the development of humoral antibody formation to thymic dependent antigens (Pepys, 1972). The addition of quite small/...

small quantities of C3b to cultures of mouse lymphocytes in vitro, is sufficient to cause transformation of B lymphocytes with incorporation of H³ thymidine into DNA. (Hartmann and Bokisch, 1975). C3, C3a and C3c were much less stimulatory, or failed to stimulate cultures at all. A role for the complement receptor has been proposed by adherents of the "two signal" hypothesis of B lymphocyte activation (Reviewed by Dutton, 1975) and some others regard the C3 receptor as a major mitotic stimulator of the B lymphocyte (Hartmann, 1975)(Moller, 1975). These concepts of B lymphocyte activation will be discussed later.

The complement receptor has been generally regarded as a superior marker of B cells than the Fc receptor, since the Fc receptor is also expressed on activated T lymphocytes. T lymphocytes do not express complement receptors, although neoplastic cells, as in some lymphoid cell lines from the mouse (Greenberg and Zatz, 1975) chicken and man (Powel et al., 1974; Stuart and Habeshaw, 1975) show characteristics of T lymphocytes and also express the complement receptor.

The complement receptor has been used to deplete lymphocyte populations of "K" lymphoid cells, indicating that in addition to possessing the Fc receptor through which it mediates the cytotoxic reaction, the K cell has a receptor for complement. However, the addition of complement to target cell monolayers pretreated by IgG antibody, did not increase target cell lysis by lymphocytes, showing that this receptor is not important to cytotoxic K cell reactions (Perlmann et al., 1975). The classes of lymphocyte expressing Fc and complement receptors were transformed by B cell mitogens/...

mitogens. It was shown that more B cells had Fc receptors than C3 receptors. After activation by mitogens (LPS) and transformation into blast cells, the C3 receptor is lost. Mitogens such as P.P.D., stimulate B cells to transform. C3 receptors are quickly lost, but Fc receptors are retained (Moller, 1974). There is also evidence that B cell mitogens themselves will activate B cells much more efficiently in the presence of complement than in its absence (Moller and Coutinho, 1975).

In addition to the presence of receptors for the clearly defined immunologically reactive intermediate such as the IgG Fc receptor, receptors for IgM, and the receptor for C3 and C4 components of complement, lymphocytes show other characteristics by which they can be identified. The receptor for the sheep erythrocyte is claimed as a specific marker for human T lymphocytes, receptors for EB virus are B lymphocyte markers, and receptors for Cholera endotoxin, also mark B lymphocytes. Receptors for measles virus have been detected on human T lymphocytes.

The phenomenon of positive binding of sheep erythrocytes to a subpopulation of human blood lymphocytes was first reported by Brain, Gordon and Willetts, (1970) (Brain and Gordon, 1971; Brain and Marston, 1973). The finding was confirmed by a number of separate laboratories (Lay et al., 1971; Wybran Fundenberg and Schlesinger, 1971; Jondal, Holm and Wigzell, 1972; Froland, 1972; Coombs et al., 1970). Before the technology was standardised, results obtained tended to be rather variable, and accurate determination of the class of reactive cell was not possible (Bentwich et al., 1973). The blocking of the sheep erythrocyte receptor/...

receptor (E receptor) was studied by Wernet and Kunkel (1973) and immunoglobulins were ineffective in inhibiting E rosetting.

It was shown that antisera prepared against human thymocytes or brain could inhibit E rosetting (Wortis, Cooper and Brown, 1973) suggesting that the cell responsible was the thymic dependent T lymphocyte. Most of the E rosetting cells are not found to have Fc IgG or complement receptors, although a subpopulation (2% or so) do express dual receptors (Steel, Evans and Smith, 1975)(Dickler, Adkinson and Terry, 1974). The most substantial early evidence for identifying the E rosetting cell as a T lymphocyte stemmed from the high levels of E rosetting obtained with thymus (Jondal et al., 1972; Silveira et al., 1972) and observations with anti-T cell sera (Yata et al., 1973; Auiti and Wigzell, 1973). E rosettes are occasionally encountered on EB virus infected B lymphoid cell lines, and occasional co-existence of E rosettes and complement receptors on the same cell do occur, but have hitherto been described only on cell lines, or in lymphoid neoplasms (Smith et al., 1975; Stuart and Habeshaw, 1975). The levels of E receptor lymphocytes, Fc IgG, complement receptor lymphocytes and B lymphocytes have been quantitated accurately in human peripheral blood (Holm et al., 1975; Habeshaw and Young, 1975)(Bobrove et al., 1974) and in spleen (Visakorpi and Repo, 1973; Stuart and Habeshaw, 1974).

Proteolysis appears to enhance E rosetting and T cell function in malignant disease (Holland, Brown and Thornes, 1975). Protease I from *Aspergillus oryzae* (Brinase) and streptokinase both were effective in increasing levels of E rosetting cells. Chapel (1973) described/...

described enhancing of E rosetting by papain and trypsin, providing treatment of the lymphocytes was mild and no loss of viability occurred. This effect was attributed to "unveiling" of E receptors by proteolysis. Phospholipase A inhibited E rosetting. E rosettes can be stabilised by the addition of glutaraldehyde after rosetting, but when this is done substantial numbers of B cells do bind sheep erythrocytes (Evans, Smith and Steel, 1975). Enhanced E rosette formation is also observed following treatment of erythrocytes with 0.143m^2 aminoethylisothiuronium bromide (Kaplan and Clark, 1974) and substantial numbers of surface Ig+ (B lymphocytes) forming E rosettes are seen, with virtually all peripheral blood lymphocytes (87%) rosetting. Similar, but not so pronounced E rosetting by B cells occurs with sheep cells pretreated with neuraminidase (Bentwick et al., 1973)(Weiner, Bianco and Nussenzweig, 1973).

In the mouse, treatment of lymphocytes with phyto mitogens (plant agglutinins) agglutinates and stimulates subclasses of B or T lymphocytes (Schnebli and Dukor, 1972). Both phytohaemagglutinin and concanavalin A will enhance E rosetting when cultured with human lymphocytes (Gergely et al., 1973). Phytohaemagglutinin is recognised as a T cell specific mitogen, while CON-A stimulates both B and T cells. Stimulation of human lymphocytes with PHA does increase both E rosetting cells, and spontaneous rosette formation with homologous human erythrocytes (gpOrh-) both functions of T cells (Sheldon and Holborow, 1975).

The effect of dextran on E rosette formation was studied by Brown, Halpern and Wortis (1975) who found enhancement. A similar effect/...

effect has been noted on human lymphocytes separated over Ficoll triosil gradients (Blackie, 1973) and on some samples of human polymorphonuclear leukocytes separated by dextran sedimentation (Hsu and Fell, 1974).

The rather strange biological phenomenon of human T cells binding sheep erythrocytes does have a species parallel in that papain treated rabbit erythrocytes are bound by guinea pig thymocytes and peripheral T cells (Wilson and Gurner, 1975).

Other receptors on human lymphocytes include the measles virus receptor on human T lymphocytes which is not a consequence of prior immunisation (Valdimarsson, Agnarsdottir, Lachman, 1975). Human B lymphocytes have a receptor for the EB virus (Jondal and Klein, 1973), which overlaps with the receptor for complement, i.e. is on the same population. Some E rosetting, thymus derived lymphoid cell lines have been described (Minowada, Ohnuma and Moore, 1972) and more recently, proof that these cell lines lack EB virus particles (which only affect B lymphocytes) has been provided (Kaplan, Shope and Peterson, 1974).

Thus in man there is good evidence that the E receptor overlaps substantially with the T lymphocyte population as defined by antisera and mitogens, and overlaps little if at all with the population expressing complement receptors. If lymphocytes are treated with proteases, or sheep erythrocytes with neuraminidase, E rosetting populations then overlap substantially with Surface Ig, or complement receptor lymphocytes.

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CHAPTER 9

ANTIGEN RESPONSE

Having examined how lymphocytes trap antigen, and having described surface characteristics related to determination of cell class and immune responsiveness, this chapter describes the principal types of interactions between cells which occur in the response to antigen.

2) Co-operation between thymic dependent (T) and thymus independent (B) lymphocytes in humoral immune responses

Lethally irradiated mice can be saved from death by an injection of syngeneic bone marrow cells. If thymectomy is carried out before irradiation, the reconstituted animals can be shown to lack cells capable of causing graft versus host disease, and such animals will also accept skin grafts from unrelated mouse strains. They have no thymic dependent lymphocytes. In investigating the response to such animals to antigens, Claman, Chaperon and Triplett (1966) injected mice reconstituted with either bone marrow cells or thymus cells, or both, with sheep red blood cells as antigens. They found a greater antibody response in bone marrow and thymus reconstituted animals than in recipients of thymus or bone marrow alone. This synergism of bone marrow derived and thymus derived cells was subsequently confirmed by Miller and Mitchell (1968) for other classes of erythrocyte as antigen, and by Taylor (1969) and Miller and Svoboda (1971) for serum proteins. Not all proteins require the presence of both T and B cells for an adequate immune response - some require only B lymphocytes; these are called thymic independent antigens, the thymic dependent antigens require both B and T lymphocytes for an adequate humoral immune response (Miller, 1971). One class of antigens, the haptene-protein conjugate, shows unusual behaviour when used to immunise animals (Rajewsky, 1969). Haptenes will only elicit an immune response when coupled to a carrier protein. If a secondary challenge with the same haptene coupled to the same carrier is given, then a good anti-haptene/...

anti-haptene antibody response is seen. If the second challenge is made with a different carrier, but the same haptene, then a poor anti-haptene response occurs. The effect is due to carrier specific thymic derived cells (T cells) and all haptene-carrier responses are thymic dependent. This was shown by Mitchison (1971) using NIP-ovalbumin, (NIP = 5 iodo 3 nitrophenyl acetyl - ovalbumin). When animals were immunised with NIP-ovalbumin and then challenged with NIP-BSA (BSA = Bovine Serum Albumin), no secondary response to NIP occurred. This was shown to be due to the generation of cells with anti-ovalbumin binding capacity during the primary immunisation. Since the subsequent challenge was with NIP-BSA and as no BSA binding cells were present the response to the haptene NIP was reduced. Raff (1970) showed that the cells which bound the carrier had the characteristics of thymus derived lymphocytes. As a rule it is now established for several haptene-protein conjugate systems that thymus derived cells "see" the protein carrier and that the antibody forming B lymphocytes "see" the haptene. For an efficient secondary response both cell types must be present. This role of the T lymphocyte is described as "the helper effect".

Such experiments have now clearly established that in the normal humoral immune response both B and T lymphocyte co-operate in antibody formation. The effects of T cells on antibody formation by B cells are more involved than enabling B lymphocytes to "see" antigen. As demonstrated by Mitchison (1968) the uncommitted "virgin" B lymphocyte can be "primed" by antigen in concentrations of 10^{-7} molar. If 10 times less (10^{-8} molar) antigen is present the lymphocyte binds it, but fails to respond to subsequent challenge/...

challenge, i.e. it is paralysed. If a "primed" lymphocyte is exposed to 10^{-10} molar concentration of antigen it will convert to an antibody secreting plasma cell. If exposed to 10^{-5} molar, i.e. 100,000 times that concentration of antigen, it will fail to secrete antibody, i.e. will become paralysed by antigen excess. Therefore a humoral immune response will occur only if antigen is present in concentrations which will "prime" or convert primed antigen reactive B cells. Where antigen is scarce, some mechanism of concentration is required, where antigen is in excess some mechanism of elimination of that excess is required or no immune response will ensue (Mitchell, Humphrey and Williamson, 1972). It has been suggested that antigen activated T cells, by binding antigen, reduce its availability for B cells in such a way as to decrease the tolerogenicity (paralysing capacity) of that antigen for B lymphocytes of high antigen binding capacity, and at the same time to prevent other B cells forming clones due to the "antigen drive" by a direct T lymphocyte mediated suppressor effect. The former activity can be interpreted as a T cell helper effect, and the latter as a T cell suppressor effect (Mitchell, 1975). Such effects can undoubtedly be shown, but requirement for another cell type - the macrophage can be demonstrated.

3) The role of the macrophage in antigen handling

During an immune response, injected antigen is trapped in lymph nodes by cells which do not themselves secrete antibody. Two types of cell are involved, the macrophage, and the dendritic cell of the germinal centre. Macrophages, unlike B lymphocytes, can bind/...

bind many antigens. The extent to which they bind antigen depends upon the degree of polymerisation of the antigen (i.e. its size) and upon the presence of specific antibody. Macrophages do not appear in any way to influence the specificity of antibody formation subsequent to antigen binding, but binding is important as it is impossible to induce tolerance by macrophage bound antigen in distinction to soluble or native antigen (Unanue, 1972). By contrast, immunisation of mice with antigen bound to macrophages invariably increases the subsequent immune response in comparison with free antigen (Unanue and Askonas, 1968). Some antigens administered in a soluble form are very poor immunogens, but are highly immunogenic when administered with macrophages (Mitchison, 1969). According to one view, macrophages act as a "focusing" device for antigen, concentrating antigen on its surface in a form where B and T lymphocytes can interact together to initiate an immune response. Feldman et al. (1975) believes that the macrophage acts as a receptacle for the T cell surface receptor. In this model the T cell interacting with antigen by virtue of its surface antigen receptor sheds the complexed receptor (consisting of 7S IgM bound to antigen) which is abstracted from the intercellular fluid by the macrophage. The macrophage possibly has receptors for IgM antibody-antigen complexes (e.g. isohaemagglutinins) (Stuart and Cumming, 1967; Lay and Nussenzweig, 1969) but this is controversial (Huber and Holm, 1975). This has been discussed. The macrophage + T cell receptor + antigen stimulates the B lymphocyte which then forms antibody, in this model. Macrophages are also ideally suited to the destruction of excess antigen, after an antigen injection less than 0.5% of the antigen remains associated with the macrophage surface/...

surface. The bulk of antigen is destroyed. Macrophages may also co-operate directly with B lymphocytes through the medium of surface fixed antigen, either directly or in the form of antigen antibody complexes.

The question of co-operation between macrophages and T lymphocytes in the initiation of graft rejection has not been settled. T lymphocytes from unsensitised rats when cultured on monolayers of fibroblasts from unrelated rats can undergo transformation and generate cytotoxic lymphocytes (Ginsberg, 1969). Evidence supporting the role of the macrophage as an antigen processing cell for T lymphocyte responses stems from the in vitro mixed lymphocyte reaction, in which leucocytes from two unrelated donors are mixed and cultured in vitro. Transformation of T lymphocytes occurs if macrophages are present. Highly purified lymphocyte preparations are said to be inactive (Bain et al, 1965). The importance of the macrophage may relate to its ability to "fix" antigen in an immunogenic form.

4) Role of the reticular cell in antigen handling in the developing germinal centre.

There are frequently observed close anatomical relationships between antigen containing macrophages and lymphoid cells, especially in homograft reactions (Sharp and Burwell, 1960; Haheln, 1971), and in the germinal centre. Macrophages preserve the immunogenic portion of an antigen (Cohn, 1962), but the bulk of ingested antigen is destroyed. The remaining immunogenic component is capable of activating B cells, either directly or through interaction with the/...

the T lymphocyte. A direct role for the macrophage in activating B cells was shown by Gallily and Feldman (1967) using shigella organisms transferred to irradiated mice. Macrophages are required for the interaction between particulate antigens and B lymphocytes (Mosier, 1967). In studying the response of the lymph node to antigen it is found that cells other than the macrophage retain antigen. Marshall and White (1950) describes these cells as irregular with finely branched processes, surrounding the follicle. They stained with silver. Similar cells within the germinal centre did not stain with silver.

At this point some explanation is required. The fact that Marshall and White failed to stain the intrafollicular cell with silver but could stain the perifollicular cell is of some interest. Both cell types were at that time regarded as forming a part of the "reticulum" of the lymph node. The smaller, dark, and silver staining cells were regarded as structural cells which had differentiated from the larger, pale, non-silver staining totipotent "reticulum" cell which gave rise to fibre-forming, lymphoid and histiocytic elements. (Marshall, 1956; Maximow, 1924). It was therefore quite natural to suppose that since the germinal centres produced lymphocytes, no fibres should be present within them, as the reticulum cell was differentiating in a "lymphoid" rather than a "fibrogenic" direction.

This observation of the "reticulum" cell of the germinal centre acquired greater importance when it was demonstrated (White, 1963) that an antigen retaining "reticular net" was present in the germinal centres of lymph nodes and spleen, and that these antigen retaining cells/...

cells belonged to the same group as the cells described by Marshall and White. Several other authors have described the antigen retaining dendritic or reticular cell of the germinal centre. The localisation of antigen within the germinal follicle was studied by radio-iodinated proteins and synthetic polypeptides, injected into animals with and without preformed antibody (McDevitt et al, 1966). Such studies indicated that follicular localisation of antigen was a function of preformed antibody, and that the amount localised in germinal follicles was dependent upon the amount of antibody given with the antigen or naturally present. It was later shown that the immune complexes formed between antigen and antibody in the germinal centre failed to bind complement (Pernis et al., 1969). Antigen localised in the perifollicular area penetrates the germinal centre rather slowly on the reticular cell processes. The movement of antigen into the germinal centre is radio-sensitive but its retention therein is not (Hunter et al., 1969). The ultrastructural features of antigen uptake by germinal centre dendritic cells show that localisation is extracellular, and is in the form of small aggregates of antigen and immunoglobulin diffusely localised to the fine dendritic processes of these cells which run between the lymphocytes and lymphoblasts (Nossal et al., 1968). These dendritic cells have "desmosomes" at points of contact and appear to contain fibrils (Maruyama and Masuda, 1964).

In summary, antigen is handled by macrophages which destroy most of it. Any they retain is immunogenic both to T and B lymphocyte populations. If pre-existing antibody is present in an animal, the antigen (or a very minor part of it) localises to the dendritic reticular cell of the germinal centre as an antigen/antibody complex. Such/...

Such antigen stimulates germinal centre development and enlargement and is unusual in that when so localised the complexed material fails to bind complement.

5) Macrophage lymphocyte interaction in the immune response

Reactivity of thymus dependent lymphocytes can be induced by contact in vitro with allogeneic cultured fibroblasts (Ginsberg, 1969). In antigen induced proliferation of immune T lymphocytes in the guinea pig complete removal of adherent cells, including macrophages, prevents the proliferative response of T cells to antigen (Waldron et al., 1973). Exposure of macrophages to antigen for 1 hr. at 37°C, washing, and subsequently mixing with immune lymphocytes produced a proliferative response. Although in the first case sensitisation of T cells directly to allogeneic fibroblasts in long term culture can occur, it appears that, upon sensitisation, macrophages are necessary to enable an immediate proliferative response to ensue. One morphological marker of macrophage induced lymphocyte proliferation may be physical adherence reactions between macrophages and lymphocytes such as those described in lymph nodes by Miller and Avrameas (1971). Mosier and Pierce (1972) have suggested that macrophages in thymus may play a part in the functional maturation and differentiation of thymocytes. Lipsky and Rosenthal (1973) have investigated the binding of guinea pig lymphoid cells to tissue cultures of macrophages. This binding of lymphocytes was independent of serum and antigen and not dependent upon the presence of surface antibody and both B and T lymphocytes were bound. Bound cells exchanged readily for free lymphocytes/...

lymphocytes in the surrounding medium. The receptor for lymphocytes on the macrophage surface can be shown to differ from the Fc IgG receptor, in its requirement for Calcium (Ca^{++}) ion and susceptibility to sodium azide.

Macrophages and lymphocytes can be physically observed to interact in immune responses. In immune reactions in the guinea pig, there is evidence that for antigen recognition by the thymic dependent lymphocyte, macrophages are required. Where the macrophage and T lymphocytes are from different strains of guinea pig, there is no antigen recognition by the T lymphocyte, but when both macrophage and lymphocyte are histocompatible, sensitisation of the T lymphocyte by antigen can occur (Rosenthal and Shevach, 1973). In responses to thymic dependent antigens, or haptene-carrier complexes, contact between the antigen carrying macrophage and the antibody forming lymphocyte was essential. The first step in such reactions - the binding of antigen by T cells and its release as an immunogenic complex (IgT/antigen) produces tolerance in a B cell population unless macrophages are present (Feldman, Schrader and Boylston, 1975).

Other important features of macrophage/lymphocyte interaction include a) the maintenance of lymphocyte viability in tissue culture, b) functional maturation and differentiation of thymocyte populations, c) the enhancement of non specific resistance by macrophages following contact with sensitised lymphocytes (Krahenbuhl and Remington, 1971), d) induction of a primary response to particulate antigens, though not to B cell mitogens, e) induction of a secondary response to haptene-carrier complexes in presence of T lymphocytes, f) the ability/...

ability for lymphocytes to proliferate in a secondary response to a particulate antigen is dependent upon macrophages (Lipsky and Rosenthal, 1975).

Equally important classes of interactions between sensitised lymphocytes and macrophages occur in which the macrophage acts as the effector cell under the influence of the sensitised lymphocyte via the lymphokines. This kind of interaction does not require direct lymphocyte macrophage contact.

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CHAPTER 10

THE PROBLEM OF B CELL DIFFERENTIATION

This chapter is concerned with the definition of differentiation, and explains on what basis the term is used in describing differentiation in the B lymphocyte system.

B Cell Activation in the Normal Response to an Antigen

The reaction to an antigen involves the ultimate production of a specific protein, the antibody, which reacts with the stimulatory antigen. In order to produce this antibody the B lymphocyte must be stimulated to proliferate expanding the population of cells capable of synthesising that antibody, and must differentiate from an inactive cell to a cell capable of manufacturing and exporting $2-3 \times 10^6$ molecules of antibody per minute.

The necessity of expanding the reactive B lymphocyte pool by mitosis is seen when the genetic control over antibody synthesis is considered. Normally, only 1 in 1,000 to 1 in 10,000 B lymphocytes express antigen binding sites which will react with a given antigenic determinant. The antigen binding sites are found in the variable portion of the immunoglobulin molecules, and the amino acid sequence in this region is determined by a set of genes (estimated at about 1,500) called V genes (Hiltschmann et al., 1969). A principle, called V gene restriction, appears to operate which states that a cell can express only one of the several million possible V gene sequences. In general terms this principle requires that one B lymphocyte can express only one kind of variable chain determined by its own unique V gene sequence (one cell one antibody rule). This rule does not affect the class of antibody secreted (IgM, IgG etc.) since this is determined by the constant or C region of the heavy chain of the immunoglobulin molecule. Thus it is possible for a B lymphocyte to "switch" from IgM antibody production, to IgG production, with the same V gene sequence, and hence antibody specificity being shared by both IgG and IgM molecules (Bell, 1975).

Factors/...

Factors which trigger an expansion of the B lymphocyte pool, by inducing mitosis in the B lymphocyte population, may not be directly related to either reaction with antigen, or subsequent antibody synthesis. It is better to regard factors producing cell division as their principal effect as producing "activation" of the B lymphocyte, and those influencing immunoglobulin production as producing "differentiation" of the B lymphocyte. Thus it is found that substances such as bacterial lipopolysaccharide will produce a great mitogenic response by B lymphocytes (Gery, Kruger and Spiesel, 1972) but this mitogenic response does not result in production of a single class of antibody directed against the stimulating substance. Those cells not expressing high affinity receptors for lipopolysaccharide proliferate in the early response, as well as those producing specific anti-lipopolysaccharide antibodies. Such a response is called a polyclonal response, and the mitogens producing it are called polyclonal B cell activators (PBA). These substances show two features of interest

- 1) They are all activators of complement and may function by interaction with the complement receptor on the B lymphocytes (Dukor et al., 1974)
- 2) In the response to such substances T lymphocytes are not required (Coutinho, 1975). These are sometimes called T independent antigens.

However, polyclonal B cell activators are a highly heterogenous group, including lipopolysaccharides, proteins, synthetic polypeptides, polymers of inosine and cytosine, (Poly 1C), and products of activated T cells and macrophages. They produce proliferation/...

proliferation, rather than differentiation and specific antibody secretion.

The modulation of the proliferating B cell into a specific antibody secreting cell has been regarded as a co-operative function of helper cells and antigen itself, adsorbed to the specific receptors on the cell surface. This, has resulted in the "two signal" hypothesis of B cell activation, one non-specific signal from T cells or macrophages activating the cell to secrete antibody, and the second specific signal by antigen inducing commitment to antibody secretion. The experimental evidence supporting this view is given by Dutton (1975), who shows that in the response to donkey red cells, antigen alone will not stimulate the B cell to synthesise antibody (as assessed by counting the number of lytic plaques made by B cells exposed to antigen alone). The addition of T cells 40 hours after exposure to antigen increases the numbers of plaque forming and hence actively secreting B cells. However, exposure to antigen alone does initiate, or prime B cells to proliferate, and the subsequent addition of T cells stimulates both proliferation and antibody secretion. In this model the "switch on" signal comes from antigen, and the "secrete" signal comes from T cells.

Proliferative responses (usually measured by the incorporation of H^3 thymidine) by B cells exposed to antigen can occur where no T helper cells are present. In experiments with synthetic antigens (e.g. (T,G)-A -L), it was shown that IgM to IgG switching, and limited proliferation of B cells occurred in the absence of T cell help. The switching from IgM to IgG production is taken as representing/...

representing a differentiation step. Yet the actual secretion of antibody did not occur (Hammerling et al., 1973). IgM secreting cells are able to proliferate in the absence of T cell help, but IgG secreting cells were produced only following the addition of syngeneic T cells (Davie and Paul, 1974). The differentiation step from IgM to IgG surface immunoglobulin expression was shown to be thymus independent.

The use of the term differentiation

Differentiation is the irreversible commitment of a cell to a particular pattern of development. Cells which secrete IgG cannot revert to IgM production, and hence are fully differentiated. They become, after development of the cytoplasmic machinery for IgG manufacture, end cells:- plasma cells. On the other hand IgM producing cells may become IgM manufacturing plasma cells (and hence fully differentiated) or may switch to IgG production. The IgM carrying B cell, which is not secreting IgM or IgG may differentiate into an IgM secreting end cell, or into an IgG carrying (but not secreting) cell, or into an IgG producing fully differentiated plasma cell. Differentiation is said to occur at the point at which commitment of the cell occurs. This is independent of cell proliferation in B cell systems, and independent of antigen exposure in T cell systems (Cantor and Boyse, 1975a and 1975b). Whether or not a committed cell synthesises its product is dependent on "activation" "modulation" or "stimulation" and is usually preceded by mitotic division. It appears that the sequence of events in the B cell system involves 1) differentiation followed by division and 2) activation followed by synthesis of antibody.

Unlike/...

Unlike the T cell system, in which cellular differentiation is either autonomous or under thymic humoral control and is largely independent of antigen, the steps of B cell differentiation are not marked by the sequential appearance or loss of easily detected surface antigens. Differentiation steps can be inferred from the behaviour of the complement and Fc receptors on immature B cells as they mature into antibody producing cells (Ramasamay et al., 1974; Gelfand et al., 1974a; Gelfand et al., 1974b), or as they develop from lymphoid stem cells in spleen and lymph node explants (Metcalf et al., 1975). B lymphocytes stimulated to proliferation by mitogens appear to lose their receptor for complement at the blast cell stage, but retain their receptor for Fc IgG (Moller, 1974). In a study of immature B cells in the neonatal mouse it has been shown that they lack C3 receptors, but possess Fc receptors, and although they express surface Ig, fail to cap and hence shed this immunoglobulin after treatment with anti-Ig sera (Sidman and Unanue, 1975a). Further investigation showed that failure to clear anti-mouse Ig immune complexes from the membrane correlated with a failure of the immature B cell to restore its surface Ig following treatment with anti Ig sera. The mature B cell capped readily, and shed this material quickly, and equally quickly restored "clean" surface Ig. If surface Ig was removed by pronase, both mature and immature B cells resynthesised surface Ig at equal rates (Sidman and Unanue, 1975b).

Early in embryology and in their development from stem cells, B lymphocytes develop certain surface features which are retained throughout their life span. These include surface immunoglobulin (Sidman and Unanue, 1975a,b; Metcalf et al., 1975) and the species specific/...

specific antigens MBLA in the mouse (Raff & Cantor, 1971) HBLA in man (Greaves and Brown, 1973), together with alloantigens PC1 (Takahashi et al., 1971) B cell antigen (Yotoku et al., 1974) and Ly4 in the mouse (McKenzie and Plate, 1974) and HL-B alloantigens on B cells in man (Winchester et al., 1975a,b; Mann et al., 1975). Apart from the HL-B alloantigens in man which are also present on monocytes, these antigens are more or less specific for all B cells at whatever stage of their differentiation. Plasma cells are reported to possess antigens not expressed on the bulk of B cells (Yotoku et al., 1974).

It was believed, by analogy with the thymus gland, that these antigens were acquired by a process of induction from the epithelial tissues of the bursa in the case of B cells. As previously mentioned, there is some evidence that congenitally thymusless mice do possess small numbers of cells expressing theta (thy 1) antigen (Raff, 1973; Loo and Roelants, 1974) and the "precursor" or stem cells of T cell lineage are actually increased in numbers. Similar findings have now been reported in Bursectomised birds (Jankovic et al., 1975) in which IgM bearing cells of B lineage were found in numbers equivalent to those in sham Bursectomised birds, the only difference being in the density of surface IgM which was greatest in the sham Bursectomised controls.

These findings must cast doubt on the process of induction - which is held responsible for the appearance of class specific differentiation antigens on B and T cells, although as Boyse and Cantor point out the thymus is necessary for the induction of TLA and Ly antigens in the mouse. As these authors have shown, the surface/...

surface antigen characteristics of T helper and T cytotoxic cells are determined intrinsically before exposure to antigen occurs (Cantor and Boyse, 1975b).

Unlike the T cell system, the B cell system has not such well characterised surface antigens, although it is possible that Ir gene determined Ia antigens in the mouse (Hauptfeld, Klein and Klein, 1973), monkey (Dorf, Balner and Benacerraf, 1975) and man (Jones et al., 1975) may yet prove able to identify functional subclasses of B lymphocytes.

There is good evidence that the differentiation of the B lymphocyte from the immature, non-capping, and weakly responsive B cell to the rapidly capping, receptor rich and, finally, secreting B cell is not brought about by intrinsic regulatory mechanisms as proposed for the T lymphocyte. The B lymphocyte in its response to antigen shows that antigen is the inductive agent, bringing about subsequent differentiation as expressed by changes in reactive cells in recirculation (Strober, 1972) and by the fact that most in vitro proliferative responses to antigen involve proliferation of T cells, rather than B cells (Alm and Peterson, 1969). The fact that antigen alone induces a change from a sessile (fixed) pro-secretory B cell to a circulating B cell in the first case, and antigen alone fails to proliferate B cells in the second case illustrates that changes in B cell behaviour and function can be influenced directly by antigen but only in certain respects. Antigen alone can change the recirculatory patterns of B cells (a surface phenomenon) or can trigger a switch in the class of surface immunoglobulin secreted (Hammerling et al., 1973), but of itself fails to trigger either proliferation or secretion of antibody by B/...

B cells. Since in antigen responses B cell differentiation is not overtly accompanied by alterations in surface antigen expression, it is debateable whether changes triggered by antigen amount to a differentiation step. However, the subsequent behaviour of B cells reactive with that antigen is permanently altered, and since such alterations are by definition differentiation steps it can be concluded that

- a) B cell differentiation is antigen driven
- b) Antibody secretion is independent of the presence of antigen.

As has been shown, there is good evidence to support the theory that proliferation of B cells, suppression of B cells, and secretion of antibody in the secondary (IgG) response are all T cell dependent, although the differentiation of the B cell prior to engaging T cells is antigen driven and independent of proliferation.

Recent evidence in support of B cell differentiation by antigen has been shown by Zauderer and Askonas (1976). In lipopolysaccharide (LPS) stimulated spleen cell cultures precursors of IgM, IgG and IgA secreting cells can be produced by the mitogenic stimulus of LPS. These "secondary" secreting B cells differentiate over a 17 day period following LPS stimulation. In high density cultures inhibitory cells are also found. The LPS stimulation, and the development of secretory B cells is independent of T cells in the culture medium. Thus an antigenic and mitogenic stimulus in vitro is quite adequate to produce differentiation from IgM bearing cells of IgM secreting and IgG secreting cells. IgA secreting cells also are derived from surface IgM expressing cells. In/...

In this system proliferation could be inhibited without effect on the development of IgG secreting cells by 6-8 days; indeed owing to the inhibition of suppressor cells IgG levels were actually higher in BUdR treated than in untreated cultures.

TABLE 6

EXPRESSION OF DIFFERENTIATION ANTIGENS AND SURFACE RECEPTORS AS A
CORRELATE OF THE FUNCTIONAL STATE OF THE LYMPHOID CELL

<u>STATE</u>	<u>SURFACE CHARACTERISTICS</u>	<u>SPECIES</u>
Stem Cell	Unidentified Alloantigens No E Receptor No Complement Receptor No Thy 1 antigen Surface Ig negative	man man mouse mouse and man
Organ Specific Lymphocyte Populations	Organ Specific Antigens; Thymus, Spleen, GALT	Rabbit. No information mouse or man
Thymus Steroid Sensitive Population	Theta (thy 1) TL positive Ly positive HTLA positive E Receptor positive H2 negative	mouse mouse mouse man man mouse
Thymus Steroid Resistant Population	TL negative Thy 1 weak H-2 positive	mouse mouse
Spleen T cell	Theta weak (thy 1) H2 strong TL negative E Rosette positive HTLA positive	mouse mouse mouse man man
B lymphocytes	Surface Ig+ MBLA positive HBLA positive Express M Locus IAD'S, B antigen, SD & LD alloantigens/Express EB virus Receptors Complement Receptor Positive Express Ia antigens Strongly Express B2 Microglobulin	mouse and man mouse man mouse man man mouse and man mouse man
Immature B Cell	Express IgM Does not Cap with Anti-Ig antibody	mouse and man mouse
Immature B Cell/...		

<u>STATE</u>	<u>SURFACE CHARACTERISTICS</u>	<u>SPECIES</u>
Immature B Cell	Complement Receptor Negative	mouse
	Has abundant Fc Receptor Inhibited by Anti-Ig	mouse
Mature (committed B cell)	Expresses IgG Fc Receptor Expresses Complement Receptor Caps with Anti IgG serum Ly 4 antigen positive Cytoplasmic IgG	mouse mouse and man mouse and man mouse mouse
Secretory B cell (Plasma cell)	Expresses Pc1 B cell/Plasma cell (PC2) antigen Complement, Fc receptor negative Intracellular immunoglobulin	mouse mouse mouse and man mouse and man
PHA responsive cells	E rosette positive Thy 1 positive	man mouse
'K' cells	Fc Receptor positive Complement Receptor positive Surface Ig negative E Rosette negative	mouse mouse mouse and man man
T lymphocyte (activated)	Fc Receptors Express SD alloantigens	mouse and man
Inducible T cell helper in T cell mediated cytotoxicity	IgM Receptors	mouse
Helper cells (Antibody formation)	Ly1 positive) Ly2, 3 negative) Theta (thy 1) positive)	mouse
Immature T cell	IgM Receptors	mouse and man
Cytotoxic T cell	Steroid Resistant Ly2, 3 positive	mouse
Suppressor T cell	Thy 1 positive	mouse

TABLE 7B Cells and their Subclasses

<u>CLASS</u>	<u>FEATURES</u>
All B lymphocytes	Surface Ig+ MBLA positive Fc receptor positive Express receptor for C4, C3d. Express M Locus LAD's Express SD and LD alloantigens B antigen (Ia antigen) LNa (Ia antigen) EB virus receptors (man) B ₂ microglobulin (man)
Immature B lymphocyte	Surface IgM No capping with anti-IgM antibody Complement receptor negative Fc receptor positive Transformation inhibited with anti-Ig antisera
Mature B lymphocyte	Fc receptor positive C ₃ receptor positive Ly4 antigen positive Surface IgM or IgG May have secretory IgG
Plasma Cell (Secretory B lymphocyte)	PC ₁ antigen positive B cell/plasma cell Pc ₂ antigen positive Fc, Complement receptors weak or absent Intracellular Ig always unresponsive to mitogens

All B cells express SD and LD alloantigens, and Ia antigens strongly. Note expression of Fc and C receptors is variable, and the paucity of differentiation alloantigens on B cells compared with T cells. B cell differentiation is antigen dependent (antigen drive).

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CHAPTER 11

SUMMARY OF PHYSIOLOGY OF
LYMPHOID TISSUE: (CHAPTERS 4-10)

The physiology of the lymphocyte and the cells associated with it in lymphoid tissue has been reviewed in some detail, and the following conclusions have been drawn.

The lymphocyte, and the macrophage are derived from stem cells originating in bone marrow, and these stem cells are probably of different classes. The lymphocyte develops initially in either the thymic cortex or the bursal equivalent tissue of the mammal, and depending upon the inductive influences of thymic epithelium or bursal equivalent tissue develops as either a non-immunoglobulin secreting cell responsible for cell mediated immunity (the T lymphocyte) or as an antibody secreting precursor of the plasma cell (B lymphocyte). These two cell classes can be distinguished on the basis of their tissue localisation, their function, their surface antigenic structure, the expression of receptors for immunological intermediates, and the secretion of surface immunoglobulin. The receptors which interact with antigen on B and T lymphocytes are antigenically similar sharing idiotypic determinants that of the B lymphocyte is immunoglobulin, the nature of the T lymphocyte antigen receptor is unknown. From the classes of B and T lymphocyte it is possible to describe subpopulations of B and T cells which differ, in the case of B cells in their expression of surface and intracytoplasmic immunoglobulin, and in T cells in the expression of subsidiary surface antigenic structure. T lymphocytes of three classes exist, the immature T cell, the cytotoxic T cell and the helper T cell. B lymphocytes of several classes exist, as defined by surface antigenicity they are either pro-secretory B cells or plasma cells, by surface Ig expression they/...

they are pro-secretory B cells of IgG, IgM, IgD, IgA, IgE classes, or plasma cells of these classes, and by functional studies they are defined as precursor B lymphocytes (virgin B cells), activated B lymphocytes (responder B cells), or memory B lymphocytes.

Macrophages in tissue, and in blood develop from a precursor cell, the monocyte, which circulates in man. The precursor of this cell is the colony forming unit, which can differentiate along either granulocytic or monocytic pathways. As far as is known it does not differentiate into lymphocytes. Macrophages exist as several subclasses determined by their tissue localisation (blood, lung, liver, etc.) but really three different types are important, the colony forming unit (series stem cell) the tissue macrophage and the monocyte (the exudate macrophage precursor). The monocyte is probably one of the cytotoxic lymphoid cells (so called K cells) responsible for antibody mediated cytotoxicity.

Macrophages can interact with T lymphocytes and with B lymphocytes in both inductive and effector phases of the immune response. T lymphocytes can act as suppressors of B cell function or as helpers. In either role they determine the capacity of the committed B lymphocyte to secrete antibody, they do not prevent commitment of the B lymphocyte by antigen. Antigen retention in lymph nodes also involves a third cell type, the dendritic macrophage, which bears an uncertain relationship to the stromal reticulin fibre associated reticular cell.

The nature of such reactions is to produce an immune response, a permanent adaptive change in the reactivity of the animal concerned, to the antigen which evoked the change.

Lymphomas/...

Lymphomas are proliferations of lymphocytes, macrophages and possibly of cells derived from the stromal component of the lymph node (reticular tumours). It has been hypothesised that such tumours can be interpreted as AN ABNORMAL IMMUNE RESPONSE IN WHICH THE RESPONDING CELLS BECOME FIXED IN AN ATTITUDE OF PROLIFERATION BY FAILING TO TERMINATE THE RESPONSE IN THE NORMAL WAY.

In the next section, the facts discovered about the lymphocytes of man in health and in neoplastic disease will be surveyed.

CHAPTER 12

T LYMPHOCYTE, B LYMPHOCYTE

SURFACE CHARACTERISTICS IN DISEASE

T Lymphocyte, B Lymphocyte and Macrophage surface Characteristics
in Man.

The concept of a dual system of immunity, mediated by two separate classes of lymphocyte has received wide acceptance, and has stimulated the extension of investigations performed on experimental animals into the field of human physiology and pathology.

Initially studies of the function of the T lymphocyte in man were limited to the assessment of PHA response (a function of T lymphocytes)(Heilman, Gambrill and Leichner, 1973) or testing indirectly for T lymphocyte function by skin testing (Aisenberg, 1966) or the use of lymphokine assay in, for example, the migration inhibition test (Dumonde, 1975). Copying the mouse work, several attempts have been made to identify specific antigens, analogous to the theta (thy 1) antigen on mouse lymphocytes, by immunising rabbits or guinea pigs with human foetal thymus (Aisenberg et al., 1973)(Ishii et al., 1975) human brain tissue (Greaves and Brown, 1974)(Takada et al., 1974) or with T cells from patients with agammaglobulinaemia (Auiti and Wigzell, 1973). Tests of these sera, employing cytotoxicity, detect the T lymphoid population with a high degree of accuracy, but may fail to achieve the same specificity when employed in the immunofluorescence technique (Habeshaw - personal observations). Similarly some workers have found that antisera against T cells give anomalous results when compared with surface receptor evaluation, either in immunodeficiency states (Yata et al., 1973; Smith et al., 1974) or with lymphoid cell lines. The discovery of the spontaneous sheep cell rosette, and/...

and correlation of this finding with the presence of E receptors on thymocytes, gave an easily adaptable technique widely used for the quantitation of T lymphocytes in man. For example, Incefy, L'Esperence and Good (1975) and Bach (1973) have used the technique to investigate the role of thymic humoral factors in the functional maturation of the human T lymphocyte, and others have used the E rosette test to distinguish "active" rosetting, i.e. to demonstrate a subpopulation of strongly rosetting "activated" T lymphocytes (Wybran, Carr and Fudenberg, 1973). The E rosette test has largely replaced the functional PHA transformation studies in the evaluation and enumeration of T lymphocytes and has lately come to be thought of as an absolute marker of T lymphocytes. This has occurred despite the findings of considerable overlap between B and T lymphocyte markers when E rosettes are made following neuraminidase treatment, or other treatments which enhance E rosetting (Kaplan and Clark, 1974). It also ignores the potential confusion of "immune" B cell rosettes and E rosettes made by T cells, especially since at least one B lymphoid cell line, PEN2, can be shown to secrete IgM antibody with anti-sheep red cell activity (Steel, et al., 1974) and also contains substantial numbers of E rosetting cells (Habeshaw - personal observations). Such objections to the absolute equation of E rosetting cells = T lymphocytes are valid, and it is preferable not to call E rosetting cells T lymphocytes (Habeshaw and Young, 1975). It is also known that under some conditions both polymorphonuclear leukocytes (Hsu et al., 1973) and hepatocytes will form spontaneous sheep cell rosettes. Other easily identifiable surface characteristics are/...

are much less well documented, and depend more on the absence of other markers, such as surface Ig and C3 receptors, than on the presence of specific markers for T cells. The surface morphology of T cells by scanning electron microscopy was thought a specific feature, since the B lymphocyte is "hairy", while the T cell is smooth (Poliack et al., 1974) but this is now no longer thought to be a foolproof method of distinguishing B and T lymphocytes (Kay, 1975). Other features of the E rosetting phenomenon, not so well known, are the blocking of the E receptor by very high concentrations of non-immune IgG, IgA and IgM antibody globulin (at levels of 1 mg/ml), and the complete failure to inhibit E rosette formation with anti-human IgG, or IgM antisera (Blackie, 1973). This demonstrates that the E receptor is not an immunoglobulin molecule, yet can be competitively inhibited by very high concentrations of non-immune globulin. It is also possible to inhibit or mask the ligand on the sheep cell surface with which the lymphocyte receptor interacts. Sheep red cells coated either specifically or non-specifically (e.g. tanned RBC) with immunoglobulin in high concentrations will fail to form E rosettes with T cells, but will react with the Fc receptor on B lymphocytes and macrophages (Fraser, 1974). Similar findings have been reported with human thymocytes (Gatien et al., 1975). In addition to the formation of E rosettes, there is some evidence that a subpopulation of human T lymphocytes can form spontaneous rosettes with human red cells, particularly if treated with PHA or neuraminidase (Sheldon and Holborow, 1975)(Baxley et al., 1973)(Sandilands et al., 1975). These observations have been extended by Tak Yan Yu (1975) who showed/...

showed that human red cell rosettes were more resistant than SRBC rosettes to inhibition with proteolytic agents, and anti-thymocyte antiserum, but that the capacity for spontaneous rosette formation with human RBC was not regenerated after trypsinisation. Using stringent rosetting criteria, including the use of 5% BSA as a supporting medium, levels of human RBC rosetting comparable to E rosetting were achieved, and most E rosetting cells bound human red cells also. The E rosette and anti-human thymocyte antisera may detect different populations of cells, since E rosettes are consistently lower than the numbers of T cells detected by anti-thymocyte antisera. Thymic extracts appear to induce the formation of both the E rosette, and human T cell surface antigen. Protein synthesis by the cell is necessary for both E rosetting and antigen expression (Incefy, L'Esperence and Good, 1975). It has also been suggested that T cells in peripheral blood have less T cell antigen on their surface than thymocytes, a situation similar to that found in the mouse (Ishii et al., 1975).

In addition to the markers for the T lymphocyte already described, there is some evidence that additional receptors on the T cell for complement, for Fc IgG, and for IgM do occur. A small percentage (2-3%) of human T lymphocytes in peripheral blood do have receptors for both sheep red cells, and for complement coated chicken red cells (Chiao, Pantic and Good, 1974). This finding has also been reported for surface immunoglobulin carrying cells and E rosetting cells (Dickler, et al., 1974) and for E rosetting cells with Fc receptors. In a murine lymphoma, cells in culture showed/...

showed the presence of surface IgG, Fc receptor and reaction with anti-theta antiserum (Grey, Kubo and Cerottini, 1972).

In the mouse such mixed receptors are frequently found, in particular the Fc receptor, frequently described as being on "activated" T lymphocytes. The expression of these receptors has been extensively studied, in particular by Gyongyossy et al. (1975). Human T cells appear to express also a receptor for IgM and this receptor is not inhibited by free IgG, which distinguishes it from the Fc receptor (Moretta et al., 1975). Human T cells can be shown to have Fc receptors (Ferrarini et al., 1975).

In summary, the distinguishing surface markers for the human T cell are surface antigen, ability to rosette with sheep, or human erythrocytes under controlled conditions, and the occasional occurrence of Fc, C3 and IgM receptors among the T lymphoid population.

The surface characteristics of the human B lymphocyte are well documented; the B lymphocyte is defined as a lymphoid cell expressing surface immunoglobulin easily detectable by staining with immunofluorescence technique. This immunoglobulin is a cell product, and is not merely adsorbed to the cell surface. There is an enormous literature relating to this surface immunoglobulin, since it was one of the first class specific lymphocyte markers detected, and also one of the most easily studied. The original description of surface immunoglobulin on lymphocytes is attributed to Moller (1961) in a study of histocompatibility antigens on mouse lymphocytes. It was established by/...

by the work of Raff (1970), Pernis et al. (1971), Unanue et al. (1971), that cells expressing easily detectable surface Ig by direct immunofluorescence were B lymphocytes. In studies of surface Ig, it was shown that the light chains were more easily detected than the heavy chain determinants which tend to remain "buried" in the cell membrane (Froland and Natvig, 1972). This is important, particularly if good results are to be obtained with anti-heavy chain antisera these should preferably be directed against both the Fc and Fd portions of the immunoglobulin heavy chain (Seligmann, Preud'homme and Brouet, 1973). These authors also established two important technical variations in surface immunoglobulin determination, that of demonstrating two sets of Ig molecules on the lymphocyte surface, and secondly the demonstration of Ig synthesis by the lymphocyte following trypsinisation. When the lymphocyte is stained by fluorescent coupled anti-immunoglobulin in the cold, and then warmed to 37°C within 10 to 15 minutes most of the immunoglobulin has "capped" to one pole of the cell (Taylor et al., 1971). If the cells are first capped with a monospecific anti-heavy chain antiserum and then restained in the cold with a second antiserum, the presence of two different surface Ig molecules can be confirmed (Preud'homme et al., 1972). This "co-capping" technique has also been used to describe the relationship between B₂ microglobulin and HLA antigen. The numbers (as percentage) of surface Ig bearing cells in peripheral blood are well documented, and range from 12%-23% (Seligmann, Preud'homme and Brouet, 1973) to between 10 and 16% (Murphy, 1975) of purified (monocyte depleted) lymphocytes. These figures are lower/...

lower than those of Habeshaw and Young (1975) of $0.5 \times 10^6/\text{ml}$ (500 cu mm), but in the latter case no loss of B cells was allowed and the technique probably slightly overestimates B cell numbers in whole blood. The more recent data for unseparated blood (Greaves and Brown, 1974) and for whole blood corrected for cell loss (Holm et al., 1975) are all compatible with these findings. In their paper, Habeshaw and Young drew attention to the fact that a small population ($0.3 \times 10^6/\text{ml}$) of peripheral blood cells undoubtedly stained for surface Ig, but failed to cap, even when treated under conditions which capped a very high proportion of peripheral blood B cells. At the time the nature of this cell was unclear, although it was thought unlikely to be a monocyte or macrophage. Subsequent published work from studies of the mouse B lymphocyte (Sidman and Unanue, 1975) identify this fluorescent non-capping cell as being an immature B lymphocyte.

Apart from this, other studies have indicated a clear "maturation" of peripheral blood lymphocytes on the basis of expressed surface immunoglobulin. Murphy (1975) showed that in the first two decades of life, cells expressing IgM surface Ig were much more numerous than those with IgG. After the second decade IgG expressing lymphocytes become more common than IgM lymphocytes. Earlier work on the lymphoid cell antigen receptor immunoglobulin (Pernis, Forni and Amante, 1971) clearly showed that cells secreting IgG antibody can express on their surfaces IgM antibody of the same light chain type, and the same allotypic specificity as the secreted IgG. Seligmann, Preud'homme and Brouet (1973) showed that in multiple myeloma they could detect on/...

on the cell surface both kappa and lambda light chain determinants, and sometimes more than one class of heavy chain, and similar findings have been described in CLL by Papamichael et al. (1971) and Piessens et al. (1973). The explanation of these findings is still not clear, indeed Piessens suggests that CLL may be a disease characterised by a polyclonal increase of B lymphocytes, not a monoclonal expansion of these cells. The most commonly accepted explanation of the finding in normal blood, of two classes of heavy chain (usually IgM, IgG but sometimes IgD, IgM) on the lymphocyte surface, with a common single light chain determinant is that of "immunoglobulin switching" as a consequence of cell maturation (Froland and Natvig, 1973) (Rowe et al., 1973). In cases where different classes of light chain occur together on the same cell, it is probable that spurious interaction of Ig aggregates with an Fc receptor may have occurred, or that the cell concerned is secreting a rheumatoid factor with anti-IgG specificity. In the latter case, co-capping experiments will show the IgG class to co-cap with an anti IgM antiserum (Seligmann, Preud'homme and Brouet, 1973). An additional possibility, encountered in some connective tissue diseases such as SLE, is that anti-lymphocyte (anti-leukocyte) globulin is actually present, and capable of inducing a spurious staining reaction (Bentwich and Kunkel, 1973).

Chronic Lymphocytic Leukaemia

The most widely studied phenomena in human lymphoid neoplasia/...

neoplasia have been the synthesis, distribution, and class of immunoglobulin on the surface of lymphocytes in CLL (Aisenberg and Bloch, 1972; Aisenberg, Bloch and Long, 1973; Grey, Rabellino and Pirofsky, 1971; Piessens et al., 1973; Preud'homme and Seligmann, 1972; Flad et al., 1973; Moroz, Shalmon and Hahn, 1973; Murphy, 1975). In the series of Grey, Rabellino and Pirofsky, most CLL cells expressed IgM, and a smaller number membrane associated light chains with no detectable heavy chain, suggesting that CLL was a monoclonal expansion of B lymphocytes. Aisenberg, Bloch and Long showed that both IgM and IgG could be demonstrated on the surface of some individual lymphocytes in CLL, and found two cases of CLL with no surface immunoglobulin present upon the neoplastic cells. A tendency for cells to become surface Ig negative with either chemotherapy or disease relapse was noted. As previously remarked, Piessens group holds the view that a proportion of cases of CLL are of lymphocytes showing a polyclonal immunoglobulin coat, and suggesting that at least a proportion of CLL cases are of T rather than B lymphocyte origin. Recent data from Seligmann's group bases on immunoglobulin resynthesis following trypsinisation suggests that this view might be correct (Brouet et al., 1975). Murphy (1975) in common with Preud'homme and Seligmann (1972) classifies most CLL as B cell tumours with monoclonal expression of IgM. Some cases express surface IgG, and occasional cases exhibit a mixed IgG/IgM pattern. Flad et al. (1973) find that in CLL there is good evidence of two abnormalities of behaviour of the CLL cell, firstly a large proportion of CLL cells fail to cap their surface Ig/...

Ig, and secondly the lymphocytes of CLL fail to recirculate in a normal fashion. An additional abnormality expressed by the membrane of the CLL lymphocytes is the susceptibility of the Ig synthetic mechanism to blocking with anti-Ig antiserum (Munoz, Shalmon and Hahn, 1973), recalling the mouse experiments of Sidman and Unanue (1975) who showed that this is a feature of the immature B lymphocyte. A similar kind of abnormality is the observation that in alpha chain disease, despite the presence of intracytoplasmic immunoglobulin there was no surface Ig, leading to the conclusion that in the absence of a light chain, there is loss of the ability to express surface Ig (Seligmann, Preud'homme and Brouet, 1973). Such features of the CLL cell compare with those described for the cell of immunodeficiency disease patients, where three classes of abnormality can be described (Wu, Lawton and Cooper, 1973); a failure of the B lymphocyte to develop the capacity to secrete Ig at all (which corresponds to the loss of surface Ig expression in CLL), the arrest of B lymphocyte development, (with failure to secrete intracytoplasmic Ig) and disordered maturation of the B lymphocyte with secretion of defective Ig or with defective Ig secretion.

In one case of CLL Bentwich and Kunkel (1973) found that the neoplastic cells bound aggregates of immunoglobulin but failed to show surface immunoglobulin. On culture, the reappearance of surface Ig was noted, and it was found that a factor in the patient's serum necessary to cause synthesis of surface Ig was lacking in this case.

In a study of 10 patients with CLL, Wilson and Hurdle (1973)/...

(1973) found 8 to be B cell in type, with 2 cases showing T cell markers. In this paper assessment of surface immunoglobulin was made by a sensitive radiolabelling assay of immunoglobulin synthesis by the neoplastic cells. In a similar study of one patient with CLL, MOROZ, Shalmon and Hahn (1973) showed that surface Ig on the CLL cells was IgM in type, and free light chain was synthesised. The synthesis of both u chain and of light chain could be blocked with anti u and anti kappa or anti lambda antisera (antigenic modulation) a feature thought to represent an immature form of B lymphocyte (compare the case with that described by Bentwich and Kunkel, 1973).

In the cases described by Mellstedt and Pettersson (1974) it was noted that the Ig bearing cells lacked C3 receptors in most cases, but C3 receptor bearing cells were active in cell mediated cytotoxicity. In most cases of CLL, the circulating cells show the presence of surface Ig detectable by immunofluorescence, and these cells frequently also express complement receptors (Ross et al., 1973) and receptor for aggregated immunoglobulin, or the Fc receptor (Ferrarini et al., 1975b). In some instances surface Ig may not be present, as in the case described in a patient with thymoma (Siegal et al., 1973) but Fc receptors may in such cases be present. Serum factors may suppress the expression of surface Ig, not only in CLL but also in immune deficiency states (Cooper, Lawton and Bockman, 1971; Wernet et al., 1973). The nature of the suppressive factor is unknown, but it is noteworthy that B cells left in contact with anti immunoglobulin antisera fail to synthesise Ig. When well/...

well washed, the bulk of B cells shed the surface bound immune complex and can recommence Ig synthesis (Loor, Forni and Pernis, 1972). Pre-incubation of cells from lymphoid cell lines in acetate buffer converted a high percentage from a non synthetic resting state to an actively synthesising state. Most of 10 cell lines expressed Kappa chain and u chain determinants, but two synthesised only light chain (Hutterworth, Litwin and Cleve, 1972). Similar findings of mainly u chain and Kappa chain synthesis by neoplastic cells in a variety of lymphoproliferative disorders were noted by Piessens et al. (1973), and by Grey, Rabellino and Pirofsky (1971).

Not all chronic lymphocytic leukaemias are of B cell origin, as previously remarked there are accounts of T cell leukaemias. In the case described by Wetter, Reis and Weert, (1973) plasma cells in peripheral blood showed E rosetting, and failed to transform when treated with anti-Ig antisera. These cells also failed to transform when exposed to pokeweed mitogen, but expressed surface Kappa chain and had the electron microscopic appearances of plasma cells. The most complete account of T cell chronic lymphocytic leukaemia (Brouet et al., 1975) showed a clear correlation of certain clinical features with the presence of a population of E rosetting cells in peripheral blood. The clinical features were those of splenomegaly, skin lesions, and neutropenia. Cells from the bone marrow (2 patients) and in the skin nodules (1 patient) rosetted with sheep erythrocytes and stained with anti T antisera prepared from brain (2 cases) and from the circulating T cells of/...

of a child with Bruton-type agammaglobulinaemia. These authors suggest that T cell CLL may be more common than supposed, since spurious B cell appearances can be given by in vivo coating of T cells with immunoglobulin and since in the absence of E rosetting the diagnosis of a T cell lymphoma required an absence of surface Ig staining and negativity of B cell markers, such as Fc or EAC receptors on the lymphoma cells. Further details of T cell CLL were given by an account of a female patient with lymphadenopathy and erythrodermia, with a high proportion of E rosetting cells in the peripheral blood and negative staining of leukaemic cells for surface Ig (Sumiya et al., 1973).

The nature of the T cell in chronic lymphocytic leukaemia is not entirely settled, since at least one section of the available literature considers the population to be reactive rather than neoplastic: for example in Infectious Mononucleosis, where it is well established that the causative EB virus infects the B lymphocyte, the morphologically abnormal infectious mononucleosis cell shows the surface features of a T lymphocyte (Sheldon et al., 1973). Circulating B lymphocytes are apparently present in normal numbers in this condition. Among those convinced that a morphological T cell equivalent of the plasma cell exists - as could be presumed from some descriptions of "T cell lymphoma" - are Lennert, Kaiserling and Muller - Hemerlink (1975) who describe by Light and EM morphology a cell equivalent to the plasma cell occurring associated with T lymphoblast in the paracortex of lymph nodes. This cell is stated not to form E rosettes. Whether or not the T lymphocyte is/...

is implicated in the chronic lymphocytic leukaemias, there are several theories about the role of the E rosetting cells found in some CLL peripheral blood samples. In a study of chronic lymphocytic leukaemia, Wybran, Chantler and Fudenberg (1973) isolated normal T cells by applying the criteria of E rosetting, negative staining for surface immunoglobulin, and responsiveness to PHA. These authors consider that the depressed PHA responsiveness of peripheral lymphocytes in chronic lymphocytic leukaemia is not due to any intrinsic defect of the T lymphocyte, but rather that it is due to "dilution" of normal responsive T cells by the neoplastic peripheral blood population. In other accounts, however, there is evidence presented which shows an apparent change in the neoplastic population from a predominantly B lymphocyte pattern, to a T lymphocyte pattern for example during a psuedomonas infection in the patient described (Nowell et al., 1975). The data relevant to the behaviour of CLL cells in vitro when stimulated with mitogenic substances is reviewed by Perera and Pegrum (1975). An interesting conclusion, drawn from over fifty reports in the literature, concerns the fact that the CLL lymphocyte will continue to proliferate in vitro by virtue of some surface property which can be eliminated by prior trypsinisation. The other surface abnormalities which CLL lymphocytes readily show are the rosetting with mouse erythrocytes (Stathopoulos and Elliott, 1974) and a failure to respond as do normal B lymphocytes when incubated with anti-immunoglobulin antisera (Daguillard, Fontaine and Tardieu, 1974). Refractoriness to stimulation by anti-immunoglobulin antisera is not generally found to extend to agents such as pokeweed mitogen/...

mitogen or phytohaemagglutinin (Schweitzer, Melief and Eijsvogel, 1973). The CLL cells do, however, fail to either stimulate or respond to stimulation in the mixed lymphocyte reaction. The proliferative response to phytohaemagglutinin could certainly be due to contamination of the cell cultures with small numbers of normal blood lymphocytes, particularly T cells. This is certainly the opinion of Cohnen, Konig, Augener, Brittinger and Douglas (1973) who found in normal blood plasma cells caused by stimulation of B lymphocytes by pokeweed mitogen. These were considerably less frequent in CLL, showing the relative unresponsiveness of CLL lymphocytes.

The effect of treatment on CLL has been considered by several authors. Smith and Ezdinli (1974) showed that in the normal course of the disease the numbers of B cells, with complement receptors rose, and the numbers of T lymphocytes (E rosetting cells) fell. If a single course of irradiation was given to the spleen, the numbers of circulating B cells rapidly fell, suggesting that a selective trapping of neoplastic B lymphocytes (expressing monoclonal immunoglobulin) was operating, and that the beneficial effects of splenic irradiation could be due to enhancement of this trapping mechanism (Astaldi et al., 1975).

Lymphoid Cell Lines

Lymphoid cell lines have been the chosen objects of study by many investigators, and there are some points of similarity between the findings here, and in some lymphomas. For example lymphoma/...

lymphoma cell lines bearing both B and T lymphocyte surface markers have been described in the mouse (AKR strain) (Greenberg and Zatz, 1975) although on prolonged culture the cells appeared to revert to a B cell clone. This has also been observed with the cell line PEN(2) (Habeshaw - personal observations) but in this case much of the E rosetting activity shown by these immunoglobulin secreting cells may be due to the production of antibody against the sheep erythrocyte (Steel et al., 1974).

In normal blood, cells bearing both B and T surface markers have been described (Dickler, Adkinson and Terry, 1974) and dual receptor neoplasms of similar type occur in man (Habeshaw and Stuart, 1975; Murphy, 1975; Haegert et al., 1974).

In one account of a T lymphoid cell line from man (Huang et al., 1974) cultures of peripheral blood lymphocytes showed that initially they possessed either T cell, or a mixture of B and T cell markers. Subsequently, 5 lines lost their T cell characteristics, and showed reversion from an abnormal (psuedodiploid) mode of Karyotype to the normal diploid mode. However, one cell line retained its psuedodiploid mode, and retained its T lymphoid characteristics over an extended (14 month) period in culture. Minowada, Ohnuma and Moore (1972) established their criteria for evaluation of lymphoid cell lines on the basis of rosette formation, and it was later shown (Kaplan, Shope and Peterson, 1974) that T lymphoid cell lines in man were all derived from children with leukaemia secondary to lymphosarcoma, and none contained the EB virus genome which is ubiquitous in B lymphoid cell lines. Hutteroth, Litvin and Cleve (1972) showed that at this/...

this date B lymphoid cell lines, whether from normal or neoplastic tissue, all contained the EB virus genome.

A recent, and intriguing, observation of lymphoid cell lines, by Jondal, Klein and Yefenof (1975) shows that virtually all normal human peripheral blood T lymphocytes will readily form rosettes between themselves and EB virus carrying B lymphoid cell lines. Blast transformed T cells never showed this phenomenon, but normal blast transformed B cells could rosette normal T cells, but rather weakly. The T cell receptor for the lymphoid cell lines was not blocked by sheep red cell membranes, and was not therefore the E receptor, was not blocked by staphylococcal protein A - which reacts with IgG present on the B lymphocytes, and was not blocked by IgG, anti-immunoglobulin antisera, anti-B₂ microglobulin antisera or anti T cell antiserum. These observations are relevant and may be crucial to the phenomena later described in Hogkin's Disease.

Macrophage cell lines have been described only once by Ralph and Nakoinz (1975) who showed a capacity of the cultured cells to phagocytose, and to be cytolytic in an antibody mediated cytotoxicity reaction.

Acute Leukaemias

In cases of leukaemia of acute lymphoblastic type most of the cell types found are receptor silent (18 out of 22). Four cases were found to have T surface markers. In chronic lymphocytic leukaemia, only 1 out of 11 cases was receptor silent, one case showed T cell markers and the other nine were of B lymphocytes/....

lymphocytes (Brown et al., 1974). An antiserum prepared against human thymus gland and adsorbed with cells from B lymphoid cell lines reacted with both normal T lymphocytes and with acute lymphocytic leukaemia cells (Smith et al., 1973) implying an origin from T lymphocytes even in the absence of other T surface markers (E rosettes).

A novel clinical syndrome in acute lymphocytic leukaemia has been described (Ritter et al., 1975) in which three patients showed both acid phosphatase positivity and E rosette formation. This interesting observation may need caution in interpretation since cells of the granulocytic series have on occasions been found to exhibit E rosetting (Hsu et al., 1974). Acute leukaemias of macrophages (monoblastic leukaemia) have been described which are positive cytochemically for peroxidase and strongly positive for non specific esterase. The syndrome is characterised by a high incidence of spontaneous disseminated intravascular coagulation (DCC) and response with heparin and Daunorubicin was obtained. (McKenna et al., 1975). The studies of Borella, Sen and Green (1974) showed that acute lymphocytic leukaemia of cells having convoluted nuclei were E rosette positive in three cases of acute lymphoblastic leukaemia and in one case of childhood lymphosarcoma. These authors conclude that there are two types of ALL in children, one of undifferentiated stem cells, and the other showing the features of lymphoblasts with cell markers of normal thymocytes, and extensive disease at presentation with mediastinal enlargement. Other authors report cases of acute lymphoblastic leukaemia in which/...

which the lymphoblasts bound sheep cells, or reacted with anti thymocyte antisera. These lymphoblasts were negative when examined for surface immunoglobulin by immunofluorescence (Kersey et al., 1973; 1974). There is a recent publication describing specific acute leukaemia associated antigens which are disease rather than cell class specific (Baker, Ramachandar and Taub, 1975). These can be confused with membrane antigens specific for human lymphoid cells in the dividing phase (Thomas and Phillips, 1973; Thomas, 1974).

A recent report by Chechik and Gelfand (1976) shows that the antigens common to thymocytes, and found in acute lymphoblastic leukaemia of T cell type, (called H Thy-L, Human Thymus Leukaemia antigen) can also be found in acute myelogenous leukaemia - in one case. Patients with E rosette negative acute myelogenous leukaemia (7 cases) failed to show the presence of H Thy-L antigen.

Solid Lymphomas

Eleven non-Hodgkin lymphomas were studied by Peter et al. (1974). These included 4 diffuse lymphocytic lymphomas, 4 nodular lymphocytic lymphomas, 2 histiocytic lymphomas and 1 epithelial thymoma. These authors judged 6 of the lymphomas to be B cell in type, 2 were T cell, 1 was a "null cell" lymphoma and 2 were of indeterminate status. One small cleaved and one small non-cleaved follicular centre cell lymphoma were judged to be of T cell type, with 72, and 77 per cent of the cells bearing T markers. Of the others, 40-55% of cells bore IgM antibody/...

antibody, 1-90% bore IgG antibody, 16-84% had kappa chain, and 4-98% had lambda chain immunoglobulins. Of the B cell lymphomas, 3 out of 4 studied had receptors for aggregated immunoglobulin (Fc receptors). Seven normal lymph nodes did not contain more than 50% of T cells or more than 50% of B cells of a single immunoglobulin class, these being the criteria for diagnosis of lymphoma. The receptor silent tumours were two; one was a diffuse lymphoma of poor differentiation, and the other an epithelial tumour of the thymus gland. Of particular interest was the discovery that only 2 IgM bearing lymphomas were monoclonal. One tumour had two classes of heavy chain (μ and γ) and two classes of light chain (Kappa and Lambda). One had two light chains (Kappa and Lambda) and one heavy chain. In two patients Ig secreting cells clearly had no Fc receptor.

One problem in classifying lymphomas by markers presumed specific for T and B cells lies in the possibility of a single class of neoplastic cell expressing both B and T cell markers. Such a case has been reported by Sandilands et al. (1974), where pancytopenia and hepatomegaly with abnormal dual receptor cells in the peripheral blood was followed by a leukaemic blood picture and death from hepatic failure. Cells with dual receptors also occur in small numbers in normal peripheral blood (Dickler, Adkinson and Terry, 1974).

In a limited number of cases reported by Murphy (1975) mixed receptors for T cells (E) and EAC figure prominently. In three cases of thymoma examined, two cases of lymphosarcoma with leukaemia and one case of chronic lymphocytic leukaemia mixed E and/...

and EAC receptors were found on four occasions. The single patient with Sezary Cell syndrome had normal blood differential, and it is remarked that in most patients with solid lymphoid tumours no monoclonal Ig secreting lymphoid population is detected in the blood.

A much broader selection of patients has been studied by the group comprising, Green, Jaffe, Shevach, Edelson, Frank and Berard (1975) who adhere to the conventional but restricted frozen section technique for demonstrating the localisation of areas of lymphoid tissue binding receptor particles, developed by Silviera, Mendes and Tonali (1972).

Jaffe et al. (1975) showed that in frozen sections of control lymph nodes, which are reacted with complement or antibody coated red cells, the follicular lymphocytes bound C3 coated red cells, but did not bind antibody coated red cells. Macrophages in the sinusoids bind both EAC and IgG coated red cells. This data indicates that in the normal lymph node follicle EAC receptors are present, but Fc receptors are absent from the follicular B lymphocyte. In their studies of six patients with nodular lymphoma the neoplastic cells expressed receptors for complement, but not for Fc portion of IgG, and were presumed to be B lymphoid in origin. In 2 cases of reticuloendotheliosis, and one case of histiocytic lymphoma, the tumour cells bound IgG-EA (i.e., had Fc receptors) but failed to express complement receptors, and were classified as neoplastic histiocytes. In three cases of diffuse lymphocytic lymphoma, only sheep erythrocyte binding cells were found, and these neoplasms/...

neoplasms were classified as T cell lymphomas.

Brouet, Labaume and Seligmann (1975) report that in 25 cases of lymphoma, especially well differentiated lymphocytic lymphoma, and acute lymphosarcoma cell leukaemia, the neoplastic cells were predominantly of B cell type. Most poorly differentiated lymphocytic lymphomas were also monoclonal proliferations of B lymphocytes. These authors did however report that in 4 cases of histiocytic lymphoma, the cells lacked receptors and other surface characteristics of either B or T cell type, and were receptor silent tumours. Not all B lymphoid malignancies expressed monoclonal surface Ig, in at least one case the surface immunoglobulin was polyclonal. In the one nodular lymphoma examined 40% of E rosetting lymphocytes were also present in addition to the neoplastic B lymphocytes, which expressed μ chain and Kappa chain (i.e. were IgM bearing cells). In a further case the malignant cells expressed both B and T cell surface markers.

Hansen et al. (1974) evaluated lymphocytic subclasses and lymphocyte function in twenty eight patients with lymphoma, 10 with Hodgkin's Disease and 18 with non-Hodgkin lymphoma. Most (80%) of Hodgkin's Disease patients showed impaired cell mediated immunity, or defective leukocyte response to PHA, and there were indications of changes in the ratio of B and T lymphocytes. In non-Hodgkin lymphoma, six patients showed monoclonal expansion of the peripheral blood B cell population. In one patient with a pleural effusion, the abnormal circulating B cells did not appear in the pleural effusion. This paper concentrates/...

concentrates exclusively on the peripheral blood mononuclears and does not report on the cellularity of the lymphoid neoplasm itself. The monoclonal B lymphocytes expressed IgM with Kappa chain.

In a similar paper, Smith et al. (1974) report on the susceptibility of abnormal circulating lymphocytes to anti-thymocyte (HTLA) and anti-bone marrow lymphocyte (HBLA) antisera directed against the HTLA and HBLA surface antigens. Many inconsistencies were noted when these antisera were used to classify lymphoma cells, or cells from patients with primary immunodeficiencies. T cells in Wiscott-Aldrich syndrome, in acute lymphocytic leukaemia, and T cell lymphocytic lymphoma, failed to form E rosettes but were classifiable as T cells on the basis of their reaction with anti-HTLA sera. In Burkitt's lymphoma, B lymphocyte lymphoma and some B cell lines, there was a spurious elevation of E rosetting cells which reacted with anti-HBLA antisera. This implies that in B lymphocyte lymphomas, especially those carrying EB virus, E rosetting by B lymphocytes can occur.

In a limited series, studied by methods similar to those used by Shevach et al. (1975) and Green et al. (1975) Dorfmann (1975) finds that the nodules of nodular lymphomas will bind EAC, but do not bind EA reagents - in other words they are B cells with C3 but not Fc receptors. He also observed that in no case of histiocytic lymphoma were receptors expressed for either Fc or C3 on the lymphoma cells. He concludes that nodular lymphomas are tumours of B lymphocytes, while the histiocytic lymphomas are/...

are of debatable origin.

Green, and co-workers (1975), using adherence of antibody or complement coated red cells to frozen sections of lymph node or spleen show that preferential binding by complement receptors occurs in the follicles of spleen and lymph node. The preparation used for the detection of the Fc receptor does not apparently bind to the B lymphocyte, but will bind with the tissue histiocyte. Histiocytes around the splenic follicle, in the marginal sinus, bound complement coated red cells, but those in the cords of Bilbroth did not. In their cases they find that "histiocytic medullary reticulosis" showed a predominance of histiocytes in the single spleen examined. and in "hairy cell" leukaemia they conclude that the neoplastic cells in two cases were also histiocytic. Nodular lymphocytic lymphomata (six cases) were tumours of B lymphocytes. In three cases of diffuse lymphocytic lymphoma the tumours were T cell in type. In four cases of chronic leukaemia and erythrodermia, three of whom had the Sezary syndrome, and three patients with mycosis fungoides, most showed elevations of T lymphocytes. In one case mixed E and EAC rosettes were detected in blood. In tissue sections the bulk of the cells detected were found to be T cell in type. There is some indication that the small lymphocytic form of Sezary Syndrome might be confused with CLL. The T cell "solid lymphomas" are of interest, particularly since the case described by Smith et al. (1973) of a malignant thymic tumour in a boy, associated with a lymphosarcoma cell leukaemia, in which both the circulating and the tumour cells expressed E receptors (were T lymphocytes).
The/...

The two varieties (small cell and large cell) of Sezary syndrome were described by Lutzner, Edelson and Smith (1973) who showed that circulating neoplastic cells of T lymphocytic class were a feature of both diseases. Additional cases were described by Broome et al. (1973) with confirmatory evidence of T cell origin of the Sezary cell, and by Kalden et al. (1974), who showed that the neoplastic cells failed to respond to stimulation with PHA.

The "Leukaemic Reticuloendotheliosis" cell has attracted attention; with Green et al., describing it as histiocytic. On the other hand Stein and Keiserling (1974) clearly show this interesting cell to have the properties of a B lymphocyte, in particular it is peroxidase and esterase negative, non glass adherent and non phagocytic. The surface Ig expressed is IgM in type. This view was supported by studies of an additional five cases of leukaemic reticuloendotheliosis (Catovsky et al., 1974).

More recent accounts of receptors in solid lymphoid tumours attempt some degree of classification of the lesions by the surface characteristics of the constituent cells. In the paper of Cooper et al. (1975) four groups of lesions are identified, six patients had B cell tumours with strong surface Ig expression, eight patients had CLL with weak expression of surface Ig, five patients had lymphomas composed predominantly of T cells, and three patients had unclassifiable lesions. In no case of T cell lymphoma were there circulating neoplastic cells, and in histology these lesions were poorly differentiated according to the/...

the Rappaport classification.

Gajl-Peczalska, Bloomfield and Sosin (1975) in their series showed that 20% of nodular lymphocytic lymphoma patients, and 17% of diffuse lymphoma patients had abnormal B cells in the peripheral blood at diagnosis. The lymph nodes in their series of nodular lesions showed 62% to be of B cell origin, and all of their poorly differentiated diffuse lymphomas were of B cell type. The patients with abnormal circulating cells of B or T type had a worse prognosis than the others. In an earlier paper in which the peripheral blood only was examined in 37 patients, abnormal B cells identified by monoclonal surface Ig were found in about 20% of cases (Gajl-Peczalska et al., 1973).

In a study of seven cases of diffuse histiocytic malignant lymphoma Habeshaw and Stuart (1975) showed that only in one case were the cells of histiocytic (macrophagic) derivation. One case showed a definite overlap of E and EAC receptors, and one a possible overlap. One case was a "receptor silent" tumour showing an absence of all surface markers. The other cases were of B cell origin. In a further paper (Stuart and Habeshaw, 1976) in which nineteen cases of diffuse and nodular lymphocytic lymphomas were examined, it was shown that most lesions were of B lymphocyte class. One diffuse lymphocytic lymphoma was of T lymphocyte type, and one was receptor silent. The nodular lymphomas all exhibited some T cells as well as B cells, and in one case the nodule cells showed overlap between E rosetting and surface immunoglobulin positive cells. The surface Ig positive cells expressed some C3 receptors, but failed to express receptors for Fc IgG.

Burkitt's/...

Burkitt's lymphomas have also been examined, and are found to be B cell in type. Jondal, Svedmyr, Klein and Singh (1975) showed that in Burkitt's lesions about 1% of T cells contaminated the predominantly B lymphoid populations. When EAC rosetting cells (B cells) were removed the residual T lymphocytes exhibited cytotoxicity towards EB virus carrying Burkitt cell lines. The Burkitt B cell failed to kill a normal cell line, K-562, in the presence of antibody, while normal B cells could kill this cell line. T cells from patients with infectious mononucleosis were also weakly cytotoxic for EB virus carrying cell lines. The clonal origin of the Burkitt cell was neatly demonstrated by making use of the G-6-PD isoenzyme pattern, by Fialkow, Klein, Gartler and Clifford (1970). The tumours in one patient expressed only one of the two isoenzymes present in the normal tissue of that patient.

Some work on the surface characteristics of animal lymphomas has reached the literature. In an analysis of the gonadal tumours in Marek's disease in the fowl, Hudson and Payne (1973) found most to be T cell in type. In the blood of chickens with T cell gonadal tumours, the circulating T cell population was reduced, while the B cell numbers showed an absolute, not relative, increase. In some cases a mixture of both B and T cells was found in the tumours, and in these cases the proportions of circulating T cells were increased. These studies made use of anti-T and anti-B antisera.

In Marek's disease, the T cells from gonadal tumours form T lymphoblast cell lines in tissue culture (Powell et al., 1974).

In Bovine lymphosarcoma it has been demonstrated that the neoplastic cells are B cell, with surface expression of gamma or mu heavy/...

heavy chain. Thymic cells from these animals failed to bind antibodies to surface Ig determinants. It was noted, however, that tumour cells expressed gamma heavy chain while blood lymphosarcoma cells expressed mu heavy chain. The neoplastic cells were also found to bind IgM non specifically, and retained it after multiple washes. (Tsuyuguchi, Ming-Ming and Karusch, 1973). The similarity of the bovine lesion to human lymphosarcoma is obvious, but the lesion of Marek's disease has no obvious counterpart in man.

Receptor studies are of some value in examining tumours other than those of the lymphoid system, and recent investigations have explored the role of macrophage content in experimental tumours of mice, and its relationship to prognosis (Eccles and Alexander, 1974). In this paper the macrophage content of the lesions is related to the T lymphocyte, since removal of T lymphocytes resulted in a decrease in the macrophage content of the tumour. BCG, which has some suppressive effect in this system raised macrophage numbers within tumours. There is also a connection between immunodeficiency states, and the incidence of lymphomas (Gatti and Good, 1971) and it is now established that the incidence, particularly of poorly differentiated lymphomas is much higher (possibly by a factor of 7) in immunodeficient as against normal persons. The reason for this may be failure of immune surveillance, which could account for the increased incidence of many kinds of tumour in immunosuppressed patients (Gershwin and Steinberg, 1973), lymphomata in particular. Since suppressor cells regulate lymphoid hyperplasia, loss of the suppressor T cell function could lead to continual stimulation of antigen reactive/...

reactive cells - with the consequence of unrestricted and hence neoplastic proliferation. Another exciting possibility lies in the identification of blocking factors which prevent lymphocyte differentiation, such as that demonstrated in a patient with thymoma (Seigel et al., 1973) or that present in thoracic duct lymph in a patient with myasthenia gravis (Hammerstrom et al., 1975).

Another factor which may operate in the biogenesis of lymphomas is that of macrophage stimulation of the growth of lymphoma cells. Nathan and Terry (1975) have clearly shown that normal mouse macrophages profoundly stimulate the growth of experimental murine tumours, especially lymphomas. BCG infected macrophages appear to stimulate much less than normal macrophages. This observation is rendered more important by demonstrating that other factors, e.g. 2 mercaptoethanol, which dissociate surface immunoglobulin also have a stimulatory effect on murine lymphomas. These observations illustrate that blocking factors, and macrophage content of human lymphomas may be crucial factors in predicting their biological behaviour.

Hodgkin's Disease

Hodgkin's Disease is usually regarded as an entity quite distinct from the other classes of lymphomatous disease. The epidemiology of the condition contrasts with that of other lymphomas in terms of bimodal age and sex distribution, and in seasonal incidence (Levine, 1974; Smithers, 1974; Kaplan, 1974). Some (Levine, 1974) argue strongly that it is of infective (virus) aetiology/...

aetiology, like infectious mononucleosis or Marek's disease in the chicken, while others (Kaplan) regard it as a true neoplasm of lymphoid tissue. The latter view is to some extent supported by the finding of monoclonal B cell expansion in the blood of some cases of Hodgkin's Disease (Hansen et al., 1974) but the absolute numbers of B cells are usually normal or low (Faletta et al., 1973). After treatment, the levels of B cells rise, and the levels of B lymphocytes are thought to correlate with the presence of active disease. Material for the study of Hodgkin's disease is readily available from laparotomy, and includes spleen and abdominal lymph nodes removed for staging purposes (Rosenberg, 1972; Farrer-Brown, et al., 1971). The disease exhibits the presence of a morphologically abnormal cell, the Reed Sternberg cell, and much of the receptor data concerns the exploration of the nature of this entity. Three views as to its origin are given; Kadin et al. (1974) regard it as B lymphocyte in type by virtue of the presence of surface and intracytoplasmic (Dutton, 1974)(Taylor, 1974) immunoglobulin. The cell is frequently found to be rosetted by T lymphocytes, and all authors agree on this point. Biniamov and Ramot (1974) discount the feeble surface Ig expression on the Reed Sternberg cell, and suggest an origin from the T lymphoblast, while Brylan, Jaffe and Berard (1974) on the basis of Scanning EM morphology would favour a histiocytic (not B cell not T cell) origin. In the involved spleen, and in involved lymph nodes (Kaur et al., 1974; Joseph and Belpomme, 1975) the numbers of T lymphocytes are found to be much higher than in uninvolved tissue. Joseph and Belpomme (1975) showed a decrease in splenic B lymphocytes but did not regard this as/...

as statistically significant. In a survey of 22 spleens from patients with Hodgkin's Disease (Habeshaw, Stuart, Young and Gore, 1976) it was shown that in male patients the total concentration of splenic lymphocytes fell, while in female patients it rose, during Hodgkin's disease. There was also evidence from male patients of a significant decrease in splenic B lymphocytes. In involved tissue, B lymphocytes are low in number, and at many different stages of differentiation, while T lymphocytes are raised in number. IgM receptors are occasionally found on cells from Hodgkin's lesions, implying T cell or macrophage involvement (Habeshaw, et al., 1976). The Reed Sternberg cell is a protean entity, which appears to exhibit both macrophage like features (esterase positivity) and expresses some surface immunoglobulin (Stuart and Habeshaw, 1976). The increase in T cells in the spleen in Hodgkin's Disease described by Kaur et al. (1974) has also been shown in the spleen of treated (Kaur et al., 1974b) and untreated patients with chronic granulocytic leukaemia (Kaur, Spiers and Galton, 1975), and may therefore not be specific to Hodgkin's Disease. However, the lesion in involved spleens involves the Malpighian follicle, leaving the periarteriolar lymphocytic sheath intact, and this would give some support for both B and T cell abnormalities in the Hodgkin's Disease spleen (Hallie et al., 1974). It has also been shown that IgG antibodies directed against T lymphocytes can be found in Hodgkin's Disease tissue, and immunoglobulin synthesis is high in uninvolved spleen (Grifoni et al., 1975; Longmire et al., 1971). Recent studies (Payne et al., 1976) show that T cell increase does occur in uninvolved Hodgkin's Disease lymph node and spleen/...

spleen, with corresponding decrease in B cells. In this study, in 6 cases out of 24, an overlap between SIg bearing cells and T cells is noted. In a study on IgM receptors in Hodgkin's Disease, it was noted that in 5 out of 32 cases, cells bearing IgM receptors were present on spleen lymph node or blood cells (Habeshaw, Young and Stuart, 1976).

INTERPRETATION OF RECEPTOR DATA FROM LYMPHOMAS

The previous survey of crude receptor data in lymphomas brings out several important new facts. Firstly tumours of lymph nodes are predominantly tumours of B lymphocytes. In this group of tumours there are several descriptions of abnormal surface Ig, both in the class of immunoglobulin expressed and its location on the cell surface. Secondly not all neoplasms of B lymphocytes express the Fc and C3 receptors normally expressed by B lymphocytes in lymph node or peripheral blood. These receptors are concerned with activation of B lymphocytes in the normal immune response, and such findings lend weight to the postulate that neoplasms could represent an abnormal or perverted immune response.

Thirdly it has been shown that in several classes of lymphoma, and in lymphocytic leukaemia, that cells thought not to be neoplastic are altered numerically or functionally in a constant manner. Increased numbers of T cells occur in some B cell neoplasms, or non neoplastic conditions. Macrophages are important in the development of and resistance to experimental lymphomas in mice.

These observations point to the possibility of cell to cell interactions in lymphomas similar to those described in the normal immune response. In this section, the data relating to abnormal Ig expression in lymphoma, changes in Fc and C receptor expression, and evidence for cellular interaction in lymphomas is briefly surveyed.

Surface/...

Surface Immunoglobulin Expression

There are consistent abnormalities of surface Ig expression associated with lymphoma. The chief of these is the failure of the cell to secrete immunoglobulin at all: a process which can be reversed by trypsinisation (Habeshaw - personal observations; Aisenberg, Bloch and Long, 1973). The ability of the B cell to express surface Ig may depend upon its capacity to synthesise light chain, since in heavy chain disease, the neoplastic cells fail to express surface Ig (Seligmann, Preud'homme and Brouet, 1973). Bentwich and Kunkel (1973) showed that surface Ig negative cells from a patient with CLL, became positive after culture in the patient's serum. The potential abnormalities of Ig secretion by neoplastic or immunodeficient lymphocytes have been reviewed by Wu, Lawton and Cooper (1973). Serum inhibition of surface Ig secretion has been described by Cooper, Lawton and Bockmann (1971), and Loor, Forni and Pernis (1972) showed how antisera against surface Ig could block new surface Ig synthesis. In lymphoid cell lines the capacity to secrete surface Ig can be restored by washing the cells in acetate buffer (Hutterworth, Litwin and Cleve, 1972). Recently, in experimental immune responses in mice, a factor secreted by T lymphocytes effectively blocks surface Ig synthesis in the responding population (Lee and Paraskevas, 1976). Milton and Mowbray (1972) have shown that Bovine $\alpha 2$ glycoprotein will reversibly inhibit Ig synthesis by rat lymphocytes. Apart from failure of the B cell to secrete immunoglobulin in the neoplasm, it is now known that in many cases the immunoglobulin secreted is structurally/...

structurally abnormal (Piessens et al., 1973; Grey, Rabellino and Pirofsky, 1971). In most cases monoclonal IgM antibody with Kappa chain (κ) is secreted, but cases secreting K chain only have been described. These primary abnormalities in surface immunoglobulin secretion have also been shown in acute lymphoblastic leukaemia, and chronic granulocytic leukaemia (Metzger, Mohanakumar and Miller, 1975). The neoplastic cells in these conditions could be killed by anti-Kappa and anti-Lambda and anti-heavy chain antisera and complement. Following trypsinisation of the cells reactivity of K-light chain antiserum was detected after 2 hours (in the case of GCL) and 8 hours (ALL). All heavy chain activity was abolished following trypsinisation, and did not regenerate.

The expression of surface Ig, and secretory Ig, and the switches from IgD IgM, and IgM IgG during the maturation of the B lymphocyte into the plasma cell have been used to formulate a classification of neoplasms of the B cell, by an assessment of its degree of maturation or "differentiation" (Salmon and Seligmann, 1974).

Salmon and Seligmann (1974) proposed that B cell lymphomas could be classified on the basis of 5 classes of B lymphocyte:- these are B0 (B stem cells), B1 lymphocyte (virgin B lymphocyte), B2 (Immunoblast), B3 (Memory B lymphocyte), B4 (Plasmacytoid lymphocyte) and B5 (Plasma cell).

The basis of this classification are the extensive studies carried out by the authors on myeloma cells. The central feature is the triggering of a clone of cells and its subsequent monoclonal expansion, by an antigen. A second or oncogenic stimulus then leads/...

leads to irreversible proliferation. B cells in a lymphoma are regarded as B cells which have become "frozen" at a point on the pathway of normal B lymphocyte differentiation. The neoplastic B lymphocyte virtually always exhibits monoclonality of the expressed surface immunoglobulin. Monoclonal immunoglobulins are found in diseases such as CLL, some - but not all lymphomas, heavy chain disease, macroglobulinaemia, and multiple myeloma. On the other hand, if the B0 stage had undergone malignant transformation, polyclonal neoplasms of B cells might be expected - such lesions are rare. Salmon and Seligmann believe that the B1 lymphocyte tumours are represented most commonly by the CLL and well differentiated lymphocytic lymphoma group. In CLL the lymphocytes are usually non-secretory and fail to show further maturation, e.g. by development of intracytoplasmic Ig. In a few CLL patients the cells are more mature, showing serum M secretory components.

The B2 cell is regarded as a derivative, by division, from the B1 cell. The B2 cell is the principal cell type of poorly differentiated lymphomas, Burkitt's Lymphoma, and some cases of Histiocytic Lymphoma. These tumours may or may not express the same class of heavy chain as the precursor B1 cell.

The B3 or memory cell has no identifiable equivalent in lymphomas to date.

From the B3 memory cell is derived the lymphocytoid plasma cell (B4) which has both surface and intracytoplasmic IgM and secretes IgM in the body fluids, the malignant equivalent is Waldenstroms Disease.

The final stage, the secretory plasma cell secretes IgG, IgA, /...

IgA, IgD, IgM or IgE.

Because the neoplastic process is continuous, in B₄ neoplasms division of B₃ cells is necessary to maintain memory cells. Thus in Waldenstroms Disease pleomorphism of the B cell series is seen. Although the bulk of the cells are secretory B₄ cells, all stages from B₁ to B₃ are seen with some B₅ plasma cells.

In addition, it is suspected that myeloma cells secrete a substance which inhibits the proliferation of normal B₂ cells. These two features - the wide involvement of B cells at several stages of differentiation and the blocking of B₂ proliferation render the classification of lymphomas by these means very complex.

In support of the hypothesis is the discovery of myeloma proteins with antibody-like activity in both mouse and man indicating the importance of antigen in triggering B cell proliferation initially, and this is the most important finding relating the neoplasm and responsiveness to antigen (Eisen et al., 1970).

It has also been shown that cell lines can secrete antibodies which react with sheep or ox red cell determinants (Steele et al., 1974).

Apart from the expression of abnormal or monoclonal immunoglobulin on the cell surface, the distribution of immunoglobulin can also be abnormal. The reaction of any ligand with the surface glycoprotein of the lymphocyte is followed by movement of the crosslinked surface protein to one pole of the cell, and this movement is dependent upon an intact cell metabolism. Incubation in the cold (Loor and Hagg, 1975) or in the presence of sodium azide/...

azide prevents capping. This phenomenon, first described by Taylor, Dufus, Raff and De Petris (1971), is also dependent upon the degree of cross linkage of membrane immunoglobulin, or membrane glycoprotein. Using anti-immunoglobulin antibodies, De Petris and Raff (1973) showed that "patching" occurred at 0°C with whole antibody, and capping at 37°C. When Fab fragments of antibody were used the labelling on the surface was diffuse at all temperatures. Looor, Forni and Pernis (1972) showed that aggregation of diffuse surface Ig into spots or dots of fluorescent complex when treated with anti-Ig-antisera, was not inhibited by sodium azide or incubation in the cold. Formation of caps following washing or warming at 37°C was followed by disappearance of Ig anti-Ig complex from the membrane by interiorisation or shedding. If surface Ig bearing cells are left in contact with anti-immunoglobulin antisera surface Ig synthesis is inhibited. The phenomenon of dot formation on the cell surface is thus NOT DEPENDENT UPON THE INTEGRITY OF THE METABOLIC PROCESSES OF THE CELL. The formation of caps can be prevented by inhibition of cellular respiration (azide) or by cytochalasin which interferes with spindle formation and intracellular microfilaments. This suggests that the ability to cap surface Ig-anti-Ig immune complexes is a phenomenon of normal and metabolically intact cells, while failure to cap implies abnormality of either the intracellular microfilaments or respiratory metabolism. Cells vary in their ability to cap surface Ig. In CLL cells often fail to cap surface Ig (Flad et al., 1973), and in mouse myeloma cells very prolonged incubation, e.g. more than 5 hours, is required for capping to occur (Bevan, 1973).
Patching/...

Patching and capping are general phenomena, not restricted solely to the B lymphocyte - for example basophils and macrophages patch and cap cytophilic antibody bound by the Fc receptor on the surface of these cells (Ferrarini, Munro and Wilson, 1973). In Hodgkin's Disease some of the surface Ig bearing cells show failure to cap, but in addition antibodies reacting with T cells may be present, implying that non capping cells might be T lymphocytes (Grifoni et al., 1975). In normal peripheral blood most of the B lymphocytes cap readily a small number, however, do not. These cells are not monocytes, since monocytic leukaemia cells show no surface Ig (Habeshaw and Young, 1975). The comparative ease with which normal lymphocytes cap, and the failure of some lymphoma B cells to cap can be used to classify B cell lymphomas (Habeshaw, Macaulay and Stuart, in press).

Expression of Fc and Complement Receptors

The work of Corte, Risso, Ferrarini and Bargellesi (1976) clearly establishes a correlation between Fc receptor expression and immunoglobulin secretion by MPC 11 myeloma cells. Only Ig secreting cells expressed the Fc receptor, or cell which had surface Ig. Cycloheximide treatment abolished both SIg expression and Fc receptor. The complement receptor is thought to be important in stimulating proliferation and secretory responses by B lymphocytes (Hartmann, 1975). The complement receptor and the Fc receptor are both important in cellular interaction, both between normal lymphocytes and between lymphocytes and tumour cells (Kerbel and Davies, 1974). In antibody mediated cytotoxic reactions/...

reactions the Fc receptor only is involved in cell killing, the complement receptor is not (Perlmann, Perlmann and Muller-Eberhard, 1975).

Cell interactions

Although there is little direct evidence implicating Fc and complement receptors in interactions between lymphocytes, or between lymphocytes and macrophages such interactions clearly do occur. In a review of macrophage/lymphocyte interaction Lipsky and Rosenthal (1975) list six classes of such interactions. These are (1) the promotion of lymphocyte viability by feeder layers of macrophages in vitro (2) functional maturation of T lymphocytes from immature thymocytes in vitro (3) enhancement, of the bactericidal function of macrophages by lymphocyte products (4) induction of primary response in B lymphocytes by macrophages (5) induction of secondary response (6) antigen initiated lymphocyte proliferation. Normal macrophages can bind thymocytes and B and T lymphocytes by a trypsin sensitive cation dependent mechanism. In the presence of antigen, such macrophage lymphocyte interactions become more stable, and binding of lymphocytes precedes lymphocyte transformation and proliferation. If antibody is present linkage between macrophages and Fc receptor bearing cells can occur through the Fc receptor. Similar spontaneous interaction between human peritoneal cells and autologous lymphocytes occurs in vitro, and is independent of serum antibody (Stuart, 1970).

T lymphocytes bind to B lymphoblastoid cell lines, and weakly to normal blast transformed B lymphocytes (Jondal, Klein and/...

and Yefenof, 1975). Similar interactions between Reed Sternberg cells and T lymphocytes occur in Hodgkin's Disease (Stuart and Habeshaw). Such binding is probably independent of the Fc receptor. These morphological interactions have been regarded as providing an alternative "second signal" to the activation of B lymphocytes, in circumstances where the complement receptor is either not present or not occupied by activated complement. In normal lymphoid cultures the complement receptor probably supplies the signal for B cell proliferation (Schumann et al., 1974). It is evident that the role of the Fc receptor on B lymphocytes is not concerned with B lymphocyte activation directly, but it may well provide for close interaction between B cell and macrophage or B cell and Fc receptor bearing T lymphocytes in the immune response (Moller, Coutinho and Persson, 1975). In this review the authors show that B cell activation is complex and multifactorial, Serum inhibitors of B cell activation can be shown. Some antigens by themselves activate B lymphocytes in a non specific manner. Synergistic responses to incomplete (haptene) antigens require the co-operation of T cells or macrophages, and antigen itself can amplify the non specific proliferative response induced by B cell mitogens.

These effects are difficult to relate to lymphomas. Habeshaw and Stuart (1976) introduced the concept of co-operative neoplasms for those tumours containing both B and T lymphocytes. In untreated CLL, a predominantly B cell neoplasm, T cells are frequently increased, although these cells are not neoplastic (Kaur, Spiers and Galton, 1975). The effect of the "neoplastic" cell/...

cell in raising the T lymphocyte population is also seen in chronic granulocytic leukaemia, and in involved nodes in Hodgkin's Disease.

So far little evidence of T cell abnormality - other than the occurrence of morphologically bizarre T cells in Sezary syndrome - has been found in lymphoma in man. Some heterogeneity of the T cell population undoubtedly exists in normal peripheral blood T lymphocytes (Woody et al., 1975).

Changes in surface antigenicity associated with lymphoma

Most of the reported variations in surface antigens of lymphoid cells concern the leukaemias rather than lymphomas. Cell membrane marker glycoproteins specific for some human leukaemias and lymphomas have been described (Billing, Rafizadeh and Terasaki, 1975). Using purified glycoproteins as immunising agents, antisera cytotoxic to leukaemia and lymphoma cells but not to normal cells could be prepared. The antigen concerned was detected in 70% of leukaemic patients, and also in Hodgkin's Disease, Burkitt's Lymphoma, and histiocytic lymphoma. In some cases free antigen was present in serum. Antisera to acute myeloid leukaemia cells have also been prepared (Pendergrass et al., 1975) and used in epidemiological investigation. In one family of seven sisters, 3 died of acute myeloid leukaemia, a fourth sister died from an undetermined cause, and a fifth presented with acute myeloid leukaemia. Antisera reacting against this sister's cells, also reacted with myeloid cells of a disease free sibling. The surface antigens of EB virus are frequently expressed in Burkitt's Lymphoma, infectious mononucleosis, and also in acute lymphoblastic leukaemia (Zorbala-Mallios/...

(Zorbala-Mallios and Sutton, 1974). The recent discovery of naturally occurring allotypic markers for the human B lymphocyte (Mann et al., 1975) has not yet been exploited in the study of B lymphoid lymphomas. Antigens analogous to the p.27 and p.30 viral proteins of murine C type viruses have been isolated from human tissues, especially in systemic lupus erythematosus, but such antigens have not so far been related to any form of lymphoid malignancy.

Conclusions

Lymphomas are tumours of the cells of lymph nodes - and can be tumours of B or T lymphocytes, of macrophages, or of the stromal reticular or endothelial components of the lymph node. The physiology of each of these cell classes has been reviewed. Lymphocytes are subclassified on the basis of their surface characteristics, the presence of specific surface antigens, expression of surface immunoglobulin, and receptors for Fc and complement or the sheep red cell. The function of B lymphocytes and T lymphocytes is to produce an immune response and in this co-operation between the lymphocyte and other cell classes occurs. The study of the surface characteristics of lymphoma cells reveals that solid tumours are predominantly of B cell class, while T cell lymphomas are rare. Lymphoma cells can express mixtures of receptors of both B and T class, and receptor silent tumours occur. Abnormalities of surface Ig expression, and distribution have been noted in lymphomas, together with anomalous expression of/...

of Fc or deletion of Fc or C3 receptor. From this data it is possible to suggest new features of lymphoma cells which might indicate defects in cellular metabolism (Failure to cap surface Ig) or co-operative (synergistic) tendencies in some neoplasms. The data reviewed clearly indicates that previous views of the pathogenesis of lymphoma grossly underestimate the complexity of the subject facing the investigator in this field. The remainder of this thesis illustrates the findings from two years investigation of the biology of lymphoma using the comparatively new and powerful techniques for investigating the cell surface.

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MATERIALS

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Chapter 13

Contents

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MATERIALSRed CellsSheep

Sheep red cells were obtained from a single animal. From a flock of control sheep (through the courtesy of Dr.'s Cockburn, Forrester and David Mellors) six red cell samples were tested for ability to form SRBC, IgG and EAC rosettes. Cells from one animal were selected, and formed the basis of most of the results obtained in this series, as well as those reported by Blackie (1973) and Fraser (1974). Antisera were prepared in rabbits (against sheep stroma prepared from slaughterhouse blood) and both IgM and IgG antibody titres standardised with reference to this animal (H88). After using these red cells weekly for nearly 2 years the animal was slaughtered. A new sheep cell donor was selected (W77) and these cells standardised against the antisera already prepared. Quite marked differences in agglutinating and complement fixing antibody titres were noted with these new cells, but when correctly sensitised results with normal blood lymphocytes for E, EAC and EAIGG rosettes were comparable with the previous red cells. A third sheep was also used for a short time, prior to the introduction of Ox red cells as the vehicle for complement and IgG. Since then several sheep have acted as red cell donors for E rosette formation: Anti-sheep red cell antisera are no longer used and the singularity of the donor is not important as virtually all samples of sheep red cell tested have equivalent ability/...

ability to rosette with human blood T lymphocytes.

Sheep red cells were collected in Alsevers solution, containing benzyl-penicillin (200 units/ml) and streptomycin (50ug/ml) and stored under plasma at 4°C. Red cells preserve their rosetting characteristics for at least 4 weeks under these conditions, but were never stored for more than two weeks.

Ox red cells

Bovine red cells were obtained from a single animal (No. 01171) kept at the District Cattle Blood Typing Field Station (Roslyn). Recently a second single animal has acted as the donor of the red cells (No. 01857). Dr. J.G. Hall kindly supplied these blood samples. The ox blood is taken directly into ACD as anticoagulant and the cells stored under plasma in sealed "Vacutainer" tubes until required. Storage for up to two weeks is possible without affecting reactivity with IgM or IgG antisera. Both animals acting as red cell donors have "low agglutinating" red cells.

Rabbit anti-sheep, and Rabbit anti-ox IgM antibody

Both antisera were prepared in a similar manner. Washed, haemoglobin free stroma were standardised to a concentration of 1mg protein nitrogen per ml of solution (Protein determinations by micro-Keljahl analysis) and used to immunise rabbits intravenously according to the schedule described by Kabat and Mayer (1961). After bleeding out, the separated serum was fractionated on/...

on Sephadex G200 gel, in glycine buffer at pH 8.0 - 8.2. On testing, the bulk of the C fixing and lytic antibody activity was found in the first peak eluted from the gel. It proved more easy to prepare and separate anti-sheep-red-cell IgM than to prepare anti-ox-red-cell IgM. Indeed with some immunisations, virtually all antibody against sheep red cells was IgM, and such anti-sera were used diluted but unfractionated. Rabbit anti-ox-red-cell IgM was never found free of demonstrable 7S IgG antibody activity in the same sera (mercapto-ethanol resistant agglutination) and rabbit anti-ox-red-cell IgM antisera were always separated by gel filtration before use. Antisera were absorbed with human AB rh+ red cells before fractionation.

The separated and titred IgM antibody was dialysed against 0.1% lactose solution (4 litres of 0.1% lactose per 40 ml first peak column effluent), freeze dried in 2 ml aliquots, and stored in sealed ampoules under vacuum at -20°C . When required, these IgM preparations were reconstituted with 1 ml of sterile 2% human serum albumin solution (Salt poor HSA provided by courtesy of Dr. J. Cash, South East Regional Blood Transfusion Service). Such standardised preparations showed reproducible activity upon reconstitution, and titration of activity in both the column effluent, and each batch of freeze dried product ensured maximum control over the sensitisation process.

Rabbit anti-sheep, rabbit anti-ox IgG antibody

Rabbits were immunised intraperitoneally with 2 ml of a suspension of ox or sheep red cell stroma, followed by a single I.M./...

I.M. injection of 0.1 ml of stroma in complete Freund adjuvant. Intraperitoneal injections were repeated at three weekly intervals, and antibody titre monitored by agglutination and complement lysis tray tests. Rabbits were bled out at the appropriate intervals, and the IgG separated by ammonium sulphate fractionation. The precipitate was redissolved and this was followed by prolonged dialysis against tap water, and distilled water, to remove ammonium sulphate, euglobulin, and reduce ionic concentration. Pure IgG was then recovered by elution from a column of DEAE or QEAE Sephadex in 0.01 Molar phosphate buffer containing 0.01 Molar NaCl. The anti RBC IgG was titrated, and stored in 0.1 ml quantities in elution buffer, at -20°C in sealed glass ampoules. Antibody was absorbed with packed human AB red cells before fractionation, to remove rabbit anti-human activity.

Human Red Cells

Human red cells were obtained from ABO typed laboratory personnel. These cells were used for the formation of mixed rosettes after sensitisation with rabbit anti human IgG to form Hu-Fc reagent. Under certain circumstances human red cells will form spontaneous rosettes with human lymphocytes. Red cells of gpOrh- were used for these tests. Human gpOrh+ red cells were used to detect the absence of C3b fixed to ox or sheep red cells. These human cells contain receptors for C3b, and absence of co-rosetting between C3 sensitised cells and human red cells was taken to indicate absence of C3b on complement sensitised sheep or ox red cells. The absence of immune adherence on C3 sensitised/...

sensitised cells indicated that the exposed determinant was C3d (C3 inactivator cleaved C3b).

Rabbit anti-human red-cell IgG

Human cells were injected intraperitoneally as 1 ml of washed 5% suspension in physiological saline weekly for six weeks. The serum was fractionated by ammonium sulphate precipitation and the fractions stored at -20°C .

Buffer Solutions

Ficoll Triosil Mixture for separation of leukocytes from samples of peripheral blood and spleen

This was prepared by mixing 10 parts of a 22.9% solution of Triosil 350 (Nyegaard and Co., Oslo) were mixed with 24 parts of a 9% solution of Ficoll (Pharmacia), sterilised by membrane filtration (Pore diameter 0.22 microns) and stored at room temperature in 10 ml quantities in siliconised universal containers. The specific gravity was 1.076.

Veronal Buffered - Gelatin - Saline with EDTA (VBS-G-EDTA)

Stock solutions of veronal buffered saline (VBS), 0.10 molar trisodium ethylenediaminetetraacetate (EDTA) and 10% Gelatin solution (G) were prepared. The gelatin was acid ossein (Credo Food Products Ltd.) of p17.1 and was devoid of anti-complementary/...

anti-complementary activity. The solution was prepared by mixing 100 ml of stock VBS with 395 ml of glass distilled water, and 5 ml of warmed stock gelatin solution to give a final gelatin concentration of 0.1%. This was then mixed in the ratio of 450 ml of VBS-gelatin with 50 ml of stock EDTA solution to give VBS-G-EDTA. The solution was prepared fresh each week, sterilised by filtration and stored at 4°C.

Veronal buffered Saline gelatin with Calcium and Magnesium ions
(VBS-G-Ca⁺⁺ Mg⁺⁺).

Stock solution of 1.0 molar MgCl₂ with 0.15 molar CaCl₂ was added to 20 ml of stock veronal buffered saline, and 1 ml of warmed 10% gelatin solution added. The volume was adjusted to 100 ml with glass distilled water. This solution was prepared fresh daily.

Additional buffers used were phosphate buffered saline (PBS) and physiological saline (8.5 litre NaCl solution). These were stored after sterilisation by autoclaving, in sterile bottles. Tissue culture medium 199 with antibiotics was used for the conveyance of lymph nodes and preparation of cell suspensions.

Complement

Several complement preparations were used, including Guinea Pig complement (Wellcome freeze dried guinea pig serum) and Human complement. Human complement was prepared from fresh human serum (group AB), adsorbed with sheep or ox red cells, and stored/...

stored in 1 ml. quantities in liquid nitrogen.

Yeast Suspension

Yeast suspension was used for adsorbing guinea pig or human complement to prepare guinea pig R₄, or human R₃ reagent. Auto-claved bakers yeast (200 gm) was washed in PBS until the supernatant was clear. The yeast was then suspended in 250 ml of PBS, and 1.7 ml of mercaptoethanol added with stirring, and incubated at 37°C for 2 hours (in a fume cupboard). The yeast was then washed until the supernatant was free of mercaptoethanol (determined by smell). Washed reduced yeast suspension in 500 ml of 0.85% NaCl was then treated with iodoacetamide (0.02 molar)(addition of 17.8 mg per 100 ml) with stirring. pH was adjusted by addition of 4.4% sodium bicarbonate to pH 7. After 2 hours incubation with stirring at room temperature the yeast was washed 3 times in 500 ml of PBS, and made up to 1 litre with VBS. 1 mg sodium azide was added as preservative and the reagent stored at 4°C.

Antisera

The following antisera to rabbit and human immunoglobulins and antisera to complement C₃ and C₄ were used:

Polyvalent rabbit anti-human Ig: This preparation contains antibodies against the major heavy chain classes of human immunoglobulin, e.g. IgG, IgA, IgM (Sera Services Ltd.).

Rabbit/...

Rabbit anti-human Kappa, anti-human Lambda Light chains: Anti-Kappa and anti-Lambda antisera from rabbits (Dakopats : Copenhagen), and FITC coupled rabbit anti-human Kappa and rabbit anti-human Lambda (Sera Services Ltd.) were used.

FITC Coupled goat anti-Rabbit-Ig: This antiserum was used as a tracer of rabbit immunoglobulin in the sandwich technique (supplied by Meloy Ltd.).

Rabbit anti-human C3, Rabbit anti-human C4, Rabbit anti-Guinea Pig C3, and Rabbit anti-Guinea Pig C4: (Meloy Ltd.)(Cordis Ltd.) These antisera were used to test the specificity of the reactions for coating red cells with appropriate complement components, either by a Coombs test on a C sensitised cells, or by sandwich immunofluorescence technique.

Immunoperoxidase Reagents (Dakopats)

Peroxidase-anti-peroxidase (rabbit): This reagent consists of horseradish peroxidase enzyme, coupled with a rabbit antiserum against horseradish peroxidase.

Swine anti-rabbit IgG: This reagent is a potent swine antiserum against heavy and light chains of rabbit 7S immunoglobulin.

Rabbit antisera to heavy chains (u, ,) and Kappa and Lambda light/...

light chains: Dakopats antisera to Kappa and Lambda chains, were used at titres of 1 in 100 to 1 in 200. Nordic rabbit anti-human heavy chain antisera, particularly anti-alpha chain, were more effective in this test than the Dakopats equivalent (Sera Services Ltd.), and were substituted.

Normal Swine Serum: This was obtained through the kindness of Professor Campbell, Poultry Research Institute, from a normal breeding herd of Landrace pigs.

Specificity:

The specificity of most antisera were tested, either by gel-diffusion or immunoelectrophoresis. Anti-complement antisera (Meloy) were tested by reaction with purified human or guinea pig complement components (Cordis reagents).

Miscellaneous Materials

Acridine Orange: This consisted of 0.02% w/v aqueous solution of acridine orange dye in distilled water (Brostoff, 1974).

Neutral Red Dye: A solution of 1% w/v of neutral red in distilled water was used as stock. This was diluted 1 in 10 with phosphate buffered saline before use (to make 0.1% neutral red solution).

Stock/...

Stock VBS Buffer: This buffer (5 x concentrate) was prepared by dissolving 83.0 gm NaCl and 10.19 gm of sodium diethyl barbiturate in 1.5 litres of distilled water. The pH was adjusted to 7.35 ± 0.05 by the addition of 1 N HCl, and made up to 2 litres with distilled water. It was stored at 4°C , for up to 2 months.

2% Solution Human Serum Albumin: This was prepared by dissolving 2 gms salt poor freeze dried human serum albumin in 100 ml glass distilled water. The solution was sterilised by filtration (0.22 μ filter).

White Cell diluting fluid: A mixture of physiological saline containing 2.0 v/v glacial acetic acid and 0.1% gentian violet was used in making white cell counts.

Stock Phosphate Buffered Saline: Stock solutions of 9.08 gms of potassium dihydrogen phosphate (KH_2PO_4) per litre, and 11.88 gms of disodium hydrogen orthophosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) were prepared and mixed in approximate ratios of 20 mls to 80 mls to give a pH of 7.4. 10 ml of this stock solution was mixed with 90 ml of sodium chloride solution (9g litre) to give phosphate buffered saline.

Human Gamma Globulin Preparations: These were used in (a) assessing specificity of antisera, (b) to coat red cells non-specifically in investigating subclass specificity of the Fc receptor/...

receptor, and (c) in rosette inhibition experiments. All fractions were prepared from myeloma or Waldenstrom Disease sera in which the class and subclass of the major immunoglobulin fraction was known. These sera were supplied by Departments of Therapeutics, and Haematology, Royal Infirmary of Edinburgh. Fractionation of IgG subclass myeloma sera was performed on DEAE cellulose (elution with 0.01 - 0.1 M phosphate saline buffer gradient). IgM containing Waldenstroms Disease sera were fractionated on Sephadex G 200 gel, followed by dilution precipitation of euglobulin, redissolving and refractionation. Fairly pure IgM samples were obtained by these means. Antisera to subclasses of human IgG were used to classify the product obtained by these means (Nordic reagents). The reagents so prepared were freeze dried and stored at -20°C .

Tissue Culture

Siliconised Wasserman tubes, sterile Leighton tubes and tissue culture flasks (Falcon Plastics) were used to hold and culture cells. Washing of cells was generally achieved in polypropylene centrifuge tubes, to which platelets readily adhere, but to which lymphocytes or macrophages adhere weakly. Macrophages stick well to tissue culture grade plastic. Tissue culture medium used was almost always medium 199, supplemented with human AB serum, or with lactalbumin supplement (Habeshaw, 1971).

Methods including technical control data

Chapter 14

METHODS, INCLUDING TECHNICAL

CONTROL DATA

CONTENTS

- 1) E Rosettes
- 2) Enumeration of Fc receptor with EA IgG and Ox Fc reagents.
- 3) IgM receptors.
- 4) Preparation of Complement rosettes.
- 5) Neutral Red Ingestion.
- 6) Detection of Surface Immunoglobulin bearing cells.
- 7) Detection of Intracellular Immunoglobulin.
- 8) Preparation of Cell suspension (from blood) and glass adherent cells.
- 9) Separation of cells from spleen.
- 10) Separation of cells from Tonsil, Lymph Node, Thymus and cell lines.

METHODSPreparation of Sheep Erythrocyte Rosettes (E Rosettes)

The following variables have been assessed in relation to the formation of rosettes between sheep red blood cells and human blood lymphocytes:-

- a) The optimal number ratio of sheep erythrocytes to human lymphocytes for efficient rosettes formation.
- b) The role of temperature.
- c) The time of incubation.
- d) Methods of lymphocytes preparation.
- e) Presence or absence of serum in the incubation medium.
- f) The use of different sheep as donors.
- g) Effect of glutaraldehyde fixation upon rosette formation.

This work formed a large part of the thesis of R.A.S. Blackie (1973) performed under the supervision of the author. The conclusions reached in this study were as follows:-

- a) Optimum practical ratio of sheep red cells to human leukocytes was 40:1.
- b) Preincubation of suspension for 5-10 minutes at 37°C, followed by centrifugation and 2 hours incubation at 4°C gave optimum numbers of rosettes.
- c) Prolongation of incubation beyond 2 hours did not increase/...

increase rosette formation.

- d) There was enhancement of E rosette formation by lymphocytes separated over Ficoll and inadequately washed. Washing three times abolished these co-agglutinates which were in part due to red cell clumping induced by Ficoll. Dextran sedimentation did not appear to enhance E rosetting.
- e) Providing all other conditions were optimal, the presence of serum did not enhance the numbers of rosettes detected. However, serum, or serum albumin did appear to stabilise rosettes making them more resistant to mechanical disruption. Balanced salt solutions were all virtually equivalent in supporting E rosetting, providing stable pH conditions and high cell viability could be achieved.
- f) Most sheep tested were equivalent in the ability of their red cells to rosette with human peripheral blood lymphocytes.
- g) Glutaraldehyde fixation (0.5-6%) after resuspension prevented mechanical disruption of rosettes. If applied to the red cell/white cell pellet, spurious rosettes were often found formed between the red cells and leukocytes.

Increasing inhibition of E rosetting in this system was shown by IgG or IgA concentrations in excess of 10 mg/100 ml;- at levels of 1 mg/ml rosette formation was almost totally inhibited.

From/...

From this information an optimal method for the preparation of sheep red cell rosettes was devised, and has been employed to date with little modification. The method summarised is as follows:

Preparation of E Rosettes

- 1) Washed leukocytes, 1×10^6 in 0.1 ml medium 199 are mixed with 40×10^6 sheep red cells in 0.1 ml phosphate buffered saline. The volume adjusted to 1 ml by addition of 0.8 ml medium 199.
- 2) Suspension warmed to 37°C , and incubated at 37°C for 5 minutes.
- 3) Centrifuged for 5 minutes at 180g, and the pellet incubated for 2 hours at 4°C .
- 4) Cells were resuspended for counting, after aspiration of 0.5 ml overlying fluid, by rotation of the long axis of the tube until resuspension is complete. Counting is assisted by the addition of 0.1 ml of 0.02% solution of acridine orange and viewing immediately under mixed phase contrast and U.V. illumination. Cells binding 3 or more sheep cells are counted as rosettes.

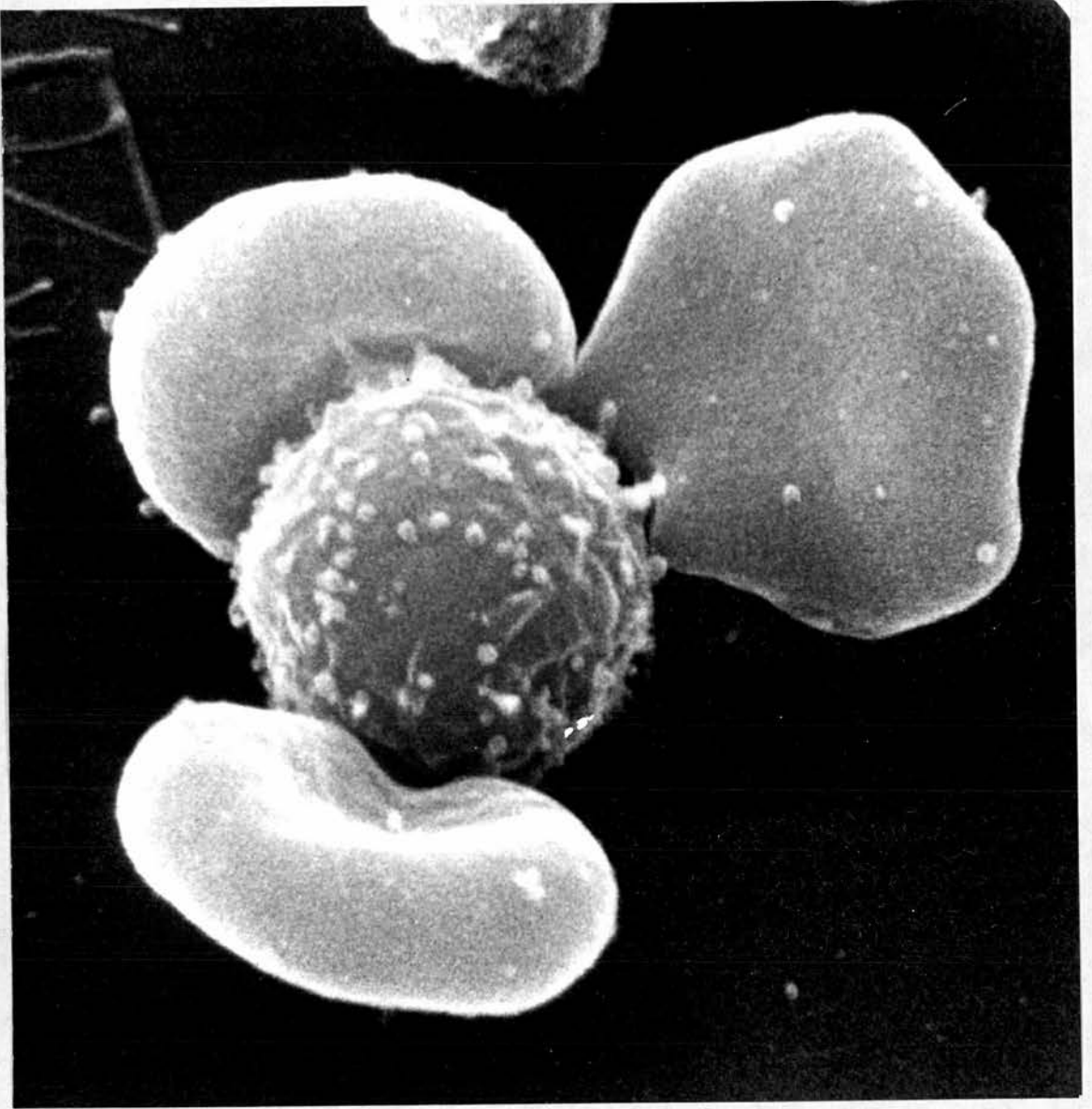


Figure 1: Rosette by Scanning Electron Microscopy

The central small lymphocyte, in this case a smooth surfaced T cell shows binding of 3 sheep erythrocytes, and thus fulfills the criteria for an E rosette.

Scanning E.M. x 12,500

(Illustration from a preparation by Dr. A.C. Parker)

Fe Receptors using EAIgG and Ox Fe

Reagents

Preparation of EA IgG or Ox Fc reagents, and enumeration of Fc receptors on lymphoid cells

In the initial series, use was made of the ability of high concentrations of rabbit anti-sheep RBC antibody to block E rosetting while at the same time allowing detection of the Fc receptors. The work which elucidated and standardised these techniques was reported by Fraser (1974). This work established that "coating" sheep red cells with IgG or IgM antibodies, or non specifically attaching IgG or IgM to the red cell surface with Chromium effectively occluded rosetting sites on the sheep red cell, and prevented them from engaging the E receptors on T lymphocytes. This difficult work in the standardisation of Fc receptors using the sheep red cell as the test particle also showed two other important features, listed below:

1) Optimal numbers of Fc receptor cells are detected only by high concentrations of IgG Fc receptor on the surface of the test red cell. Low concentrations of antibody on the red cell surface detect only macrophages or monocytes and fail to react with B lymphocytes. This is shown by the fact that Hu red cells coated with rh antisera form rosettes only with monocytes.

2) The reaction with the Fc receptor is subclass specific; only some subclasses of human IgG will react with the Fc receptor on the macrophage or lymphocyte surface.

It was previously shown that a concentration of 100 mg per 100/...

100 ml of IgG or IgA immunoglobulin was required to block E rosetting when such immunoglobulin was present in the incubation medium, but not upon the red cell surface. Titres of anti-sheep IgG of 1 in 2048, were found to inhibit E rosetting when reacting with cells from sheep H88. Titres below this, i.e. 1 in 4056 were rather less effective, and titres above this, i.e. 1 in 1024 caused agglutination of the sheep cells. If it is assumed that this titre is equivalent to 1ml/ml of surface red cell bound IgG and assuming M.W. of 150,000 Daltons, the minimum number of molecules per red cell for inhibition of E rosetting or for optimal sensitisation for Fc detection is 1.25×10^6 molecules per RBC. Concentrations of 0.625×10^6 molecules per red cell are inadequate for inhibition of E rosette formation (with sheep cells) and concentration greater than 2.50×10^6 molecules/red cell cause agglutination. It must be stressed that such calculations apply only to the individual antibody/sheep red cell combination used, and to no other, but it serves as a useful guide to adequate sensitisation of the red cell whether sheep or ox red cells are used as the antibody carrier.

In terms of the subclass specificity of the rosetting reaction, if red cells are passively sensitised with Cr coupled IgG of known subclass, it is found that IgG1, and IgG3 will bind to equivalent numbers of cells, but that IgG4 subclass is not bound by a substantial number of Fc rosetting cells. Note that this effect is not due to variations in numbers of bound IgG molecules, but to a true difference in receptor specificity.

TABLE/...

TABLE

Peripheral Blood Lymphocyte Rosetting by IgG bound specifically or non specifically to the sheep erythrocyte surface. (Partially derived from data of Fraser, 1974).

<u>Protein Concentration</u>	<u>Coupling (specific S non specific NS)</u>	<u>Fc Rosettes (Percent)</u>
10 mg/ml	NS	32
1 mg/ml	NS	29
0.1 mg/ml	NS	(no inhibition of E rosettes)
Titre (Anti-sheep-RBC IgG)		
1 in 2048	S	28
1 in 4056	S	31
1 in 800	S	Agglutination
1 in 12,000	S	62 (no inhibition of E rosettes)
NIL	-	62

The absolute numbers of Fc receptor expressing cells in normal human blood is 0.75×10^6 per ml, but if cells coated with IgG₄ subclass are used, only 0.45×10^6 per ml are found to have Fc receptor. Furthermore if human Orh+ red cells are sensitised with immune IgG anti-rh antibody from pregnant patients with/...

with rhesus sensitisation; the numbers of Fc receptor bearing cells are found to correspond only to these cells which are phagocytic, i.e. to monocytes. No B cell receptors for Fc are detected in this system (rh anti-rh).

From this data, optimum methods for the sensitisation of sheep cells and rosette formation were developed, those given here apply to sheep H88 and rabbit 7S antibody.

1) Washed sheep red cells were sensitised by incubation in 1 in 2048 dilution of rabbit anti-sheep RBC IgG in PBS for 1 hour at 37°C.

2) The cells were washed twice and mixed with 1×10^6 leukocytes in a ratio of 40 red cells to 1 leukocyte. The mixture was incubated for 10 minutes at room temperature in siliconised Wasserman tubes, in a total volume of 1 ml.

3) The red and white cells were pelleted by centrifugation at 180 x g and incubated for 2 hours at 4°C.

4) Resuspension was by vigorous end over end inversion of the Wasserman tube (x 5). The rosettes were either counted directly, using a cell counting chamber (Improved Neubaur), followed by an accurate white cell count, or the percentage of cells binding 3 or more sensitised sheep red cell determined directly after the addition of 0.1 ml of 0.01% acridine orange solution, by mixed U.V. and phase contrast microscopy.

In evaluating the Fc receptor a number of experiments were performed using incubation for 2 hours at 37°C, and overnight incubation at 4°C. Results were comparable whichever method was used.

Owing/...

Owing to the difficulties in standardisation of sensitisation of sheep cells, especially if different donor animals were used, the ox red blood cell, and rabbit anti-ox-red cell 7S antibody system were used in later experiments. At present the most stable Fc receptor is obtained with the use of Formalised ox red cells (Fox) (washed ox red cells which are stored in 0.5% solution of buffered formalin) and rabbit antibody. Using either formalised ox rbc or washed ox rbc the technique employed is as follows:-

- 1) Washed ox (or formalised ox) red cells were sensitised by the addition of rabbit-anti-ox-rbc IgG in predetermined optimal titre. They were incubated for 30 minutes at 37°C.
- 2) After washing twice in PBS cells were adjusted to a concentration of 400×10^6 /ml and mixed with 1×10^6 leukocytes in a total volume of 1 ml.
- 3) Cells were incubated together for 10 minutes at 37°C and centrifuged at 180 g for 5 minutes. They were incubated at 4°C for 2 hours.
- 4) Rosettes were resuspended by end over end inversion of the incubation tube, stained by the addition of 0.1 ml of 0.02% solution of acridine orange, and counted using incident U.V. and transmitted phase contrast illumination.

Comparison of rosettes made with sheep, ox, or formalised ox red cells sensitised with rabbit antibody showed no significant differences in numbers of rosettes detected.

IgM Receptors

IgM receptors

An essential precursor to the development of complement sensitised sheep or ox red cells was the preparation of good IgM antibodies for the sensitisation of red cells. If IgG containing antisera are used for complement sensitisation the criticism that IgG Fc rather than C3 receptors are detected is valid. The use of IgM has other advantages: in the sheep red cell system, cells coated with IgM antibody to the exclusion of E rosetting determinants show little or no background rosette formation when tested with normal human leukocytes, as shown in the accompanying table.

TABLE

Percentage of IgM rosettes formed between Rabbit IgM-coated sheep red cells and normal human peripheral blood lymphocytes.

<u>Individual</u>	<u>Percent Rosettes</u>
JR	0.26
EG	0.17
RH	0.22
AC	0.75
AB	0.37
JAH	1.76
ER	1.91
ED	<u>1.63</u>
Mean Value	<u>1.17 (Range 0.17-1.9%)</u>

IgM antibodies are also very efficient at complement fixation, an important point in producing maximum possible concentrations of complement on the red cell surface. The production of rabbit IgM antibody to sheep red cells was easily achieved, and sheep red cells sensitised with anti-sheep IgM did not form rosettes with normal human peripheral blood leukocytes. Preparation of ox red cells sensitised with rabbit anti-ox IgM antibody was more difficult, and IgM sensitised ox red cells gave somewhat higher background counts than IgM sensitised sheep cells, as shown in the accompanying table.

TABLE

IgM receptors on human peripheral blood mononuclear cells detected with IgM sensitised ox red cells.

Individual

1	8%
2	NIL (400 cells counted)
3	2%
4	3%
5	1%
6	0.75%
7	2.0%
Mean Value	<u>2.8% range 0.1% to 8%</u>

IgM coated ox red cells were used as control particles for complement rosetting, since IgM receptors are detected occasionally/...

occasionally and can be mistaken for complement receptors unless IgM controls are included.

IgM receptor bearing cells were enumerated as follows:-

1) Sheep or ox red cells used sensitised by incubation in the appropriate antibody for 1 hour at 37°C , and were then washed twice.

2) Sensitised cells, at a concentration of 400×10^6 ml, were mixed with white cells in ratio of 40×10^6 red cells to 1×10^6 white cells, and the volume of suspension adjusted to 1 ml. Cells were incubated for 5 minutes at 37°C , centrifuged at 180g for 5 minutes and incubated at 4°C for 2 hours.

3) Rosettes were counted in the resuspended material (end over end inversion) following the addition of acridine orange, or more commonly by direct rosette counts followed by white cell counting (because of the infrequency of IgM rosettes in normal samples).

The Complement Rosette

Preparation of complement rosettes

The preparation of test particles for complement rosetting gave considerable difficulty, resulting in the adoption of dual sensitisation technique for sheep red cells in the production of EAC reagent. This technique was found essential to (a) detect optimum numbers of complement rosetting cells and (b) to eliminate cross reactivity between E and EAC receptors. The principal way of avoiding the second (b) drawback was by the manufacture of an adequate IgM antibody. Sheep red cells were sensitised in different dilutions of IgM antibody, and optimal sensitisation determined by blocking of the E receptor as in the example shown below.

<u>Antibody Titre</u>	<u>% Rosettes</u>
0	55.6%
1/512	Agglutination
1/1024	Agglutination
1/2048	1%
1/4056	1.63%
1/8112	25.71%

In this case the titre chosen for IgM sensitisation was 1 in 2048. The agglutination resulting at titres of 1 in 512 and 1 in 1024 illustrates the potentiation of sheep cell agglutination by IgM antibody in the presence of leukocytes, and this is a major drawback to the use of sheep cells as a vehicle for complement components/...

components.

The sensitised sheep cells were then washed in VBS-G-Ca⁺⁺ Mg⁺⁺, cooled to 0°C and mixed with ice cold human or guinea pig complement diluted 1 in 10 in VBS-G-Ca⁺⁺ Mg⁺⁺ and held over ice for 30 minutes. Lysis occurs if the cells are warmed above 0°C. This step allows slow fixation of C1₄, and C2. Following a wash in ice cold VBS-G-EDTA, the cells were warmed to 37°C in a 1 in 10 dilution of human or guinea pig complement in VBS-G-EDTA and incubated for 30 minutes at 37°C. Often a trace of haemolysis is observed. This step allows fixation of C3, and inactivation of fixed C3b to C3d by C3 inactivator. Some C1, C2 and possibly IgM antibody may elute at this stage. The cells were then washed once in VBS-G-EDTA, and subsequently resuspended in phosphate buffered saline. This reagent is the EAC reagent used in most of the experiments described.

EAC receptors were quantitated by reacting 40×10^6 EAC cells with 1×10^6 leukocytes in siliconised Wasserman tubes in a volume of 1 ml in PBS or tissue culture medium 199. The tubes were incubated for 10 minutes at 37°C, centrifuged for 5 minutes at 180g and incubated for 2 hours at 4°C. Rosettes were resuspended by end over end inversion of the tube, and counted in a white cell counting chamber. White cell counts, performed in a standard manner, enable calculation of the percentage of rosetting cells.

Checks were made on the influence of incubation temperature on the numbers of EAC rosettes, E rosettes, and IgM rosettes as shown in the example below.

Temperature/...

	<u>Temperature of Incubation</u>		
	<u>37°C</u>	<u>20°C</u>	<u>4°C</u>
E Rosettes	0.4%	12.35%	63.51%
EAC Rosettes	21.9%	23.5%	23.3%
IgM Rosettes	0.91%	1.16%	4.4%

Despite the care taken to ensure reproducible sensitisation of sheep cell with complement, the technique has inherent difficulties. The principal drawback is the agglutination of red and white cells together, resulting in suspensions which cannot yield accurate rosette counts. This is due to the enhancement of agglutination of sensitised sheep cells by human leukocytes already referred to. Complement coated cells can be prepared only with great care, to avoid agglutination of suspension or lysis of red cells during sensitisation. Complement coated cells must be prepared fresh each day. These difficulties resulted in the adoption of the ox red cell as a vehicle for complement, and a sensitisation procedure based on the use of R reagents prepared from guinea pig or human serum. Not only is the technique more straightforward, but there is less variability in the results obtained.

Preparation of C3 reagent using Ox Red Cells

Ox red cells were sensitised by 30 minutes incubation in 2 ml of rabbit anti-ox red cell IgM reagent (rabbit anti-ox red cell IgM in 2 ml of human serum albumin solution). Half these cells were/...

were washed and were used to make IgM control rosettes. The remainder were treated with human R3 reagent to form C3d sensitised cells, or with guinea pig R4 reagent to form C14 cells. R3 reagent was prepared from fresh or liquid nitrogen stored human serum, R4 reagent (rarely used) from guinea pig serum. The human serum was diluted 1 in 10 (1 ml + 9 ml VBS-G-Ca⁺⁺ Mg⁺⁺) and to this was added 2 ml of reduced acetylated yeast suspension. The mixture was incubated for 30 minutes at 37°C and the yeast removed by high speed centrifugation. IgM sensitised red cells were added to the R3 (or R4) reagent and incubated for 30 minutes at 37°C. The red cells were washed once, and 40×10^6 red cells incubated with 1×10^6 leukocytes in 1 ml for 10 minutes at 37°C. The mixture was then centrifuged at 180g and incubated for 2 hours at 4°C in a siliconised Wasserman tube. Rosettes were resuspended by the end over end inversion of the tube and counted following the addition of 0.1 ml of 0.2% acridine orange by mixed phase contrast and U.V. microscopy.

Formalised ox red cells can be used to make F(ox)C3. The numbers of C3d rosetting cells found are equivalent with the three reagents EAC, OxC3, and F(ox)C3, under the same conditions. Reagents using formalised ox red cells are considerably more stable than with fresh ox red cells, and keep for 3-4 days with no detectable deterioration.

Neutral Red Ingestion: a phagocyte
marker.

Neutral Red Ingestion

The phagocytic cells were quantitated as cells concentrating neutral red dye in a coarse granular form from a dilute solution in physiological saline. Preliminary studies showed that of several "phagocytic" markers investigated (colloidal carbon, Pelikan Werke, Hanover, trypan blue, opsonised polystyrene, or starch particles) neutral red dye was the most reliable and the simplest marker to employ.

Cells (1×10^6) were incubated for 30 minutes in a mixture of 0.1 ml of a 0.1% w/v solution of neutral red and 0.9 ml of medium 199 or balanced salt solution, in siliconised Wasserman tubes. The cells were then washed in medium 199 or balanced physiological salt solution, resuspended in 0.1 ml volume, and sealed under a coverslip. Neutral red ingesting cells were quantitated as cells showing large vacuoles of neutral red dye in the cytoplasm. Minimal small vacuolar uptake, often in the uropod, is found in some lymphocytes, and myeloid cells can show quite marked diffuse uptake into small vacuoles. In practice mononuclear phagocytes are easily distinguished from lymphocytes (Fig. 2).

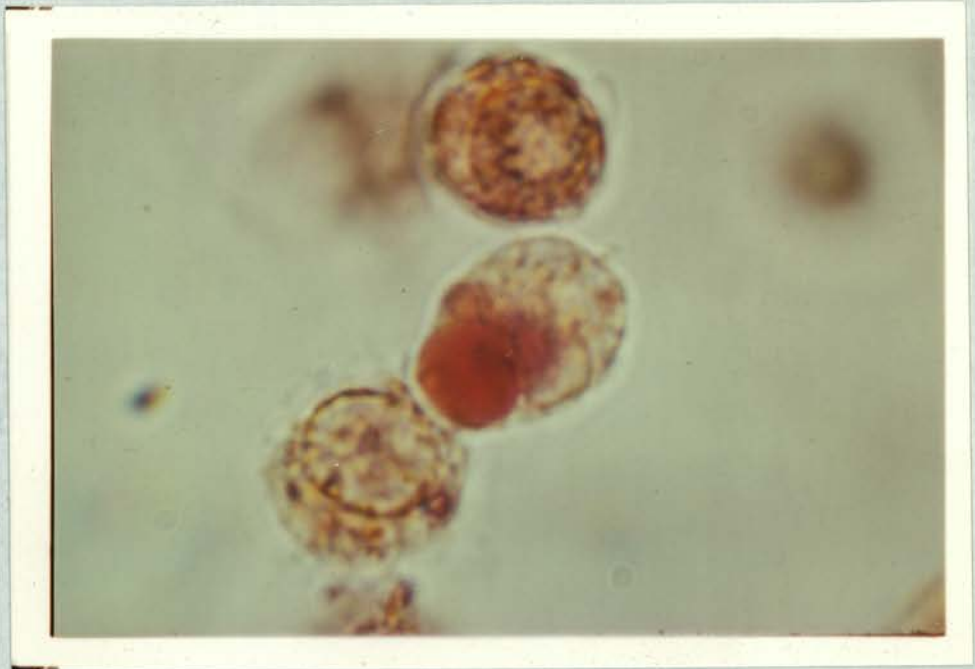


Figure 2: Neutral Red Dye Test

The preparation shows a monocyte and two lymphocytes supravivally stained with neutral red dye. The coarse vacuolar uptake of dye by the central monocyte contrast with the single vacuole containing dye seen in the cytoplasm of one of the lymphocytes (lower left of centre). In practice this method of identifying mononuclear phagocytes proved simple and accurate.

Transmitted light/phase contrast x 1,200

Surface Immunoglobulin bearing

Cells

The Detection of Surface Immunoglobulin Bearing Cells

The method used was developed from the simple detection of cells with surface Ig, into a test which determines the behaviour of the cell surface following the complexing of anti-Ig antiserum with the surface Ig. Initial studies involved the reaction of cells with FITC coupled anti-IgG, IgM or IgA antisera, by direct staining for 10 minutes at 37°C. It was noted that both capping and non-capping cells were present, and a spectrum of complete, partial, and non-capped cells was detected. It was decided to investigate the conditions of reaction leading to the capping phenomena. It was shown that some cells did not cap despite prolonged incubation, that caps were shed with prolonged incubation times, and that incubation for 10-15 minutes at 37°C detected optimal numbers of capping cells. It was also found that aggregates in the immunoglobulin antisera would produce spurious reactions with Fc receptors producing a capping reaction due to aggregates of Ig. These could be shown to react even with some thymus cells.

Method of detection of surface Ig

Washed leukocytes (2×10^6) in 0.2 ml of PBS were reacted with a 1 in 10 dilution of polyvalent rabbit anti-human immunoglobulin (anti-G,M,A chain), for 10-15 minutes at 37°C. Cells were then washed three times in PBS, cooled in ice, and then stained for 30 minutes with 1 in 10 or 1 in 20 dilution of Goat anti-rabbit Ig. The caps are formed during the incubation at 37°C, and small patches (non-capping surface Ig) are formed mostly/...

mostly during incubation at 0°C. Even prolonged (2 hr) incubation at 37°C does not induce significant capping in the non-capping population, but some aggregation of surface dots of Ig anti-Ig complex occurs, and this can be shown both at 37°C and at 0°C, and in the presence of sodium azide which inhibits capping. Caps are also shed by prolonged incubation, and can be observed to reform on the capping population if it is subsequently washed and reincubated in anti-Ig antiserum. Different forms of surface staining are illustrated (Fig.).

Specific Antisera

Antisera to human kappa and lambda light chain, and anti-IgG, IgA, IgM and IgD coupled to FITC or Rhodamine were used to determine the class of antibody expressed on the cell surface. These antisera were used at dilutions from 1 in 20 to 1 in 40, reacted with white cells for 15 minutes at 37°C, followed by washing twice in PBS. These reactions were not employed upon every occasion; as a result cells expressing light chains only on the surface might not have been detected in every case.

Synthesis of Immunoglobulin

The absence of stainable immunoglobulin does not necessarily mean that the cell concerned cannot manufacture Ig. It has been found that trypsinisation can restore the capacity of some cells to react with anti-Ig antisera. The fact that a positive staining reaction for surface Ig occurs on a cell does not necessarily/...

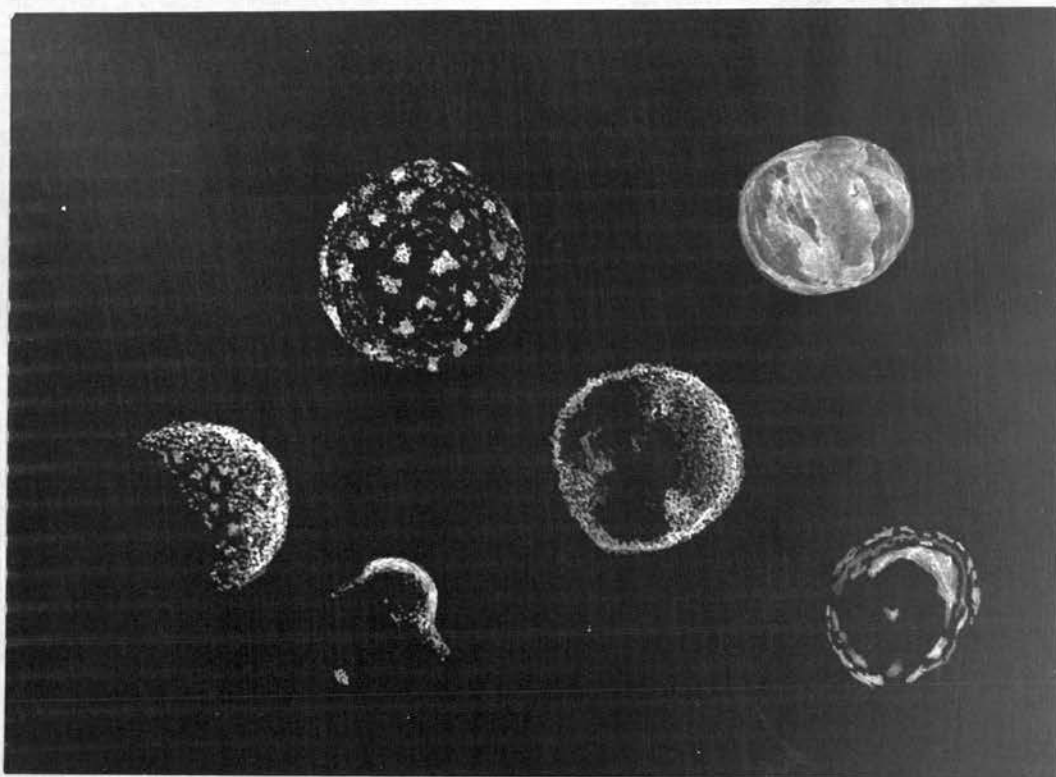


Figure 3: Patterns of Distribution of Surface Ig by Immunofluorescence.

Top right diffuse fluorescence, top left non-capping fluorescence, centre right confluent ring fluorescence, centre and lower left early and late capping fluorescence. Bottom right the appearance of intracytoplasmic fluorescence, in ethanol fixed smear, of a plasma cell. The diagram was prepared from ink sketches of fluorescent patterns made at the microscope, and printed from a reversal transparency to produce a more realistic effect.

necessarily imply a capacity for Ig synthesis. Mast cells, polymorphonuclear leukocytes, platelets, and monocytes can all be shown to react with anti-Ig antisera, but no resynthesis of surface Ig occurs following trypsinisation. Cells from carcinomata likewise show non-specific staining for surface Ig in many cases, but do not resynthesise Ig following trypsinisation. The technique used to trypsinise cells was to treat 40×10^6 cells in 1 ml of medium 199 with 1 ml of 0.25% solution of pancreatic trypsin of bovine origin for 30 minutes at 37°C . The reaction was stopped by rapid dilution and washing of cells in a large excess of prewarmed medium 199. Viability was assessed and the cells then cultured overnight in medium 199 + 10% foetal bovine serum. Before culture the absence of surface Ig was shown by staining with anti-Ig antisera. Resynthesis is present by 6 hours, and is fully developed by 24 hours, in normal control samples.

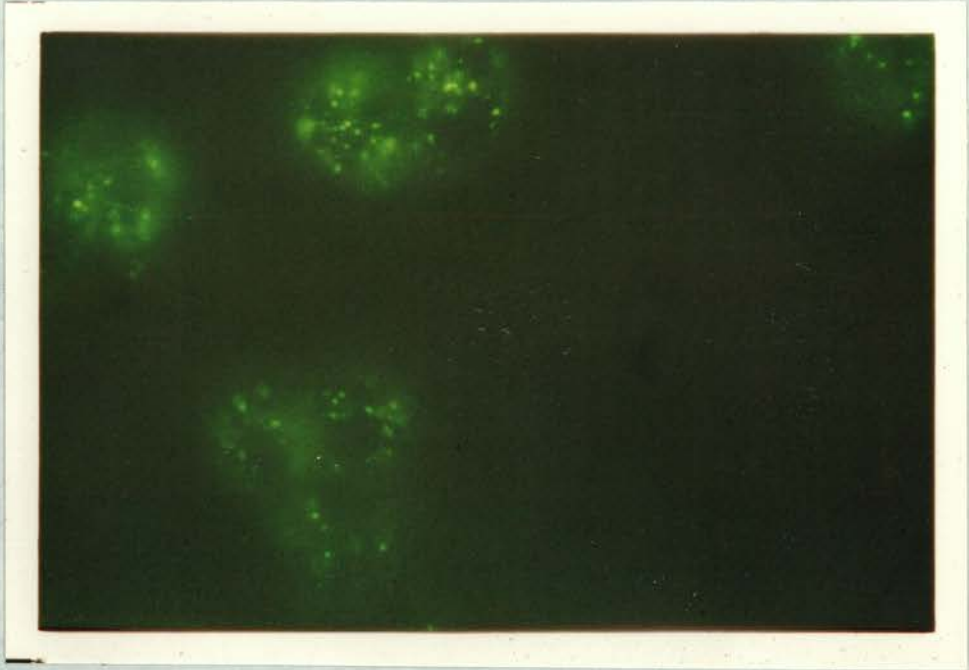


Figure 4: Patterns of Surface Ig distribution

Non-Capping Cells

The typical appearance by immunofluorescence of non-capping immunofluorescence. The tiny dots of surface fluorescence snap in and out of focus while under view, giving rise to a glittering effect. Note the absence of any capping in the cells illustrated. This appearance characterises lymphomas of B1 or B2 subclasses.

Immunofluorescence of living cells, incident UV.(Fluor) illumination, FITC conjugated goat anti-rabbit following polyvalent rabbit anti-human antiserum in sandwich technique.

x 1,200

Detection of Intracellular
Immunoglobulin

Intracellular Immunoglobulin

Initially, intracellular immunoglobulin was detected by reacting ethanol-fixed, air-dried lymph-node dabs, or cell suspension-smears, with the appropriate specific antiserum coupled to FITC. Background staining was troublesome, and was removed only by prolonged washing and by staining with specific rabbit antisera in a large excess of normal rabbit serum. In general the immunoperoxidase technique is now preferred, since it can be performed on formalin fixed lymph node which preserves the anatomical relationships of the cytoplasmic Ig secreting cells. The technique is described below: (the author is indebted to Mr. R. Hogg for refining the technique and for the excellence of his preparations).

Immunoperoxidase Technique (Peroxidase anti-Peroxidase: PAP)

In essence this technique reacts an unlabelled rabbit antibody to human immunoglobulin with the tissue section, followed by a reaction with excess swine anti-rabbit serum. This reaction leaves an excess of swine anti-rabbit immunoglobulin sites free to interact with a tracer complex of rabbit anti-peroxidase antibody and horseradish peroxidase presented as an immune complex. Sites of binding of peroxidase are revealed by a di-amino-benzidine reaction (DAB) which gives a brown precipitate at the site of peroxidase binding. A number of extremely important precautions must be taken to procure a controlled and specific result, and these are indicated below:

1)/...

- 1) Fixation is achieved in 10% formal saline (not buffered). Lymph node slices must be thin (2 mm) and fixation should not be unduly prolonged (36-48 hours).
- 2) Embedding: throughout the tissue processing the temperature must not be allowed to exceed 60°C. The wax used for impregnation should have a melting point below 60°C, and sections must be dried in an oven thermostatically controlled to 56°C.
- 3) inactivation of endogenous peroxidase. This is achieved by treating de-paraffinised sections with 1% of fresh 30 vol. solution of hydrogen peroxide. Controls incubated directly with DAB are essential to prove inactivation of endogenous peroxidase.
- 4) Blocking of non-specific (background) reactions with normal swine serum is absolutely essential. It has been found necessary to carry out the reactions with the various antisera in a 1 in 20 dilution of normal swine serum to avoid background.
- 5) The optimum dilutions of antisera must be deduced by trial and error. Too high concentrations result in heavy background, are wasteful of expensive antisera, and do not increase the sensitivity of the reaction. Titres of 1 in 40 to 1 in 200 or more are usual in this staining reaction.
- 6) The hydrogen peroxide and the DAB reagents must be of high activity and good quality. Development times MUST BE FIXED (2-3 minutes are adequate for most tissues).
- 7) Controls. The following three controls are necessary for every/...

every sample.

- a) Inactivation of endogenous peroxidase must be proven.
- b) Slides treated without addition of specific rabbit anti-human immunoglobulin sera; normal rabbit serum is substituted.
- c) Slides treated with DAB, but with omission of the peroxidase anti-peroxidase reagent.

In addition to these negative controls, each batch of antiserum must be treated for a positive reaction with a myeloma section secreting immunoglobulin of known subclass.

- d) In some sections the presence of iron containing phagocytes can lead to the mis-identification of immunoglobulin containing cells: a PBR stain on such sections is helpful.
- e) Mast cells in tissue section often stain by these methods, and must be distinguished from Ig containing plasma cells.

The method adopted is outlined below:

- Step 1) Tissue is fixed in 10% formaldehyde solution in saline.
- Step 2) Conventional dehydration and embedding at temperatures below 60°C.
- Step 3) Sections are cut at optimum of 4 microns.
- Step 4) Bring sections to ethanol.
- Step 5) Treat sections with 1% hydrogen peroxide (30 vol) in methanol to inactivate endogenous peroxidase: control sections/...

sections are necessary.

- Step 6) Wash the section in Tris buffered saline, three times for 5 minutes each.
- Step 7) Treat with normal swine serum (1 in 20 dilution in Tris Saline).
- Step 8) Introduce specific immunoglobulin; rabbit anti-human Kappa, or anti-human Lambda at dilutions of 1 in 100, anti-human IgG, IgA or IgM at 1 in 50. These dilutions are made in Tris buffer, or in 1 in 20 normal swine serum.
- Step 9) Wash well in three changes of Tris saline, for 5 minutes each wash.
- Step 10) Treat sections with a 1 in 20 dilution of swine anti-rabbit IgG, diluted in 1 in 20 normal swine serum. Incubate at room temperature for 30 minutes.
- Step 11) Wash sections in three changes of Tris saline, 5 minutes each.
- Step 12) React the sections with peroxidase anti-peroxidase immune complex at a 1 in 100 dilution, either in Tris saline or in 1 in 20 normal swine serum.
- Step 13) Wash sections in three changes of Tris saline, 5 minutes each.
- Step 14) Stain sections in Di-amino-benzidine reagent (DAB) in concentration of 6 mg/10 ml Tris Saline (This solution is made up fresh, is colourless and must be used immediately) with the addition of 2 drops of 10 vol. solution of hydrogen peroxide. Incubate for a constant/...

constant time (3-4 minutes). Staining for longer in the hope of increasing sensitivity will only increase background staining to unacceptable levels.

Step 15) Wash sections well in distilled water, counterstain with haematoxylin, blue up in STWS (differentiating if required).

Step 16) Dehydrate, clear in xylol and mount in DPX.

By this technique, sites of immunoglobulin productions are coloured an intense golden brown. The background should be colourless. Plasma cells show uniform or reticular cytoplasmic staining, proplasma cells (Mature B₄ lymphocytes) show localisation of Ig to the "hof", the clear perinuclear region occupied by the Golgi apparatus. Most lymphocytes do not stain at all in tissue sections, and there is no correlation between pyroninophilia and presence of intracytoplasmic Ig in germinal centre cells.

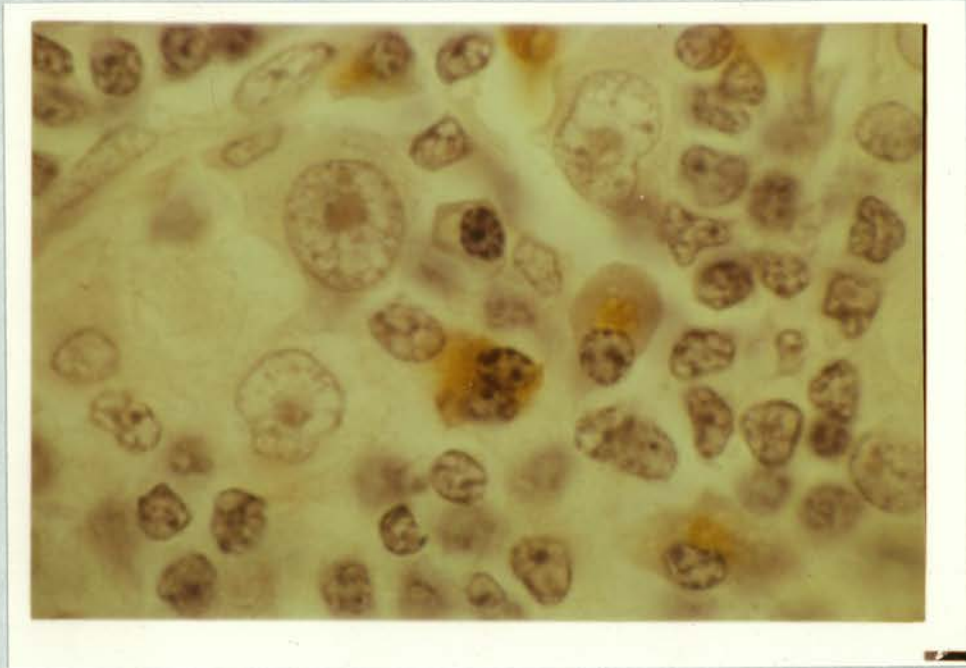


Figure 5: Detection of Intracellular Immunoglobulin by
the Immunoperoxidase (P.A.P.) technique

The illustration shows the typical appearances of plasma cells in a lymph node from a patient with Hodgkin's disease stained by the P.A.P. technique for gamma heavy chain (IgG). Note the localisation of staining to the Golgi area in two cells, and the absence of staining in surrounding cells, including one plasma cell, which is not secreting gamma heavy chain.

P.A.P. (rabbit anti-human gamma chain, swine anti-rabbit serum, and rabbit anti-peroxidase/peroxidase immune complex) Formalin fixed tissue, counterstained haematoxylin.

x 1,000

Preparation of Cell Suspensions

Preparation of Cells

The aim in producing cell suspension from several sources is to recover lymphoid cells from lymph node, spleen, blood and thymus whilst removing contaminating red cells and polymorphonuclear leukocytes. All methods of removing contaminants result in some loss of lymphoid cells, and it is important to ensure that such loss does not favour the selection of one class of cell over another. Once a cell suspension is obtained it is necessary to prove that no selective cell loss has occurred. For this reason it is essential to measure the loss of cells, and to examine smears of cell suspensions before, and after separation to prove the presence of all the initial types of lymphoid cells in the suspension. In the following section the methods of separating cells from blood, spleen, thymus, tonsil and lymph node are described.

The following table shows the recovery of white cells from Ficoll/Triosil separation carried out on normal and pathological blood and spleen.

<u>Sample</u>	<u>Numbers of samples</u>	<u>Recovery as percentage of all white cells</u>	<u>Recovery as percentage of Mononuclears</u>
Normal Blood	28	12%	31%
Patients Blood	24	16%	40%
Normal Spleen	17	-	22%
Pathological Spleen	29	-	23%

As/...

As can be seen, mononuclear cell recoveries are roughly the same order for normal and pathological samples. With a few exceptions, differential cell loss is acceptable, but occasional pathological samples can lose most of the abnormal cells and recoveries must be calculated for each.

Separation of Mononuclear cells from venous blood

Blood was taken into ACD, EDTA, or lithium heparin as anticoagulant. EDTA is the preferred anticoagulant since it prevents platelet clumping and gives cleaner cell suspensions. A white blood cell count was performed, and smears made for differential counting. The blood was then diluted with an equal volume of physiological saline, and layered on Ficoll/Triosil mixture (density 1.076). The bottles were cooled to 4°C, and centrifuged at 1,800 g for 30 minutes at 4°C. The red cells, and nearly all the polymorphonuclear cells clump, and collect at the bottom of the tube. The white cell layer was removed in toto, and washed in medium 199, with care being taken to recover all the white cells. These were finally resuspended in 1-2 ml of medium 199, and counted. Smears were also made for differential counts. The WBC in original volume divided into WBC in recovered mononuclears/x 100 gives the percentage recovery of white cells. The differential count of polymorphs/lymphocytes/monocytes in whole blood, and in the separated cells, indicates which classes of cell are lost, and also enables the mononuclear cell recovery to be calculated:-

$$\text{Mononuclear cell recovery} = \frac{\text{Recovered mononuclears} \times 100}{\text{Total mononuclears in blood sample}}$$

Average/...

Average figures for peripheral blood are 30-40%, with a range of 17-70%.

The separated cells were washed 3 times in medium 199, and finally resuspended in a concentration of 1×10^6 cells/ml.

Viability was determined by trypan blue dye exclusion technique. Using such techniques it is possible to calculate not merely the percentages of cells having a particular receptor, but their absolute numbers in normal and pathological blood. An example is given below:-

MAIR R. Atypical blood, diagnosed as A.L.L. two years ago: No treatment.

WBC count 2.3×10^6 /ml. Differential: neutrophils 10%, Eosinophils 4%, Myelocytes 1%, Monocytes 1.5%, Lymphocytes 11%, atypical lymphocytes 46%. Mononuclear cell count 2.78×10^6 /ml.

Recovery from Ficoll Triosil 44.6%, differential - all mononuclears present.

<u>Rosettes</u>	<u>Percentage</u>	<u>Absolute numbers/ml</u>	<u>Normal Values/ml</u>
E Rosettes	59	1.63×10^6	$1.01 \pm 0.23 \times 10^6$
Fc Rosettes	9	0.25×10^6	$0.74 \pm 0.23 \times 10^6$
C3 Rosettes	9	0.25×10^6	$0.55 \pm 0.26 \times 10^6$
IgM Rosettes	Nil	Nil	0.03×10^6
Neutral Red Phagocytes	4	0.1×10^6	$0.38 \pm 0.12 \times 10^6$
Capping Cells	30	0.82×10^6	$0.55 \pm 0.26 \times 10^6$
Non Capping Cells	Nil	Nil	$0.31 \pm 0.11 \times 10^6$

Comment: There is some increase in T cell numbers, and decreased Fc and C3 receptor bearing cells. Non-capping cells are absent, this is/...

is unusual, but B lymphocyte numbers are normal.

Method of Study of Glass Adherent Cell Receptor Profile

Human peripheral blood mononuclear cells from 25-30 ml of venous blood were separated on Ficoll/triosil and absolute mononuclear cell counts determined (nos. of cells per ml). These cells were washed three times, counted, and mixed with 20% human AB serum or 10% lactalbumin supplement (Habeshaw, 1971) in medium 199. Each batch divided into two equal samples:

Sample 1 was incubated for 6 hours at 37°C in siliconised tubes, gently agitated. It was then washed and receptor profile determined on these cells. This was the profile of normal mononuclear cells.

Sample II was incubated at 37°C for 2 hours in contact with glass, and the non-adherent cells eluted by washing in warmed medium 199. The non-adherent cells were quantitatively recovered from the washings and counted. Since the size of the original inoculum was known, the proportions and numbers of adherent cells could be calculated. Receptors were then quantitated in a standard manner in the normal cells, the non-adherent population, and the adherent cells.

Separation of Cells from

Spleen

Methods of Separation of cells from Spleen

The suspension made by teasing spleen tissue contains numerous red cells. In normal spleen up to 1% of cells recovered are polymorphonuclear, and in Hodgkin's Disease, these proportions can be exceeded. Ficoll/triosil density gradient centrifugation was the method chosen to remove non-lymphoid contaminants from spleen cell suspensions, although frequently recoveries of mononuclear cells were of a low order (mv 27%). Because of its variable cellular nature, and consistency, preparing cell suspensions by mechanical rather than enzymic means proved the most successful. The method was to tease out small fragments with a scalpel blade, followed by repeated washing with pipetted tissue culture medium. Stromal cells (fibroblasts, and endothelium) are probably not represented in the spleen cell suspensions so prepared.

In order to compare diseased spleens with normal spleens, some parameter was required which would accurately reflect alterations found in disease. It was therefore decided to measure accurately the spleen weight and total spleen cell count, and from these values to derive the quantity "spleen cell concentration" (number of cells per gm of wet spleen weight). In establishing the methods of spleen cell concentration measurement some control determinations were required, and these are now presented.

When a spleen is removed at operation, the organ contains a variable amount of blood, as a consequence of the surgical techniques employed. The blood removed with spleen can be estimated/...

estimated by (1) weighing the spleen in toto, (2) slicing the spleen finely into 2mm thick sections and allowing the slices to drain, (3) collecting the blood and weighing again. Subtraction of the weight of blood from the total weight of the spleen gives true spleen weight, which is the value used in these estimations. The average normal spleen in this series yielded between 25 and 60 grams of blood, which contains about $2-3 \times 10^6$ white cells per ml; i.e. 50×10^6 to 180×10^6 white cells, for a mean spleen weight of about 150 grams. Since the spleen contains about 500×10^6 recoverable white cells per gram of spleen weight, the blood contained in the spleen does not significantly affect the figures for normal total spleen cell count (which are of the order of $4-8 \times 10^{10}$ cells).

The determination of the total spleen cell count ideally requires that the whole spleen should be reduced to a single cell suspension after weighing, and representative counts made from the resultant suspension. This is impractical for the routine determination of cell counts on pathological spleens where some tissue may be required for other purposes. To investigate alternative methods of quantitation the following experiment was performed:

The spleen was weighed and sliced. From the organ about 1 gm of tissue was removed, at random, avoiding areas containing much vascular or connective tissue. The remaining spleen was placed in an homogeniser and reduced to a single cell suspension, the volume carefully measured, and cell counts done on the suspension. The cell count per ml multiplied by the total volume then gave the total recoverable white cell count.

The/...

The 1 gm of tissue removed before homogenisation was very accurately weighed in a covered Petrie dish to avoid evaporation and then rendered into a single cell suspension in a loose fitting all glass tissue grinder. The residue was washed carefully to recover all white cells quantitatively and the cell count and total volume accurately measured. The total cell count was obtained by multiplying the spleen weight (in grams) by the cell count per gm of spleen tissue. Comparison of the values so obtained on 5 spleens showed them to agree with $\pm 10\%$, which is of the same order as the "counting error" inherent in the white cell counting technique ($\pm 6\%$). It was thus shown that the total spleen cell count can be reliably determined from as little as 1 gm of spleen tissue. This method was subsequently adopted as standard technique.

Comparison of total cell counts obtained by total or subtotal spleen homogenisation, with counts estimated from small amounts of splenic tissue. Material consisted of 4 P.M. spleens, and one (3) Therapeutic Splenectomy.

No.	Spleen cell count (Homogenised)	Spleen cell count (Estimated Total)	\pm Difference %
1	4.14×10^{10}	4.70×10^{10}	$\pm 11.9\%$
2	6.59×10^{10}	6.01×10^{10}	$\pm 8.8\%$
3	1.025×10^{10}	1.07×10^{10}	$\pm 5.4\%$
4	9.70×10^{10}	9.08×10^{10}	$\pm 6.39\%$
5	6.04×10^{10}	5.44×10^{10}	$\pm 9.94\%$
		Mean variation	± 8.43

Cell Recovery

The effect of different methods of separating mononuclear cells from the whole spleen cell suspension was assessed as follows:

The spleen cell suspension, diluted in medium 199 + heparin (10 units/ml) was layered over Ficoll/triosil mixture, and centrifuged at 1,800 g for 30 minutes at 4°C. Smears were made from the initial suspension and from the recovered white cells, and differential counts (polymorphonuclear and mononuclear cells) were made on both. The initial cell count, divided into the cell count of recovered white cells, x 100 gave the white cell recovery as a percentage of the starting suspension. Recoveries were variable but much lower than those in peripheral blood (Mean Value 27% range 17-47%). In order to prove that selective loss of one cell class had not occurred, lysis of red cells by ammonium chloride was performed. In this case, red cells only were destroyed, and polymorphonuclear leukocytes were present with mononuclear cells in the recovered suspension. There were no significant differences in the numbers of T cells and B cells so obtained, but values for Fc and C3 receptors and phagocytes were markedly different, depending upon the polymorphonuclear contamination.

Knowing the white cell recovery from Ficoll/triosil and the differential counts before and after separation it proved possible to calculate the mononuclear cell recoveries, and these were of the same order as previously determined (22%). These values are listed in the previous section.

The technique of preparing spleen cell suspension is shown/...

shown below.

1) The spleen was cleaned of adherent fat and blood clot, and accurately weighed in a 4 litre sterile glass beaker.

2) The organ was then sliced into 2-4 mm thick slices using a sterile skin graft knife blade. The blood running from the cut surface was collected on impervious "Benchcote" material (Waterproof side up) and returned to the weighing beaker.

3) The beaker + blood was reweighed, and by subtraction the true spleen weight was determined.

4) From the spleen slices (from several separate areas of the spleen) small chunks of tissue were gouged out avoiding major vascular, capsular and trabecular tissue. These were placed in a pre-weighed Petrie dish which was covered to avoid evaporation and accurately weighed. A cell suspension was then made by grinding the tissue gently in medium 199 + heparin (10 units/ml) and the residue washed well to ensure quantitative recovery of cells. The cell count was performed and the numbers of cells per gram of spleen weight were determined. This value multiplied by the true spleen weight then gave the total spleen cell count.

5) From the remaining spleen, blocks for (a) histology, (b) special histology, e.g. E.M. and immunoperoxidase and (c) cell suspensions, were removed. (a) and (b) were appropriately fixed.

6) The tissue for cell suspensions was teased apart with fine pointed scalpel blades, and squeezing, slicing, and washing in medium 199 + heparin, rendered into a single cell suspension. Large/...

Large clumps of cells, and residue were removed by filtration through fine stainless steel gauze (80 or 120 mesh, Granton Wire Works, Edinburgh). Viability was assessed by trypan blue dye exclusion.

7) Smears for differential counts were made, the total cell count determined, the suspension was layered over Ficoll/triosil and centrifuged at 1,800 g for 30 minutes at 4°C.

8) The white cell layer was recovered from the Ficoll/triosil washed three times in medium 199, and the total white cell count determined. Smears were made for differential counting.

9) From these results, the following values were calculated and noted: (a) total spleen cell count, (b) spleen cell concentration (cell number per gram spleen weight), (c) cell recovery and mononuclear cell recovery and (d) viability. The cell suspension was now used to prepare rosettes in the standard manner.

Separation of Cells from
Tonsil, Lymph Node, Thymus and
Cell Lines

Preparation of Lymphoid Cells from Human Tonsil

Tonsils were collected fresh from the operating theatre into sterile containers holding medium 199, with penicillin (2,000 i.u.) and streptomycin (10 ug) per ml. The tonsil and medium were then immersed in melting ice for conveyance to the laboratory.

On arrival the tonsil was sliced into flat sections 1 mm - 2 mm in thickness, and laid in a sterile Petri dish. The surface was moistened with medium 199 and using a fine scalpel blade, and where necessary a dissecting microscope, the foci of lymphoid tissue were lifted out on the tip of the blade. Care must be taken to avoid (a) bacterial and fungal colonies commonly found in tonsillar crypts and (b) squamous epithelium which is in close proximity of the lymphoid follicles. The lymphoid cells separated were pipetted through 80 mesh stainless steel gauze to remove cell clumps and disrupt germinal centres. They were then washed three times. Viability was assessed by trypan blue dye exclusion, and the cells reacted with red cells to form rosettes as previously described.

Control sections from material dissected from tonsil showed that the bulk of the tissue removed was of germinal centre lymphoid type with a small addition of stromal lymphocytes and connective tissue. The receptor profile of tonsil is regarded as being similar to germinal centre in lymph node.

Separation of cells from Lymph Node

Lymph nodes were collected fresh from the operating theatre, and/...

and immediately transferred to a sterile container containing medium 199. The node and medium were then cooled in melting ice for conveyance to the laboratory. On arrival the node was sliced using a skin graft knife blade, into 2 mm thick sections. Care was taken to cut the node as evenly as possible. Sections for routine histology, and for immunoperoxidase technique were fixed in formalin. Special E.M. histology was taken and fixed in glutaraldehyde (3%) in cacodylate buffer. The slice for making cell suspensions was teased in medium 199, and the suspension filtered through 80 mesh stainless steel gauze to produce a single cell suspension. Smears of this suspension were made, and the cells washed three times in medium 199. Viability was assessed by trypan blue dye exclusion, the cells counted and receptor profiles determined as previously described.

Dabs, fixed and unfixed, were prepared from the freshly cut lymph node surface, and stained with Giemsa stain, or used for histochemistry.

Lymph nodes showing viability of less than 60% were not used for study.

Separation of cells from Thymus Gland

Thymus glands collected either at autopsy from newborn or from stillborn children, or glands removed in children undergoing cardiac surgery were collected into medium 199, and cooled in melting ice for conveyance to the laboratory. On arrival they were sectioned and teased into a cell suspension. In order to prevent cell clumping, this was generally done in 10-20% fresh human/...

human Ab serum, or in pancreatic DNA'se to remove DNA which tends to gel the resulting cell suspension. Once the cells have been separated and washed, subsequent steps can be carried out in serum free medium 199. Viability of the suspension was assessed, and rosetting tests performed as before. Sections of thymus were examined for the presence of germinal follicles.

Cells from Lymphoid cell lines

Lymphoid cell line cells were grown in RPMI medium, or medium 199, with 10% foetal bovine serum. Cells were harvested at a density of $1-2 \times 10^6$ cells/ml at the end of the rapid log growth phase. This ensured that most cells were not in the pre-mitotic phases of the cell cycle, and hence that the expressed receptor pattern was as stable as possible under culture conditions. Cells were washed in medium 199, the viability assessed, and rosettes set up as previously described.

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RESULTS

CHAPTER 15

The Receptor Profile of Normal Lymphoid Tissues

- 1) Receptor profile of glass adherent and non-adherent cells
- 2) Receptor profile of normal blood mononuclear cells
- 3) Receptor profile of cells from normal spleen
- 4) Receptor profile of cells from normal tonsil
- 5) Receptor profile of cells from normal thymus
- 6) Receptor profile of lymphoid cell lines
- 7) Receptor profile of normal and reactive lymph node.

The term receptor profile is used to describe the overall pattern of surface features expressed on cells derived from a particular tissue.

In the author's experience, and as shall be demonstrated, no normal tissue containing lymphoid cells produces a profile consistent with there being only one class of lymphoid cell present. The result of quantitating cells with different surface features in blood and in lymph node is to produce a value for the proportion of cells with each surface feature which is more or less characteristic for the tissue examined. This is called the receptor profile of that tissue. The purpose of the investigations carried out in this thesis was:-

a) to establish receptor profiles for each normal tissue examined, for example blood, spleen, thymus, lymph node and tonsil, and to compare these profiles with those resulting from similar tissue in lymphoma. The degree of difference in the profile of the normal as against the lymphoma lymph node allows the comparison of normal with neoplastic cell population.

b) To use such techniques of comparison to investigate whether lymphomas of one particular histological type produced a receptor profile characteristic of that class of lymphoma.

c) To deduce from the study of normal versus lymphoma receptor profiles, whether certain profiles could be correlated with the subsequent clinical behaviour of the tumour. The control studies necessary to achieve these aims are reported below.

Normal Receptor Profiles

1)/...

1) The receptor profiles of adherent and non-adherent mononuclear cells from blood was studied. The results indicate that a proportion of glass adherent cells expressed Fc and C3 receptors and that the majority of such cells ingested neutral red dye in a characteristic fashion. This demonstrated that phagocytic cells in blood exhibited the pattern of neutral red ingestion, glass adherence, and expression of Fc and C3 receptors.

2) The receptor profile of blood, using both proportional and numerical quantitation of receptors, showed that E rosetting cells formed the majority population and that in common with previously published results the expression of this receptor correlated with the presence of cells with similar receptor profiles derived from the thymus gland. These cells are T lymphocytes, known to be the major population of recirculating lymphocytes from animal experiments. The B cell population, identified by the presence of surface immunoglobulin was shown to consist of at least two classes of cell, based on the differences of surface behaviour following complexing of fluorescein labelled antibody with the surface Ig. The B cells showed either the capacity to cap surface Ig, so called capping cells, or failed to cap their surface Ig, so called non-capping cells. This proved to be an important phenotypic difference between B cell classes.

Fc and complement receptors on peripheral blood cells were expressed on the phagocyte population - a proportion of which always expressed Fc receptors and complement receptors - and the B cell population. The non-capping B cell population expressed Fc/...

Fc rather than complement receptors in blood.

3) The spleen lymphocyte population in health is drawn mainly from the blood, as shown from animal experiments discussed in the introduction. As expected, receptor profile of spleen reflects the profile of normal blood, save that more B lymphocytes are present and the concentrations of lymphoid cells are very much greater. The same classes of cell are present as in blood, with T lymphocytes, phagocytes, and capping and non-capping B lymphocytes being identified. The most important discovery was that in "normal" spleen, despite identical proportions of B cells T cells and macrophages in the spleen in each sex, the quantitative distribution indicates a clear sex difference. Males have more cells in the spleen than females - a fact quite unknown until the present study.

4) An important constituent of lymphoid tissue is the germinal follicle. Experimentally it has been shown to be a focus of lymphoblast transformation in the later stages of the secondary response, although its functions are quite unknown. It was considered possible that the lymphoid population of this organelle might be unique, and probably B cell in type. The difficulty of obtaining germinal centre tissue was overcome by using tonsil, which because of its unique structure, lacks many features characteristic of lymph node. Among these are the absence of lymphatic sinuses (a macrophage rich area), and medullary islands (where most primary response B lymphocytes occur) and the paracortex (a T cell area). The receptor profile of tonsil therefore should reflect the profile of transformed lymphocytes/...

lymphocytes of secondary response type:- the precursors of secondary response antibody forming B cells (plasmablasts), or the recirculating "memory" B lymphocyte. As the relevant section shows a "follicular" receptor profile did indeed characterise tonsil, and this was unlike the profile of normal blood or spleen. The follicular B lymphocytes of tonsil are either (1) a mixture of capping B lymphocytes expressing complement but not Fc receptors, with a minority of non-capping B lymphocytes expressing Fc receptors or (2) a mixture of a minority of capping B lymphocytes which express Fc receptors with a non-capping B lymphoid population expressing the C3 receptor. Either of these alternatives is possible, and it is thought may reflect the reactive state of the follicle at the time of sampling.

5) The receptor profile of the normal thymus gland, as expected shows a preponderance of E rosetting cells (T cells). A significant but small non-T cell population composed of B cells and macrophages also occurs: these may represent the medullary lymphoid populations known from experimental studies of thymic lymphopoiesis.

6) The receptor profile of several B lymphoid cell lines was studied as a test of the capacity of the cell in culture to retain surface phenotype. It was noted that although within any single line, the expression of receptors was rather variable, the features of capping and non-capping of surface Ig were present. C3 and Fc receptors were expressed, only by capping cell lines. In one cell line of non-capping cells only Fc receptor was expressed. This data confirmed the findings relating capping and non-capping behaviour to expression of Fc and/...

and C3 receptors, as found in studies of blood, and tonsil.

7) The receptor profile of normal and reactive lymph nodes served as a model for assessing the lymphoma. Here the degree of admixture of cells, T and B lymphocytes and macrophages indicated possible ways of classifying lymphoma. The characteristic receptor profile of blood, and of tonsil, were seen in normal and reactive nodes. In addition three nodes showed mature capping B cells with no Fc or C3 receptor. On the basis of the receptor profiles for each of the normal tissues and for the lymphoid cell lines a scheme whereby lymphoid populations could be assessed in a single node, and that node classified by means of its receptor profile, was developed. It is this scheme, applied to lymphoma lymph nodes, which was adopted as a method of classifying malignant lymphoma by the surface phenotype of its constituent cells. A sufficient experience of normal material enabled accurate comparison between normal and lymphomatous tissue, irrespective of the primary origin of the lymphoma. The following sections deal with the data from normal lymphoid tissue.

- 1) Receptor profile of Adherent
and Non-Adherent Cells.

Receptor Profile of Glass Adherent Cells

When peripheral blood mononuclear cells, obtained in a high degree of purity, are separated and adhered to glass in the presence of human AB serum, a mean 24.6% of cells adhere and $76\% \pm 7.73\%$ of cells are non-adherent.

The adherent population shows the presence of neutral red phagocytes ($91.38 \pm 4.65\%$) which also express receptors for sheep cells ($1.0\% \pm 1.7\%$) IgG ($46 \pm 12\%$) and EAC ($59 \pm 17\%$). A proportion of glass adherent cells also exhibit fluorescence by the sandwich technique showing diffuse surface fluorescence with occasional confluent rings or caps (6.0%).

The act of adhering cells to glass alters the total numbers of cells expressing receptors when total numbers of each receptor in the glass adherent and non glass adherent populations are summed, and compared with the numbers in the original suspension. The chief gain is in cells expressing phagocytic potential. Upon exposure to glass nearly all adherent cells exhibit the capacity to ingest neutral red. In the residual population a variable but diminished number of cells still exhibit neutral red phagocytic capacity ($6.8\% \pm 6.0\%$). Exposure to glass diminishes the numbers of cells, compared with the original sample, that express E receptors. The proportions of cells expressing Fc and complement receptors are increased. Thus the exposure of peripheral blood mononuclear cells to glass,

- 1) stimulates uptake of neutral red
- 2) increases proportions of EAC and Fc receptor cells
- 3)/...

3) diminishes numbers of E receptor cells.

TABLE

Receptor profile of Glass Adherent, Non Adherent, and Whole Blood Mononuclear Cells. (From Ficoll/triosil separated mononuclear cell suspension).

	<u>Adherent</u>	<u>Non Adherent</u>	<u>Whole Blood</u>
Proportions	24.6 ± 7	76.4 ± 8	100%
Phagocytes	91 ± 5	5.8 ± 6	14.7 ± 3.0
Fc Receptors	46 ± 12	32 ± 15	24 ± 8.0
EAC Receptors	59 ± 17	35 ± 9	24 ± 6.0
E Receptors	4.0 ± 1.7	36.6 ± 6	56.8 ± 8.0
Surface Ig Cells	6.0 ± 1.3	28.7 ± 8	29.0 ± 10

This experiment shows that following depletion of adherent cells, the non adherent population is relatively enriched for B lymphocytes (surface Ig bearing cells). Many E rosetting cells fail to rosette following glass adherence. The presence of adherent T cells and B cells, admittedly in small numbers is surprising. The fact that adherence to glass also appears to change in a subtle manner the reactivity of the population, enhancing Fc and EAC rosetting, and increasing from 14% to 20% the absolute proportions of neutral red ingesting cells precludes the use of glass adherence as a method of purifying lymphocytes. This work was instrumental in influencing the decision to quantitate receptors on unfractionated cell populations from lymphomas, thus disturbing/...

disturbing receptor patterns as little as possible. It should be noted that not all glass adherent cells express Fc or C3 receptors, despite phagocytosis of neutral red.

2) The Receptor Profile of
Blood Mononuclear Cells.

Receptor Profile of Cells from Normal Blood

The receptor profile obtained from healthy individuals was remarkably reproducible, even when mean cell recoveries from Ficoll/triosil were low (30% or so). Variation in individual normal values is of the order of 20%, while the accepted normal mean variation for white cell counting (by the chamber method) is $\pm 6\%$. The numbers of B lymphocytes sheep cell rosetting, and phagocytic cells can be obtained by calculation from the mononuclear cell recovery, the differential count, and the white cell count. This has obvious value in investigating the blood in lymphoma or leukaemia, providing the test is well understood, and its drawbacks known.

The receptor profile of peripheral blood mononuclear cells is shown in the accompanying table.

Receptor of peripheral blood mononuclear cells.

	<u>Percentage</u>	<u>Absolute values</u> <u>($\times 10^6$/ml)</u>	<u>Range (SD)</u>
E	46.9%	1.01	0.23
Fc	34.4%	0.74	0.23
G3	25.6%	0.55	0.26
IgM	1.40%	0.03	-
Phagocytes	17.7%	0.38	0.12
Capping	25.6%	0.55	0.26
Non-capping	14.4%	0.31	0.11
Total Fluorescence	40.0%	0.86	-
Total Cells	104	2.15	± 0.43

The/...

The figures shown are derived from the calculation of numbers of rosettes per 10^5 white cells. The mean recovery of mononuclear cells was 34.39% for FT separated blood, and 46.82% for dextran sedimented blood: the mean values shown were obtained by combining data from the two separation procedures. The levels of B cells are higher, and T cells lower in dextran separated blood than those for Ficoll/triosil separated blood in which E rosettes form 56.2% and surface Ig positive cells 30% of the separated mononuclears. The standard error of counting is assessed as $\pm 6\%$, percentage variations less than this are probably of little significance.

Several observations are noteworthy and these are listed below:

a) The numbers of complement rosetting, and surface Ig capping cells are virtually identical, and this obtains for individuals as well as over the whole series.

b) The numbers of neutral red ingesting and surface Ig bearing non-capping cells are also nearly identical, and, at first this suggested that non-capping cells were monocytes or macrophages. Data from the glass adherent cell population (which was virtually all neutral red positive and surface Ig negative) proved that the non-capping population did not overlap substantially with the neutral red ingesting population.

c) The non-capping B lymphocytes population expresses the Fc receptor not the receptor for complement, since Fc receptor bearing cells exceed complement receptor bearing cells by a similar degree as capping cells exceed non-capping cells.

Assuming/...

Assuming that the phagocyte population is separate and expresses both Fc and C3 receptors (as suggested by the adherent cell data), the excess of Fc receptor must be (i) either on T cells or (ii) upon non-capping surface Ig bearing lymphocytes.

d) The figures obtained for neutral red ingesting glass adherent cells consistently exceeded the numbers of morphologically identifiable "monocytes" in the peripheral blood smears. This finding suggests the existence of a population of cells, cytologically lymphocyte like which ingest neutral red dye, and are potentially glass adherent phagocytes. Studies with anti-monocyte serum show similar proportions (i.e. 15% or so) of peripheral blood mononuclears expressing monocyte specific surface antigens as were obtained in this study with neutral red ingestion (Stuart, A.E., personal communication).

These findings suggest a modification of the strict sub-classification of peripheral blood mononuclears into phagocytes, T lymphocytes and B lymphocytes. It is evident that two classes of surface Ig bearing cells are present, one showing capping surface Ig and expressing both complement and Fc receptors, and one showing non-capping behaviour and expressing Fc receptor.

The blood mononuclear cell population demonstrated by these experiments, with the potential size of each cell pool, is shown below. Note that in Ficoll/triosil separated blood T cell proportions are higher, and B cell proportions lower than those given here, by about 10%.

T Lymphocytes	- 47%
B lymphocytes	- capping surface Ig, Fc and C3 receptors/...

- receptors 26%.
 - non-capping surface Ig,
Fc but not C3 receptor
14%
 - adherent neutral red
ingesting, Fc, C3 receptor
positive cells 17%
- Phagocytes
- Total cells by addition of the above - 105%

It is therefore unlikely that any significant "receptor silent" or "null receptor" population exists in normal blood. B cells are of at least two classes which differ in respect of surface Ig expression, and in subsidiary expression of C3 receptor.

3) The Receptor Profile of
Normal Spleen.

Cellularity and receptor profile of normal spleens

The study contains, naturally, rather few examples of "normal" spleens. The spleens referred to as "normal" have two things in common: an arbitrarily selected maximum weight of 350 gms, and they satisfy histological criteria of normality.

Those investigated were

- 1) Presence of germinal centres: few or none is normal.
- 2) Presence of stainable iron: a little is normally present.
- 3) Absence of necrosis or fibrosis of the red pulp.
- 4) An absence of polymorphonuclear leukocyte infiltration of red pulp.
- 5) An absence of major plasma cell infiltrates or of granulomata.

Normal Spleen

The spleen weight is in grams, cell concentration is in numbers of cells per gram wet weight of spleen tissue.

<u>Patient</u>	<u>Age</u>	<u>Sex</u>	<u>Spleen Wt.</u>	<u>Cell concentration</u>	<u>Diagnosis</u>
N1	57	M	92 gms	716.3 x 10 ⁶	P.M. - MI.
N2	26	M	151	907.3	P.M. - drowned
N3		M	121	838.8	P.M. - RTA
N4		M	272	466.9	Laparotomy N.
N5		M	103	599.0	Ga Stomach
N6	14	M	185	432.4	Traumatic
N7	63	M	118	497.5	Traumatic
N8	47	M	150	594.7	Traumatic
N9/...					

<u>Patient</u>	<u>Age</u>	<u>Sex</u>	<u>Spleen Wt.</u>	<u>Cell concentration</u>	<u>Diagnosis</u>
N9		M	150 gms	100.0 x 10 ⁶	Cystic Spleen
N10	68	M	128	765.6	Laparotomy
N12	63	F	160	377.5	P.M. - MI.
N13		F	160	254.4	P.M.
N14	35	F	107	486.0	Traumatic
N15		F	97	458.8	Traumatic
N16	38	F	318	184.6	Chondroma
N17	43	F	95	442.1	Traumatic

These criteria removed from the "normal" spleen series most of the spleens from patients with haemolytic anaemia, or portal hypertension. It was found that spleens from patients with carcinoma of stomach showed decreased numbers of T lymphocytes and lower cellularity than "normal" spleens whilst showing no abnormal histological features (Habeshaw and Stuart, 1974). All except one carcinoma of stomach spleens were excluded from the series. It was demonstrated by statistical analysis (the author would like to thank Miss S.M. Gore who performed these tests) that significant differences were obtained when spleens were compared by sex of the spleen donor. These features are shown in the accompanying table.

Receptor Profile of Normal Spleen; comparison male with female.

Percentage/...

	<u>Percentage</u>		<u>Cells/gm</u>		<u>Total Cells</u>	
	<u>M</u>	<u>F</u>	<u>M</u>	<u>F</u>	<u>M</u>	<u>F</u>
E	40%	40%	233.6×10^6	187×10^6	3.4×10^{10}	2.99×10^{10}
Fc	34	30	200.9×10^6	111×10^6	2.95×10^{10}	1.73×10^{10}
C3	32	28	199.7×10^6	106×10^6	2.77×10^{10}	1.66×10^{10}
IgM	-	-	-	-	-	-
Neutral red Phagocyte	10	17	60.08×10^6	62×10^6	0.88×10^{10}	0.97×10^{10}
F capping	33	29	193.0×10^6	108×10^6	2.83×10^{10}	1.69×10^{10}
F.N. caps	12	12	72.8×10^6	44.6×10^6	1.06×10^{10}	0.68×10^{10}
Total cells	95	98	591.5×10^6	367.6×10^6	8.37×10^{10}	4.97×10^{10}
Weight	166 gm	156 gm				

Note difference in cells/gm and total cells between males and females are significant (P 0.05). Females have fewer T cells and comparatively more macrophages in the spleen than do males. Data from 17 spleens, 11 males and 6 females.

The use of the cell concentration as a major variable in assessing spleen cellularity is forced upon the investigator by the variability of the organ itself. It is accepted that the biological significance of spleen cell concentration is not known, but the fact that significant differences in this parameter for males and females is shown suggests that the variable measured is a real biological phenomenon.

4) The Receptor Profile of Tonsil.

Receptor Profile of Tonsil tissue

The receptor profile of tonsil was regarded as being representative of germinal follicular lymphoid tissue, as material separated from tonsil was largely of germinal centre origin. (The examples quoted form a part of the thesis of Mr. Paul Wilson (1976) under the supervision of the author.) There is wide variation in the results from tonsil lymphoid tissue receptor studies, as could be expected from the nature of the material under study. Nonetheless a characteristic receptor profile has emerged from the tonsil, as shown in the accompanying table.

Receptor Profile of Germinal Centre Cells (Tonsil) 32 Samples.

	<u>Percentage</u>	<u>Range of Values</u>	<u>SD</u>
E	36	(18 - 53)	$\pm 8\%$
Fc	9	(1 - 18)	$\pm 5\%$
C3	20	(6 - 35)	± 11
IgM	1	-	-
Phagocytes	3	(1 - 7)	± 2
Capping	17	(4 - 32)	± 4
Non Capping	15	(1 - 35)	± 9
Total B cells	32		

Total Cells 71% Mean Viability 70%

(E + NR + FL)

Note that in Tonsil non-capping cells are nearly as common as capping cells, and in a few instances non-capping exceed capping cells.

As/...

As in blood, two classes of B lymphocytes are present, those with capping surface immunoglobulin and those with non-capping surface immunoglobulin. The proportion of non-capping surface immunoglobulin bearing B cells is much greater in tonsil than in normal blood, spleen or lymph node. There is an excess of complement expressing B cells over those expressing Fc receptors. The major immunoglobulin class associated with tonsil germinal centre B lymphocytes is IgG rather than IgM as with peripheral blood B lymphocytes, and these cells may have their Fc receptors already occupied by their own, or other cells surface IgG. The immunoperoxidase stain has failed to show significant intracellular immunoglobulin in any germinal centre in either tonsil or lymph node.

In the absence of a paracortex in the tonsil, the T lymphocyte proportions are surprisingly high, and must reflect the presence of T lymphocytes within germinal centres themselves, or within the closely attached corona of lymphocytes which surround the germinal centre. Nevertheless T cells are a constant population, and must be regarded as a normal component of follicular lymphoid tissue.

Studies of the secondary immune response in experimental animals show the follicle to arise in B lymphocyte areas of the lymph node cortex. Germinal follicles localise antigen following an IgM primary response, or in a secondary immune response. Antigen penetration is slow, and is accompanied by progressive lymphoblastic transformation of follicular cells. The follicular cell profile is not derived from any single lymphocyte class but from these studies it is a mixture of four sub-classes of B lymphocytes.

1)/...

- 1) Capping, with Fc and C3 receptors
- 2) Capping, with C3 receptors and no Fc
- 3) Non-capping expressing Fc receptors
- 4) Non-capping expressing C3 receptor.

Results from individual tonsils support the existence of these classes and the "follicular profile" is commonly found in lymph node. The follicle profile arises from a mixture of several sub-classes of B cell.

The follicular profile is either (a) or (b).

- a) Capping B cells with Fc and C3 receptor as a minority population associated with a non-capping B cell expressing C3 receptor as the majority population.
- b) Capping B cells with C3 receptors and no Fc receptor as a majority population, with non-capping B cells expressing Fc receptors as the minority population.

5) The Receptor Profile of Thymus
Gland.

Receptor Profile of Human Thymus Gland

Some difficulties were encountered when assessing the receptor status of human Thymus gland cells. One feature was the way in which receptor profile varied with the viability, when viability was low, proportionally fewer T cells and more B cells were encountered. No attempt was made to separate thymic lymphoid populations into cortical and medullary components, and the finding on at least two occasions of medullary germinal centres in histological sections of the sampled thymus gland indicates that thymic lymphocytes may not be as "pure" as some published work might suggest. In the data presented about 10% of the cells in human thymus were non-T lymphoid (B cells) or phagocytes. The receptor status of medullary epithelial cells is not known, and they were sparsely represented in the samples studied.

Receptor Profile of Human Thymus Gland

(19 Determinations, 7 foetal, 12 child thymuses; No adult)

	<u>Mean Percentage of Total Cells</u>	<u>Range</u>
E Rosettes	74	(31.8 - 92.5)
Fc	9.6 (15.2)*	(1.9 - 40.4)
C3	5.24(10.18)*	(0.2 - 25.3)
Phagocytes	1.7	(1 - 3.5)
Surface Ig	14.23 (19.32)*	(0.2 - 57.5)
Mean Viability	83.5	(67 - 99)

The receptor profile of the thymus gland shows a preponderance of E rosetting cells, and relatively few B lymphocytes. As shown by/...

by * two thymus samples in the series showed presence of at least one germinal centre; the figures in brackets show the effect of including data from these cases. As a consequence of poor red cell sensitisation, in some cases the levels of EAC or EA₁gG and E rosettes detected exceeded total viability. Data from these cases has not been included.

6) Receptor Profile of Some Lymphoid
Cell Lines.

The receptor profile of several lymphoid cell lines was studied with two aims in view:- firstly to see if distinctive receptor profiles were present in lymphoid cell lines and secondly to see if any of the receptor profiles identified in blood, or tonsil lymphocyte populations were stable in tissue culture over a large number of cell divisions.

Receptor Profile of Lymphoid Cell Lines

Figures given are percentages of total cells.

<u>Cell Line</u>	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>IgM</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Total Surface Ig</u>
Jijoye	5.0	0.4	0.4	0	0	Nil	78
GSI	6.0	20	11.0	0	11.0	Present	68.6
BLAI	3.0	57.0	13.0	3.0	27.3	Present	72.0
EB4	Nil	5	0.3	0.2	0.8	Present	76
RUS2	2	0.7	4	0	5	Nil	56
DAUDI	3	32.0	3	3.0	21	Nil	52
RAJI	8	16	2.0	0	8.0	Nil	68

The results for each cell line were rather variable and dependent upon culture conditions. Expression of surface Ig was enhanced by washing in acetate buffer. The cells classed as "phagocytes" showed coarse often single granule uptake of neutral red. Fc and C3 receptors are only expressed in cell lines showing capping behaviour. The expression of Fc receptors by the DAUDI cell line is rather variable; this cell line has no capping cells. Expression of surface Ig, in most instances was diffuse and non-capping rather than "dotty" in distribution. The data is the mean/...

mean values of 5 separate determinations of receptors on each cell line.

The cell lines showed some interesting characteristics:-

a) The cells composing them all secreted Ig on their surface, and the expression of surface Ig was enhanced by washing in acetate buffer (as reported by Hutteroth, Litwin and Cleve, 1972).

b) Of the cell lines reported 4 showed no capping surface Ig bearing cells whilst three showed the presence of capping cells.

c) Two out of three capping cell lines expressed complement receptor.

d) No non-capping cell line expressed complement receptor.

e) One non-capping cell line expressed the Fc receptor strongly (DAUDI) and another (RAJI) weakly. EB4 cell line (derived from Burkitt's lymphoma) did not express Fc or C3 receptors, but contained capping surface Ig bearing cells.

Surface Ig expression was difficult to demonstrate, but with the immunoperoxidase technique the cell line EB4 was shown to have weak intracytoplasmic immunoglobulin (γ heavy chain only) with surface Kappa chain. This is illustrated.

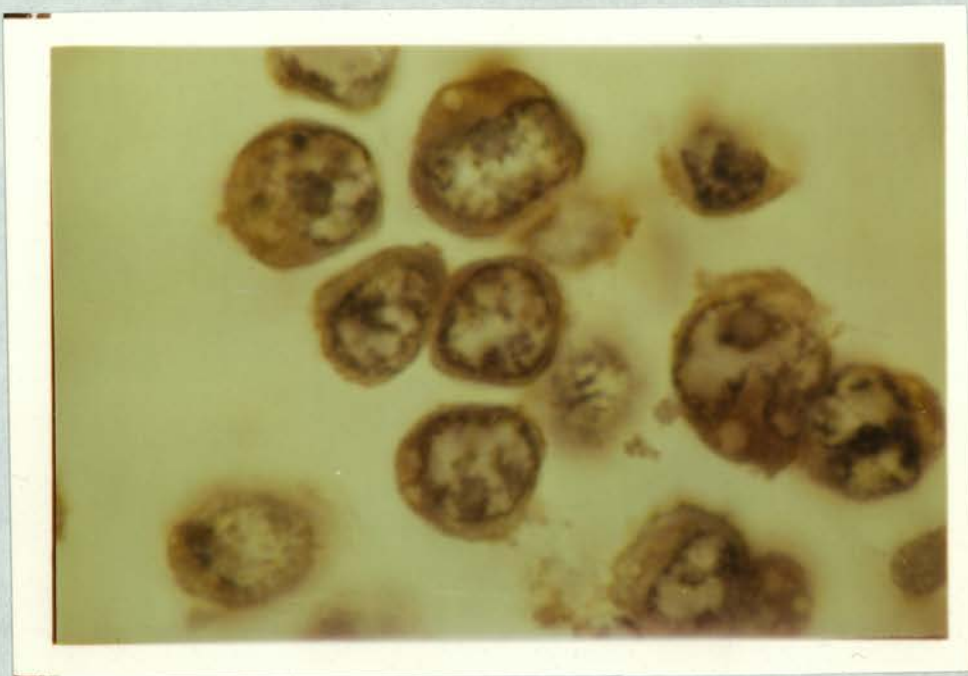


Figure 6: Detection of Intracellular Immunoglobulin by the Immunoperoxidase (P.A.P.) technique

Cells of the EB₄ cell line, illustrated here, showed capping of surface Ig detected by immunofluorescence with polyvalent antiserum, but had no Fc or C3 receptors. Immunoperoxidase reaction revealed gamma heavy chain intracellularly, reactions with Kappa, lambda, IgM, IgA were negative. This profile was unlike that of B1 lymphocytes (non-capping and intracytoplasmic Ig negative) found in other cell lines cultured from Burkitts lymphoma. The cells would thus be classified as B₄.

Formalin fixed cell deposit stained for gamma chain by P.A.P. technique. Counterstained Haematoxylin.

x 1,000

RESULTSThe receptor profile of normal and reactive lymph node cells

The results of receptor studies on normal and reactive lymph node cells is shown in the accompanying tables. Study of these receptor profiles show some interesting features listed below:

a) In most instances the numbers of fluorescent capping cells, i.e. those which cap surface Ig, are in predominance over the non-capping cells. In some reactive nodes this effect is not seen, especially in two cases where non-capping cells equal or exceed capping cells (cases Blair and Rae.).

b) In some nodes showing follicular hyperplasia, the receptor profile is similar to the receptor profile of tonsillar lymphoid tissue where most cells are derived from germinal centre. In these cases, the numbers of complement rosetting B cells exceed the numbers of B cells expressing Fc receptors.

c) In nodes with marked plasma cell reactivity, the numbers of capping B cells are generally high, the complement and Fc receptor values are low, and a receptor silent population is present. This is seen from the data where E rosettes, phagocytes and total fluorescent cells are summed. When this total is less than the viability, receptor silent plasma cells are found to be present. In such cases this is confirmed by histological examination.

d) In nearly all cases there is no clear predominance of one class of cell. A few cases show an excess of T lymphocytes over/...

7) The Receptor Profile of Normal and Reactive
Lymph Nodes.

over B lymphocytes and macrophages, but usually no single cell class is predominant.

RECEPTOR PROFILE OF 17 NORMAL LYMPH NODES

Figures given are as a percentage of total cells present

Name	E rosettes	Fc rosettes	Complement rosettes	Phagocytes	Capping	Non Capping	Viability	Comment
Scott D.	57	38	-	-	24	9.0	87	Paraortic Node
Muir	31	29	49	7.0	17	10.0	67	Child 12 years Groin Node
Findlayson	30	16	20	12.0	20	7.0	60	Groin L. Node
McGovern P.	53	38	29	1.4	24	3.0	84	Child age 12 Normal Node
McGovern R.	18	17	22	4.0	25	5.0	85	Child age 11 Normal Node
Coleman	38	13	52	27.0	26	9.0	80	Sinus hyperplasia
Cowe	41	24	31	1.0	26	11.3	76	No details
Dryburgh	50	45	-	27.0	13	9.4	76	Aged 10 Mesenteric lymph node Normal
Fee	48	56	36	20.0	13	3.0	85	No details
Ramsay	59	26	15	5.0	29	5.1	80	Frozen section L. node Normal
Tait	35	29	33	7.0	31	2.0	74	Axillary node Normal
Clark	51	7	5	11.0	9	4.0	84	Plasma cell reaction
Scott A.	25	12	-	-	29	8.0	87	Sinus hyperplasia
Cameron	32	11	24	10.0	38	16.0	-	Groin Lymph Node Normal
Williams	9	1	4	11.0	30	13.0	66	Mesenteric node follicular hyperplasia
Simpson	48	22	40	11.0	30	5.0	-	Follicular hyperplasia plasma cell reaction
Thompson	54	8	24	2.0	17	1.0	73	Follicular hyperplasia

The criteria of "normality" were 1) histology showed preservation of normal architecture 2) lymphadenopathy, if present, was limited to single group of nodes 3) nodes removed from patients with no disease of lymph nodes, but in which nodes formed an incidental part of a laparotomy or biopsy specimen. Most nodes in this group show "sinus hyperplasia" "follicular hyperplasia" or "moderate plasma cell reaction" and nearly all were enlarged.

RECEPTOR PROFILE OF 12 REACTIVE LYMPH NODES

Figures given are as a percentage of total cells present.

Name	E rosettes	Fc rosettes	C3 rosettes	Neutral red	Capping	Non Capping	Viability	Comment
Dixon	56	21	33	7.0	45	12	64	Dorfman's Sinus Histiocytosis with Massive Lymphadenopathy
Dewar	22	11	27	2.0	32	4	70	Generalised lymphadenopathy sinus hyperplasia with granuloma formation
Rusby	37	27	16	11.0	21	13	70	H.D.N.S. but node studied not involved; follicular hyperplasia
Scott	44	24	-	-	24	7	75	HD uninvolved node with polymorph infiltration, follicular reaction
Muir	55	12	35	5.0	10	5	71	Plasma cell reaction, follicular reaction, granulomata - toxoplasmosis
Murdoch	34	20	6	9.0	18	3	91	Marked plasma cell reaction + histiocytosis lipomelanotic reticulosis
Hoss	55	18	40	27	28	4	95	Shows sinus histiocytosis lymphadenopathy and some necrosis, no lymphoma
Edwards	41	40	-	26	14	8	82	Node shows mixed follicular and paracortical hyperplasia
Rae	35	5	33	1.0	15	15	64	Follicular pattern, L. node uninvolved, from patient with H.D.
White	52	9	-	8.0	16	5	68	Groin L. node. Dermatopathic Lymphadenopathy
Maule	18	7	2	7.0	48	3	78	Pronounced follicular hyperplasia
Blair	56	29	-	-	12	17	-	Hodgkins node, not involved showing mixed sinus and follicular hyperplasia

Criteria of "reactivity" were 1) histology showed gross distortion of normal architecture 2) lymphadenopathy if present was generalised 3) nodes from patients with a known primary disorder of lymph node (e.g. Hodgkin's Disease) but without involvement and 4) nodes showing the characteristic diagnostic appearances of extreme reactivity, e.g. lipomelanotic reticulosis.

The interpretation put upon these phenomena is as follows. The normal or reactive node contains B lymphocytes at all stages of development as they mature from recirculating small lymphocyte to the antibody forming plasma cell. B lymphocytes in blood exhibit four qualities:

- a) capping surface Ig
- b) the presence of Fc and complement receptors
- c) a sub population of non-capping B cells
- d) a lack of intracytoplasmic immunoglobulin.

The normal lymph node would therefore be expected to contain cells with these qualities derived from blood, admixed with a resident population derived from the germinal centre, composed of cells which have complement rather than Fc receptors, and almost equal numbers of capping and non-capping cells. The resulting B lymphocyte profile will therefore depend upon the ratio's in which these B cell subclasses are mixed within the node. Some nodes exhibit a profile very similar in proportion to that in normal blood, save that T lymphocytes proportions are lower, and B lymphocyte and macrophage proportions generally higher than in normal blood.

Lymph nodes showing follicular reactivity have the profile of germinal centre cells, rather than blood lymphocytes.

One normal and two reactive nodes (Clark, Williams and Maule) show a profile which is distinctive and unlike that of normal blood, or the germinal centre. In these nodes there are large numbers of capping B cells, a small proportion of non-capping cells, and few rosettes formed with either Fc or complement coated/...

coated red cells. Histologically these nodes show cells with small amounts of cytoplasmic immunoglobulin and often numerous plasma cells.

The receptor profiles obtained in cell lines show several types of B lymphocyte: the chief distinction being between cells which cap surface Ig, and cells which fail to cap surface Ig. Lines GSI and BLAI show capping behaviour, and can express Fc and complement receptors, and are similar to B lymphocytes of normal blood. The DAUDI cell line shows non-capping behaviour but possesses Fc receptors, and cell line EB₁ shows capping behaviour but has neither Fc nor complement receptors.

The experiments described lead to the conclusion that several subclasses of B lymphocyte are normally present which differ in their ability to cap surface immunoglobulin, and in the expression of Fc and complement receptors. These subclasses are:

1) Capping, Fc and complement receptor bearing B lymphocytes, found most commonly in lymph nodes and blood and constituting the major class of B lymphocyte. The surface Ig class expressed is nearly always IgM but there is no intracellular immunoglobulin.

2) Mixtures of capping and non-capping B cells, expressing complement receptor and little or no Fc receptor, and containing no cytoplasmic immunoglobulin. This is the follicular B lymphocyte profile.

3) A B lymphocyte showing capping behaviour, with little or no Fc or complement receptor expression, often associated with plasma cells. Found mainly in late stages of follicular reactivity and similar in phenotype to the EB₁ cell line.

As/...

As previously mentioned in two reactive nodes from cases (Blair, Rae) of Hodgkin's Disease in which the nodes were not involved, non-capping B cells expressing the Fc or complement receptor are found. The non-capping Fc receptor type is also found in the DAUDI cell line. They probably represent additional B lymphocyte subclasses. Finally a cell type is present in lymphoid cell lines (RUS2, JIJOYE) which has non-capping surface Ig and fails to express Fc or complement receptors.

The expression of these surface features can be interpreted as a sequence of "differentiation steps" in the B lymphoid population. These subclasses can be arranged in a logical sequence from immature to mature B lymphocyte forms. The sequence envisaged is as follows:

B1 Lymphocyte, non-capping surface Ig, no intracytoplasmic Ig, no Fc or complement receptors, expressing IgM or IgD surface immunoglobulin.

B2 Lymphocyte, non-capping surface Ig, no intracytoplasmic Ig, expresses the Fc receptor, or more rarely the receptor for complement, and probably IgM surface immunoglobulin (B2.C, B2.Fc).

B3 Lymphocyte, which shows capping surface Ig, and can exist in three forms: B3.1 - blood lymphocyte with complement receptor and Fc receptors, B3.2 - the follicular B lymphocytes and B3.3 - "stromal" lymphocyte, found in perifollicular tissue, expressing Fc receptor but not complement receptors. Surface Ig is presumed to be IgM (B3.1, B3.3) or IgG (B3.2).

B4 Lymphocyte, which shows capping surface Ig, and little or not Fc or complement receptor. It occurs in association with plasma/...

plasma cells. Surface Ig is probably IgG.

B5 Lymphocyte, with large amounts of intracytoplasmic Ig, and no Fc or complement receptor.

The classification of normal and reactive nodes by this scheme is shown.

B Cell Class of Normal and Reactive Lymph Nodes

<u>Name</u>	<u>B cell class</u>	<u>Normal (N) or Reactive (R)</u>
Scott	B3.7	N
Muir	B3.1	N
Finlayson	B3.1	N
McGovern P.	B3.1	N
McGovern R.	B3.1	N
Coleman	B3.2	N
Cowe	B3.1	N
Dryburgh	B3.?	N
Fee	B3.3	N
Ramsay	B3.1	N
Tait	B3.1	N
Clark	B4	N
Scott	B3.?	N
Cameron	B3.2	N
Williams	B4	N
Simpson	B3.2	N
Thompson	B3.2	N
Dixon	B3.2	R
Dewar	B3.1	R
Rusby/...		

<u>Name</u>	<u>B cell class</u>	<u>Normal (N) or Reactive (R)</u>
Rusby	B3.3	R
Scott	B3.?	R
Muir	B3.2	R
Murdoch	B3.2	R
Ross	B3.2	R
Edwards	B3.?	R
Rae	B2.C	R
White	B4	R
Maule	B4	R
Blair	B2.Fc?	R

Further divisions of lymph nodes into those showing B or T cells, or macrophages as the predominant cell type, and those which show a mixture of such cells were devised to assist in the breakdown of lymphoma lymph nodes into useful categories.

The criteria employed were as follows:-

If one class of cell was proportionally greater than the sum of the other cell classes combined, then the node is said to show predominance of that cell class.

If no single class of cell is proportionally greater than the sum of the other cell classes combined, the node is said to be of "mixed" receptor profile. This is illustrated graphically (Fig. 7) where, it should be noted, small corrections for viability have been made.

Most normal lymph nodes are T cell predominant, or mixed in class, and T cell predominance is slightly more common in normal nodes. Few normal or reactive nodes are B cell predominant. Thus/...

Thus the classification of normal and reactive nodes shows

1) Nine normal nodes are T cell predominant with B cell subclasses being B3.1 in 4, B3.2 in 3, undetermined in 2, and B4 subclass in one.

2) Two normal nodes are B cell predominant, one of follicular lymphocyte subclass (B3.2) and one of prosecretory lymphocytes (B4).

3) Six normal nodes are of mixed class, three of B3.1 subclass and two of subclass B3.2, and one undetermined B3.

4) Five reactive nodes are T cell predominant, with B cell subclasses of two B3.2, one B4, one B2 and one unclassified B3.

5) One reactive node showed B cell predominance, this being of B4 subclass.

6) Six reactive nodes showed a mixed profile, one of B3.1 subclass, one of B3.2 follicular lymphocytes, two were of B3.3 subclass and one was of B2 subclass, one was unclassified B3.

Summary Table showing receptor profiles of Normal Lymphoid tissue

Figures shown are percentage of total cells present.

<u>Class of Cells</u>	<u>E Rosettes</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-Capping</u>
Adherent	4	46	59	91		6.0
Non Adherent	37	32	35	7		29
Whole Blood (FT)	57	24	24	15		29
Whole Blood (D + FT)	47	34	26	18	26	14
Spleen (Male)	40	34	32	10	33	12
Spleen (Female)	40	30	28	17	29	12
Tonsil	36	9	20	3	17	15
Thymus	74	10	5	2		14
L.Node (unreactive)	39	24	28	11	24	8
L.Node (reactive)	41	19	24	10	24	8

Notes. Receptor profiles are distinctive in that Spleen profiles resemble normal blood, and reactive lymph node profiles are similar to tonsil. The exception is the thymus which shows high levels of E rosetting cells.

FT = Ficoll Tricosil separated mononuclear cells

D = Dextran sedimented blood mononuclears.

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RESULTS

The lymph node in lymphoma

Chapter 16

Histological and receptor classification

Contents

- a) **Histological classification**
- b) **Classification by surface receptors**

The study and classification of lymphomas is currently strongly based on Histological (Rappaport, 1966) or on cytological (Lukes and Collins, 1975) classifications of the affected lymph nodes. These classifications, which are summarised in Tables I and II, are of practical use in applied pathology, and that of Rappaport has achieved very wide acceptance.

Morphological classifications, however, suffer from some drawbacks, and are generally unsuitable for experimental investigation since they are based on clinical and therapeutic effectiveness as their main criteria, and are not intended as an attempt to explore the biology of lymphomatous disease. The last real attempt to synthesise the then known facts about the biology of the lymphoid system, and to apply these facts into a scheme which included lymphomas, is met with in the illfated classification proposed by Robb Smith (1938). This failed, not because the idea was wrong, but because knowledge of the functions of lymphocytes and related cell types was very incomplete. The failure of such schemes was responsible for the "hard line" taken by Gall and Mallory (1942) in their insistence on therapeutic and clinical effectiveness as the final justification of any classification scheme. Since that time the pathological diagnosis of lymphomas has drifted away from the mainstream of biological investigation of the lymphoid system, and only recently has the isolationist trend of the pathologist been reversed, with attempts once again being made to unite the pathological appearances with biological reality.

The chief architects of this reversal in policy have been Lukes/...

Lukes and Collins, who, since 1968 have with increasing influence, proposed that lymphomas should be classified according to the cytological features expressed by lymphocytes during their development and maturation. In the latest variant of their proposed scheme, Lukes and Collins (1975) classify the lymphoid cell tumours under T cell lymphomas, B cell lymphomas, Histiocytic lymphomas, and lymphomas of undetermined class.

In the category of T cell lymphomas, three variants are proposed, (a) Mycosis Fungoides and Sezary's Syndrome, (b) Convolutated T lymphocyte, and (c) Immunoblastic T lymphoid sarcoma. The first category forms a special case in which the neoplastic cells show the presence of convoluted nuclei in some instances, but are classified as T lymphoid on the basis of E receptors. The other category of convoluted lymphocyte lymphoma is stated to be frequently associated with a mediastinal mass and occurs in the young. The marrow is frequently involved and the condition is often found to be leukaemic, in which instance it is sometimes called "Sternberg Sarcoma" after the author of the first accounts of the co-existence of mediastinal lymphoma with leukaemia (Sternberg, 1916). More recently a single case, reported by Smith et al. (1973) was shown to have T cell markers on the tumour cells, and the leukaemic lymphocytes. Lymphomas of convoluted lymphocytes are said to be non-cohesive, infiltrative cellular neoplasms, arising initially in the paracortical area of the lymph node. The larger cells show a characteristic "chickens foot" appearance of the nuclei. Such neoplasms are steroid and radiation resistant.

The/...

The classification of the B lymphocyte neoplasms (B cell lymphomas) depends upon the "Follicular Centre cell" concept. In this, Lukes and Collins attempt to relate the cytological features of neoplastic lymphocytes to the appearances of normal transforming lymphocytes in the germinal centre of the human tonsil, and to lymphocytes transformed in vitro by mitogens such as PHA or Lipopolysaccharide. The normal germinal centre contains histiocytes (tingible body macrophages) dendritic reticular cells, and B lymphocytes. These are of two fundamental types:- type I with cleaved nuclei and basophilic or pyroninophilic cytoplasm. This small cleaved lymphocyte, under the influence of the antigen retaining dendritic cell, undergoes transformation and further nuclear cleavage producing the second class of B cell. By enlarging developing cytoplasmic RNA and thus acquiring a rim of pyroninophilic cytoplasm the small cleaved cell becomes the large cleaved cell. Following this step, the cleavage disappears, the nucleus develops a prominent nucleolus or sometimes two nucleoli along the nuclear axis - and the cell reaches the large non-cleaved stage. The small and large categories of lymphocyte are divided according to whether their nuclei are smaller or larger than the nuclei of the accompanying non-neoplastic histiocytes. The end result of the small cleaved - large cleaved - large non-cleaved is the plasma cell secreting Ig.

This pattern of lymphocyte development, from the dormant small lymphocyte to the large transformed germinal centre cell is supported by the experimental evidence of lymphocyte transformation/...

transformation. Experiments with flash labelling of germinal centres shows that label tends to appear first in the mantle zone of small lymphocytes, and later in the lymphoblasts of germinal centres (Mitrou et al., 1969). This evidence demonstrates that germinal centres are foci of antigen retention where B lymphocytes undergo transformation to lymphoblasts.

Lukes and Collins found that in the majority of nodular and diffuse non-Hodgkin Lymphoma the neoplastic cells conformed to the morphology of follicular centre cells. In nodular lymphomas the nodules were composed principally of cleaved or non-cleaved cells, or were mixtures of these two cell types. Diffuse lymphomas were of similar constitution, but showed more variability in cell size. On the basis of these findings it was proposed that the follicular centre cell neoplasms be graded into small cleaved, large cleaved, small non-cleaved and large non-cleaved types. If mixtures of cells occurred, classification is made on the basis of the predominant cell type, although prognosis depends on the frequency of the large non-cleaved cells which are the determining factor.

In other types of lymphomas, notably the T cell lymphomata histiocytic lymphoma and diffuse lymphomas of B cell type (Burkitts type of lesion) the characteristics of follicular centre cells are absent.

The important features of this classification scheme are that an attempt is made to relate the normal features of the reactive lymph node to the neoplasms of lymph nodes, and secondly it introduces the concept of "maturation block" of the B/...

B lymphocyte as an explanation of the accumulation of lymphocytes of one particular type in a lymphoma. The advantages of the concept of "maturation block" as an explanation of the phenomenon are

- a) it is known that B lymphocytes experimentally can be arrested in some phase of their differentiation, as for example the arrest of antibody secretion by committed B cells by suppressor T cells.
- b) it explains why continuous production and turnover of cells within a tumour can occur, while the morphological features and surface phenotype of the cells remain constant.
- c) it explains the fact that tumours of B cells rarely progress into tumours of functional plasma cells, an event certain to occur unless B cell differentiation was arrested or "blocked" at some stage.

TABLE IClassification of Rappaport 19661) Malignant Lymphoma, Undifferentiated (U.L.)

Definition:- a malignant tumour of reticular tissue that is composed of primitive reticular cells showing no appreciable histiocytic or lymphocytic differentiation.

1a) Malignant Lymphoma of Burkitt (B.L.)

a multifocal malignant lymphoma of poorly differentiated lymphocytic type. A characteristic features is the presence of histiocytes containing nuclear fragments (Tingible Body Macrophages) evenly scattered throughout the tumour.

2) Malignant Lymphoma, Histiocytic (Reticulum Cell Sarcoma)(D.H.L.)
(N.H.L.)

Definition:- a malignant tumour of reticular tissue composed predominantly of neoplastic histiocytes in varying stages of maturation and differentiation.

3) Malignant Lymphoma: Mixed Cell (Histiocytic-Lymphocytic)(MLH + L)

Definition:- a malignant tumour of reticular tissue that is characterised by proliferations of neoplastic histiocytes and lymphocytes without appreciable predominance of either cell type.

4)/...

- 4) Malignant Lymphoma, Poorly Differentiated Lymphocytic (DPDL)
(NPDL)
(Lymphoblastic Lymphosarcoma)

Definition:- a malignant tumour composed of lymphocytes that show varying degrees of nuclear atypia and immaturity.

- 5) Malignant Lymphoma, Well Differentiated, Lymphocytic (NWDL)
(DWDL)
(Lymphocytic Lymphosarcoma)

Definition:- a malignant tumour that is composed of lymphocytes which have the morphological features of mature cellular forms, and which do not possess the usual cytological features of malignant cells.

Note that in the category of Histiocytic Lymphoma Rappaport lays stress on the importance of intercellular reticulin as a diagnostic feature.

TABLE IILukes and Collins Classification

1) Undefined

2) T cell

a) Mycosis Fungoides and Sezary's Syndrome

b) Convolute Lymphocyte

c) Immunoblastic Sarcoma T cell type

3) B cell

a) Small lymphocyte (SL)

b) Plasmacytoid lymphocyte: associated with CLL
or Macroglobulinaemia. (PcL)

c) Follicular Centre Cell (which may be follicular,
diffuse, follicular and diffuse, or sclerotic).

i) Small Cleaved (SC)

ii) Large Cleaved (LC)

iii) Small non-cleaved (Burkitts Lymphoma)(variable
size non-cleaved cell) (SNC)

iv) Large non-cleaved. (LNC)

d) Immunoblastic sarcoma of B cells.

4) Histiocytic type (HL)

5) Unclassifiable (UL)

Classification by surface receptors

The technique of classifying lymphoid tumours by the surface phenotype of the tumour cells is new. The available data from published reports, summarised in the introduction, shows that most lymphoid tumours are composed of B lymphocytes. Tumours of T lymphocytes occur as well as macrophage tumours but these are rare. Furthermore examples of tumours bearing both B and T lymphocyte markers, and tumours which fail to show any surface markers have been described. Any useful phenotypic classification should recognise these classes of tumour. An allowance must be made for the preponderance of B lymphocyte tumours, by devising some reliable method of subclassifying B lymphocyte tumours since otherwise the majority of tumours discovered will be allotted to the same class, even though of different histological pattern and prognosis. The questions asked in devising an effective classification were:-

1) Which markers should be employed to detect the different classes of cell? The markers chosen must characterise the T cell, B cell, and phagocyte, and must be sufficient to identify subclasses of B lymphocyte.

2) Can the surface phenotype studies identify a neoplastic population if the lymphoma resembles normal lymphoid tissue in failing to show a single population of lymphoid cells?

3) What rules are needed to interpret the data to produce a consistent and reliable classification?

4) How to prove that such a classification relates to the histological/...

histological appearances and the clinical behaviour of the tumours concerned.

1) The markers to employ

The definition of the subclass T lymphocytes is by enumerating rosettes formed with the sheep erythrocyte. B lymphocytes are defined as cells which synthesise surface immunoglobulin detectable by an immunofluorescence technique. Phagocytes are defined by their ability to ingest colloidal neutral red dye.

These three markers alone allow the identification of T lymphocytes, B lymphocytes and phagocytes, but do not allow the identification of tumours bearing more than one class of surface marker, and are insufficient to detect subclasses of B lymphocyte. Experience with normal tissues has shown that the additional markers for complement and Fc receptor, and the capping or non-capping distribution of Ig anti-Ig complex on the B cell surface are necessary to subclassify B lymphocytes.

2) Identification of the neoplastic population

Experience with normal lymphoid tissues show that no tissue, with the possible exception of thymus, is composed solely of a single class of lymphoid cell. Normal or reactive nodes contain approximately equal numbers of T lymphocytes and B lymphocytes. The germinal follicle shows a unique receptor profile in which both capping and non-capping B cells, and T cells occur. There is expression of Fc and complement receptors, but complement receptors are present on more cells than the Fc receptor. The resulting/...

resulting profile is interpreted as a mixture of subclasses of B lymphocytes. In blood, capping B lymphocytes and non-capping B lymphocytes are present, and the Fc receptor is present on more cells than the complement receptor. This profile is interpreted as a mixture of subclasses of B lymphocyte. Several different expressions of surface Ig, Fc and complement receptors are possible. From normal data, and the published accounts of B lymphocyte differentiation the following classes of B lymphocyte can be identified:-

- B1 Non-capping surface Ig
- B2 Non-capping surface Ig with Fc receptor (B2.Fc) or complement receptor (B2.C)
- B3 Capping surface Ig with Fc and C3 receptors (B3.1) with complement receptor only (B3.2) or with Fc receptor only (B3.3)
- B4 Capping surface Ig with no Fc or C3 receptor
- B5 Lacks surface markers, but has intracytoplasmic immunoglobulin.

It is obvious that no "neoplastic" expression of these normal lymphocyte markers can be detected in tumours of B lymphocytes, and there is thus no possibility of differentiating "normal" from "neoplastic" B lymphocytes on the basis of these surface features alone. In the absence of specific markers of neoplasia the diagnosis of lymphoma remains firmly in the province of the histopathologist. The receptor classification can indicate an anomalous receptor profile - as when a follicular B lymphocyte receptor profile is seen in blood B lymphocytes - and this may suggest/...

suggest a lymphoma but is never diagnostic. In lymphomas too the degree of admixture of cells may not allow any single class of cell to be selected as the neoplastic population on the basis of its predominance. It was therefore recognised that the "receptor profile" of the tumour is probably of more fundamental importance to the classification scheme than the individual characteristics of the cells it contains.

3) The rules of classification

The rules required to produce a reproduceable classification on the basis of the receptor profile are straightforward. They are listed below:-

- 1) The proportions of each class of cell in the tumour are examined, and that tumour classified as a tumour of the predominant cell class. There are six divisions allowed (1) T cell predominant, (2) B cell predominant, (3) macrophage predominant, (4) predominance of receptor silent cells and (5) tumours in which no cell class exceeds the sum of the other two. There are tumours (6) in which the sum of B cells, T cells and macrophages, as characterised by the surface markers specific for these cell types exceed substantially the total viable cells in the suspension examined. These are classed as "receptor overlap". The first classification step therefore divides all lymphomas into six classes

- 1) T cell predominant
- 2) B cell predominant
- 3) Macrophage predominant
- 4)/...

- 4) Predominance of receptor silent cells
- 5) Mixed tumours in which no single class is predominant
- 6) Receptor overlap.

The scheme is illustrated in Figure 7.

Since no subclasses of T lymphocytes or macrophages can be identified by the techniques employed, no further useful classification of T cell predominant or macrophage predominant tumours is possible. Similarly receptor overlap, and receptor silent tumours cannot be further subdivided. This leaves tumours of mixed cell type and tumours showing B lymphocyte predominance as these capable of being further subclassified.

a) Tumours of B lymphocyte predominance

These are subdivided on the basis of the B lymphocyte subclass of the cells they contain. There are eight possible subdivisions of this group. The first and most important step is to decide whether capping or non-capping B cells are in the majority. If capping B cells are more common than non-capping B cells the tumours are of B3 or B4 class. If non-capping cells are the greater population the tumour is of B1 or B2 subclass. The subsidiary classification depends upon the expression of Fc and C3 receptors.

If the greater population is non-capping and expresses no Fc or C3 receptor the tumour is classified as B1.

If the greater population is non-capping, but expresses either Fc or C3 receptors the tumour is classified as B2.Fc or B2.C.

If/...

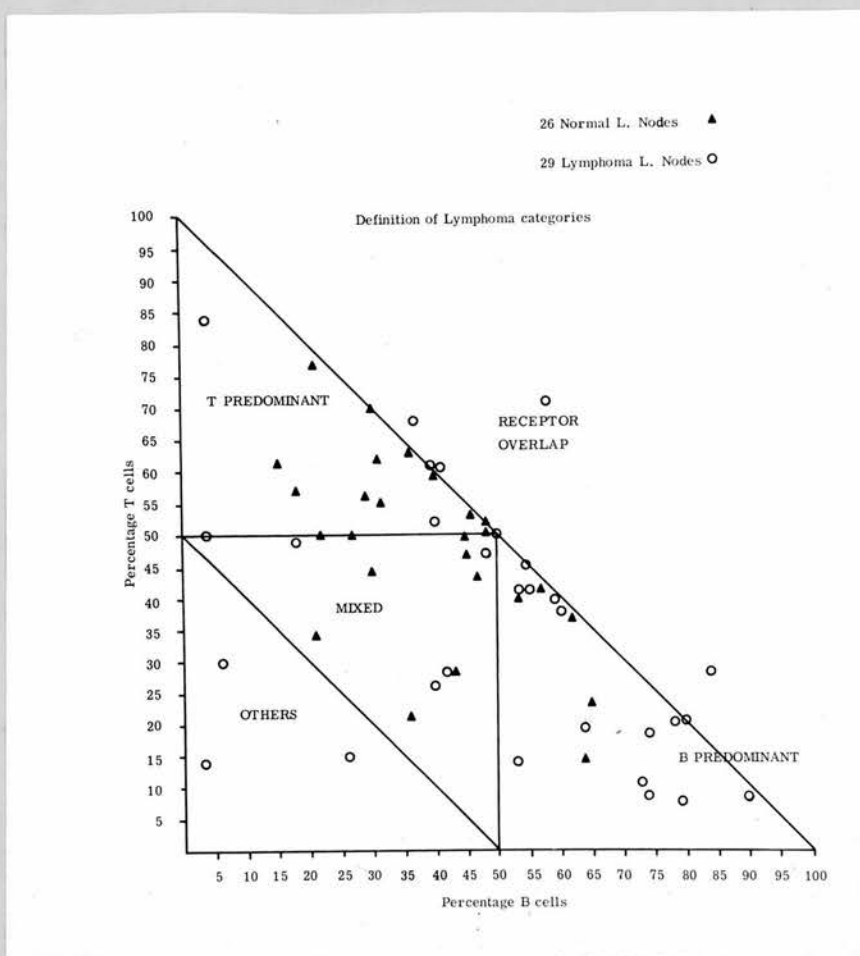


Figure 7: Definition of Categories of Lymphoma by Cell Class

The diagram is constructed as a plot of percentage T cells against percentage B cells, as percentages of Total Viable Cells. Lines are drawn to indicate B cells + T cells less than 50% to give the triangle at lower left, in which the lesion is composed of receptor silent cells or macrophages. Lines drawn at T cells = 50% and B cells = 50% of viable cells then enclose a central triangle (Mixed) in which neither B nor T cells predominate. Finally the line T cells + B cells = 100% separates receptor overlap tumours (to the right) from T cell predominant (Upper triangle) and B cell predominant (lower triangle). To use the diagram the constituent cells of the tumour are expressed as a percentage of total viable cells and the proportions of T cells and B cells then determine the area of the plot in which the tumour lies. Some of the lymphomas (open circles) and normal or reactive nodes (solid triangles) have been plotted. The distribution clearly shows (a) the extent of B cell predominance in lymphomas (b) the tendency of normal values to fall in the T predominant or mixed categories. No normal nodes show receptor overlap.

If the greater population is of capping B cells and they express

- a) C3 and Fc receptors - it is classified as B3.1
- b) C3 but no Fc receptor - it is classified as B3.2
- c) Fc but no C3 receptor - it is classified as B3.3
- d) No Fc or C3 receptor - it is classified as B4.

If the tumour is mainly composed of cells containing intracytoplasmic immunoglobulin it is classified as B5.

b) Tumours showing no predominance of any cell type (Mixed tumours)

In these tumours, there is a largest population which may be T lymphocytes or B lymphocytes. Macrophages have never formed the largest population in a mixed tumour. These tumours are classified on the basis of the B cells they contain. Obviously it cannot be argued that they are B cell tumours but T cell "tumours" cannot be usefully subdivided, whereas B cell tumours can. Mixed tumours are therefore classified as for the B cell predominant tumours. Most of the mixed tumours do show B lymphocytes to be the largest single cell class.

Lymph Node

<u>Receptor Predominant</u>	<u>Receptor Overlap</u>	<u>Mixed Receptor</u>
T lymphocyte predominant	(T cell + Surface Ig)	B lymphocyte largest single cell class
Macrophage predominant	(T cell + Fc receptor)	T lymphocyte largest single class
Receptor Silent	(T cell + C3 receptor)	
B lymphocyte predominant		Macrophage largest single class
Capping Surface Ig	Non-capping Surface Ig	Capping Surface Ig
B3.1 - Blood lymphocyte	B1 surface Ig alone	B3.1 - Blood lymphocyte
B3.2 - Follicular lymphocyte	B2.Fc surface Ig + Fc receptor	B3.2 - Follicular lymphocyte
B3.3 - Parafollicular lymphocyte	B2.C surface Ig + C3 receptor	B3.3 - Parafollicular lymphocyte
B4 - Pro-secretory lymphocyte		B4 - Pro-secretory lymphocyte
B5 - Plasma cell		B5 - Plasma cell

A small number of tumours may remain unclassifiable according to this scheme:- e.g. those expressing C3 or Fc receptor only. No mixed tumours of macrophages were seen, and only one of T cells (Patient Thompson, nodular lymphocytic lymphoma).

Table of Receptor Predominant and Mixed Tumours in the series
(31 cases).

<u>Node</u>	<u>Predominant</u>	<u>Mixed</u>	<u>Cell class</u>	<u>Histology</u>
Robertson H.	+	-	B3.1	NPDL
Robertson A.	+	-	B3.2	NPDL
Munroe A.	+	-	B4	NPDL
Smith E.	-	+	B3.1	NPDL
McKenzie	+	-	B2.Fc	NPDL
Thompson	+	-	T(B3)	NPDL
Dobie	+	-	B4	NPDL
Gaffney	+	-	B4	NPDL
Muir	+	-	B3.1	NPDL
Velzian	+	-	B1	DWDL
Moodie	+	-	B2.Fc	DPDL
Robertson J.	+	-	B2.C	DPDL
Fraser	+	-	B3	DWDL
Bathgate	-	+	B3.1	DWDL
Swanson	-	+	B3?	DPDL
McGrail	-	+	B3.3	DPDL
Cairns	+	-	B5	DWDL
Wilson	+	-	T	DPDL
Kerr	+	-	Receptor Silent	DPDL
Laverick	No	No	Overlap	DM(H+L)
Reid	+	-	B2	DWDL
Adams	+	-	B1	DHL
Hall/...				

<u>Node</u>	<u>Predominant</u>	<u>Mixed</u>	<u>Cell class</u>	<u>Histology</u>
Hall	+	-	B1	DHL
Hogg	+	-	B1	DH/DPDL
Smith	+	-	B3.2	DHL
Ross	+	-	Macrophage	DHL
Oliver	+	-	Receptor Silent	DHL
Ritchie	+	-	Receptor Silent	DHL
Baird	+	-	Overlap	DHL
Moyes	+	-	Silent	DHL
Johnstone	+	-	B1	DHL

As shown 5 nodes in this series were of mixed class and 15 showed predominance of B cells, four were receptor silent, two nodes showed T cell predominance (one only by a very small margin) and two showed receptor overlap. There was one macrophage tumour. Three of the diffuse lymphocytic lymphomas (DLL) and two nodular lymphocytic lymphomas (NLL) showed mixed receptor profiles. All B1 tumours, and all B2 tumours were B cell predominant, the only mixed tumours being tumours of B3 lymphocytes.

In the description of receptor profiles in non-Hodgkin lymphoma, it is apparent that there is not necessarily any correlation between the functional characteristics of the cell (as assessed by membrane receptors) and its morphological appearance. Where such a divergence is apparent, the preferred classification of the cells is that based on the receptor profile.

Some means of classification of B lymphocyte and mixed tumours was required, and that adopted was based on the study of normal tissues, lymphoid cell lines, and from the available literature. The primary division was between cells which showed "capping" surface Ig, and those which failed to cap. When "capping" against "non-capping" cells for B lymphocyte lymphomas and normal lymph nodes are graphed (see Fig. 8) it is apparent that in most mixed tumours, and normal nodes, capping cells exceed non-capping cells substantially. In lymphomas, particularly the B lymphocyte predominant tumours, it is not unusual to find non-capping cells exceeding capping cells in number. Non-capping lymphomas were allotted to categories B1 or B2, depending upon the expression of the Fc and C3 receptor. Non-capping lymphomas were generally not found to express complement receptors.

The capping lymphomas of B cell predominant or mixed type were allotted to categories B3, B4 depending upon the expression of Fc and C3 receptors in the same manner as normal or reactive lymph nodes were classified. B lymphocytes of the B3 class could be further subclassified on the basis of their receptor profile:- the subclassification adopted is according to whether the/...

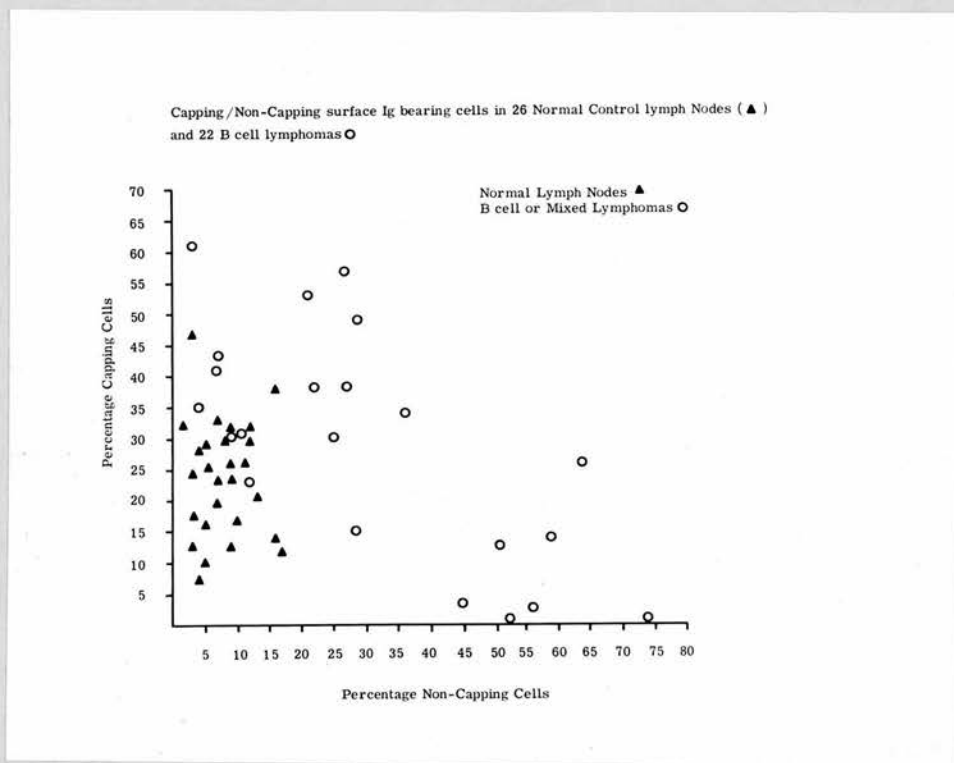


Figure 8: Plot of Capping B lymphocytes against Non-capping B lymphocytes in 26 Normal or Reactive nodes and 22 B cell or Mixed lymphomas

The normal or reactive nodes (solid triangles) show in all but 2 cases a predominance of capping cells. Six of the lymphomas (open circles) express a normal B cell profile (clustered to the left). In the central group the proportions of non-capping cells are above normal, but are still exceeded by capping cells. To the right, the group of 8 B1 and B2 lymphomas are quite clearly distinguished. The tumours shown were classified as B cell predominant or mixed.

the B lymphocytes exhibited the profile of blood B lymphocytes (B3.1) germinal centre B lymphocytes (follicular type B3.2) or the profile of lymphocytes exhibiting capping behaviour and Fc receptor only (parafollicular type B3.3).

The possible B lymphocyte subclasses, described by these tests are B1, B2 with Fc receptor, B2 with C3 receptor, B3.1, B3.2, B3.3, B4 and B5. In practice, the profile B2.C was encountered rarely:- in leukaemic blood, and in Hodgkin's disease. The profile of B5 was encountered only once.

Intracytoplasmic immunoglobulin

As an aid to classification, sections of lymph node tumour were examined for intracytoplasmic immunoglobulin by the immunoperoxidase technique.

Material was available from 20 of the 31 lymphoma cases, of which four showed positivity.

Cases showing positive immunoperoxidase reactions

<u>Patient</u>	<u>Histology</u>	<u>Receptor Classification</u>	<u>Class of Intracytoplasmic Ig.</u>
Gaffney	NPDL	B4	IgA Lambda chain
Smith E.	NPDL	B3.1	Weak Lambda chain only
McGrail	DPDL	B3.3	IgM only
Cairns	DWDL	B5	IgM, weak IgG, Kappa chain

Of the other tumours no material was available from Munroe (B4) and in Dobie (B4) some cells showed weak IgM and Kappa reactivity, but most cells were negative. No lymphomas of B1, B2 or receptor silent class showed any positivity. The distinct pattern of strong staining of Cairns stood in contrast to the other cases, only Gaffney showed unequivocal staining of most of the tumour cells and the reaction was much weaker than that of Cairns. The results showed that intracytoplasmic Ig is expressed in tumours of B4 and B5 class in the majority of cells, and in a minority of cells in B3.1, B3.3 and B4 classes. Borderline positivity is difficult to assess in all but B5 tumours.

Intracytoplasmic/...

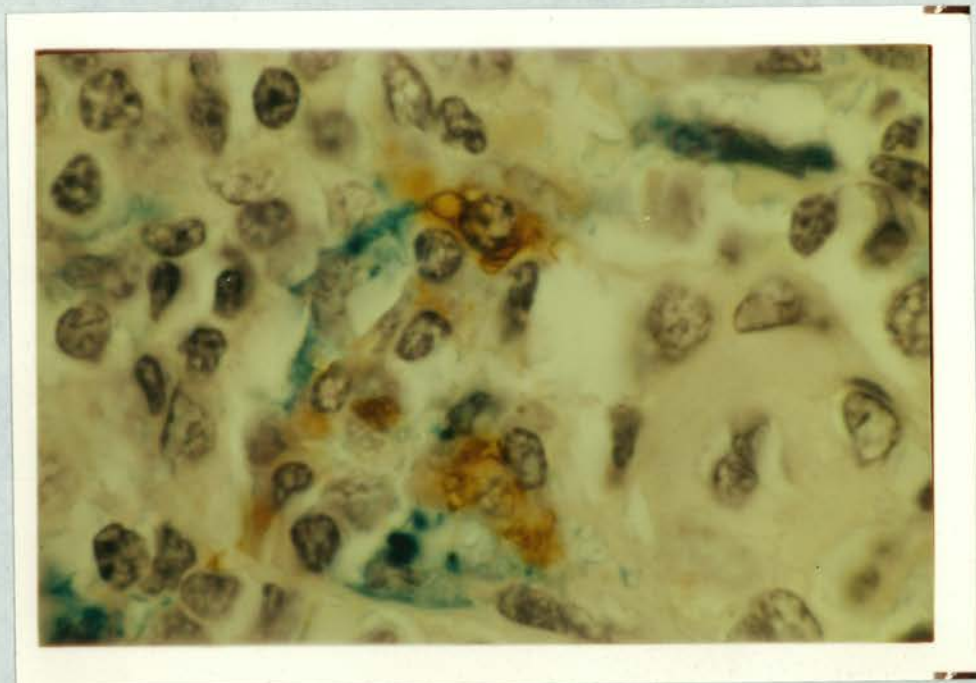


Figure 9: Schweigger Seidel sheath from the Spleen in Hodgkin's Disease

The illustration shows the macrophages of the sheath, (blue) containing iron in close relation to IgG containing cells (brown). The arteriole is present at lower right.

P.A.P. Immunoperoxidase and Prussian blue reaction.

x 630

Intracytoplasmic immunoglobulin of IgG class was found most frequently in sections from Hodgkin's disease nodes and spleen. In spleen particularly the numbers of Ig positive cells were markedly increased in comparison to normal spleen. Dual staining for iron (PBR reaction) and for intracytoplasmic Ig showed IgG containing plasma cells of both Kappa and Lambda chain type to be closely associated with iron containing splenic macrophages especially in the ellipsoids (Schweigger Seidel sheaths) of the red pulp. This striking association (Fig. 9 & 10) probably explains the observations of Order (1971) that in Hodgkin's disease antibodies to ferritin can regularly be found. The Reed Sternberg cell in Hodgkin's disease exhibited a variable pattern of staining with some cells showing unequivocal positivity for IgG or IgM with Kappa chain, while others showed no reaction. The findings strongly suggest that the Reed Sternberg cell is a derivative of B lymphoid cells but that a great variability in amount of intracytoplasmic Ig secreted is encountered.

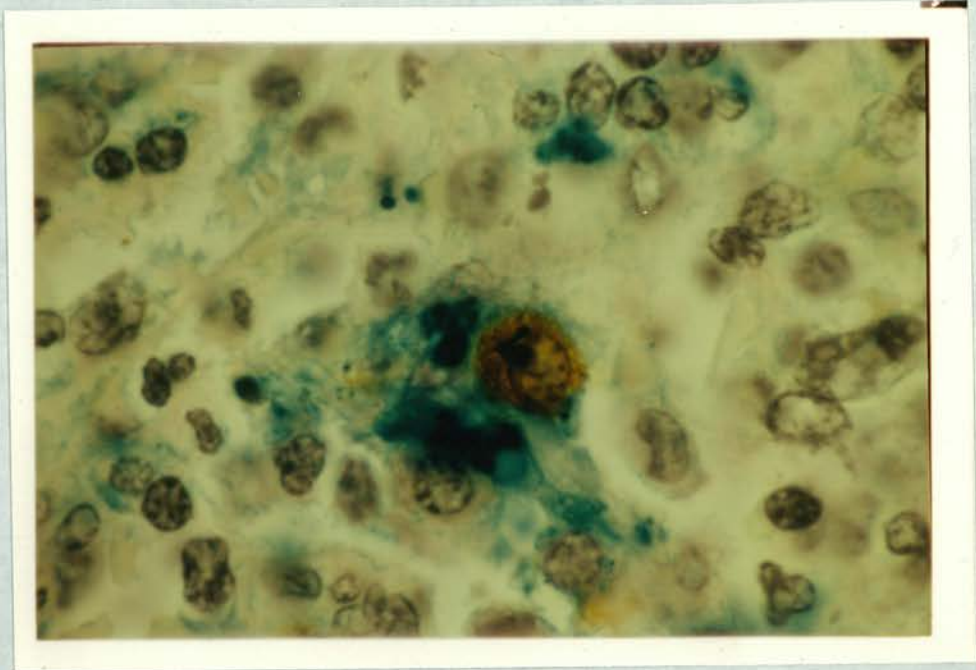


Figure 10: Immunoperoxidase/PER reaction in Hodgkin's Spleen

A single IgG containing cell (brown) in intimate contact with the cytoplasm of an iron containing macrophage (blue)

P.A.P. Immunoperoxidase and Prussian blue reaction

x 630

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RESULTS

The Lymph Node in Lymphoma

- a) In the nodular lymphocytic lymphoma.
- b) In the diffuse lymphocytic lymphoma.
- c) In the histiocytic lymphoma.
- d) In leukaemia.

RESULTS

Chapter 17

Receptor profiles in nodular lymphocytic lymphomas

Nodular Lymphocytic Lymphomas

All patients in this group had nodular lymphocytic lymphoma of poorly differentiated cell type by Rappaport's classification. In all but one, the diagnostic biopsy was lymph node. In one patient the diagnosis was made on involved spleen. In several patients, blood receptor profiles were studied at time of biopsy and at monthly intervals thereafter with the patients on treatment. Only one patient in this group is dead. The B lymphocyte subclasses in nodular lymphoma are mature in form. Two patients had, in addition, chronic lymphocytic leukaemia at the time of diagnosis. Two patients in this group have had splenectomy, in one (Muir) it was the only material available for study, in a second (Robertson A.) it was removed during laparotomy for disease progression.

Nodular Lymphocytic LymphomaClinical and Pathological Data

<u>Name</u>	<u>Sex</u>	<u>Age</u>	<u>Histology</u>	<u>A or D</u>	<u>Survival (months)</u>	<u>Stage at Diagnosis</u>	<u>Class</u>
Robertson H.	F	54	NPDL	A	10	IV	B3.1 Mixed
Robertson A.	F	-	NPDL	D	2	IV	B3.2 Predominant
Munroe A.	F	73	NPDL	A	26	III	B4 Predominant
Smith E.	F	72	NPDL	A	14	III	B3.1 Mixed
McKenzie	M	35	NPDL	A	9	III	B2 Predominant

Thompson/...

<u>Name</u>	<u>Sex</u>	<u>Age</u>	<u>Histology</u>	<u>A or D</u>	<u>Survival (months)</u>	<u>Stage at Diagnosis</u>	<u>Class</u>
Thompson	M	63	NPDL	A	12	II	B3 T Predominant
Dobie	M	71	NPDL	A	13	IV	B4 Predominant
Gaffney	F	43	NPDL	A	15	III	B4 Predominant
Muir	F	71	NPDL	A	24	III	B3.1 Predominant

The receptor profiles reveal two nodes of "mixed" status, both exhibiting B3.1 subclass of B lymphocytes. Of the remainder, three are of B lymphocyte predominant B4 subclass, one of B3.2 subclass (follicular subclass) and one is T cell predominant with B3 lymphocytes of uncertain subclass. Only one patient in this group has died, and this patient is discussed in the text. The node of McKenzie was the only B2 tumour in this group.

Receptor Profiles in Nodular Lymphocytic Lymphoma

The figures displayed are as percentages of the total cells present.

<u>Name</u>	<u>E</u>	<u>Fc</u>	<u>G3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
Robertson H.	30	20	15	8	35	4
Robertson A.	41	21	44	6	30	25
Munroe A.	8	6	4	17	38	27
Smith E.	48	23	29	1	23	12
McKenzie (Nod)*	21	22	5	2	26	64
McKenzie (Paranod)*	27	25	32	1	14	57
Thompson	52	41	-	6	31	10
Dobie/...						

<u>Name</u>	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
Dobie	28	23	17	6	57	27
Gaffney	26	8	7	2	38	2
Muir (Spleen)	26	38	29	3	33	7

(In McKenzie *, the lymphoma nodules were separated from the adjacent lymphoid tissue and their profiles studied separately. The nodules show an excess of B2.Fc lymphocytes, while in the paranodular tissue B2.C lymphocytes are the major cell class. On this basis the tumour was classified as B2.)

Patients with nodular lymphocytic lymphomas occasionally show chronic lymphocytic leukaemia as in the examples given below. Percentage figures are given as percentage of total cells, the quantitative data is shown in millions of cells per ml.

<u>Munroe A.</u>	<u>Node Percent</u>	<u>Blood (1) Percent (x 10⁶ ml)</u>		<u>Blood (2) Percent (x 10⁶ ml)</u>	
E	8	31	2.25	38	1.09
Fc	6	36	0.99	28	0.795
C3	4	26	1.73	7	0.19
Phagocytes	17	22	1.43	11	0.32
Capping Cells	38	17	1.11	14	0.41
Non-capping Cells	27	18	1.20	14	0.41
Differential Count					
Polys.		25	-	64	-
Monos/...					

<u>Munroe A.</u>	<u>Node Percent</u>	<u>Blood (1) Percent (x 10⁶ ml)</u>		<u>Blood (2) Percent (x 10⁶ ml)</u>	
Monos.		75	-	36	-
Mononuclear count		-	6.65 x 10 ⁶	-	2.88 x 10 ⁶

In this patient, at the time of biopsy a circulating abnormal cell population was present. These cells were of (B2) rather than blood type (B3.1). One month later, after treatment, the white cell count was lower, but the circulating cells were still of B2.Fc rather than blood class (B3.1). This patient did well, and still survives 26 months after biopsy. The lymph node pattern was B predominant B₄ class. Macrophage numbers were elevated in node and blood, and T lymphocyte numbers were raised (1) or normal (2).

By contrast the patient Robertson, showed an abnormal blood receptor profile, and no response to treatment. The original nodular lymphoma progressed rapidly and at autopsy was of diffuse rather than nodular type. The receptor profile of the node at diagnosis was B lymphocyte predominant and of follicular type (B3.2). The data is shown below. Percentage figures are percentage of total cells, and the quantitative data are presented as millions of cells per ml.

<u>Robertson A.</u>	<u>Node Percent</u>	<u>Blood (1) Percent (x 10⁶ ml)</u>		<u>Blood (2) Percent (x 10⁶ ml)</u>	
E	44	41	0.37	48	0.64
Fc	21	28	0.25	39	0.53
G3	44	3	0.02	37	0.51
Phagocytes	6	14	0.13	8	0.1
Capping/...					

<u>Robertson A.</u>	<u>Node Percent</u>	<u>Blood (1) Percent (x 10⁶ ml)</u>		<u>Blood (2) Percent (x 10⁶ ml)</u>	
Capping	30	19	0.17	8	0.1
Non-Capping	25	27	0.24	12	0.16
Receptor Silent	-	Nil	-	24	0.32
Mononuclear Cells		36%	2.4	38%	3.8
B Cell Class	B3.2	B2.Fc		?	

Receptor silent cells appeared in the blood within 1 month of diagnosis. The receptor profile of blood B cells was B2.Fc. With treatment (blood sample 2 obtained 1 month before death), the blood showed the presence of receptor silent cells (24%). Non-capping cells exceeded capping cells, both Fc and C3 receptors were expressed on the B cell population, and on some of the cells which were surface Ig negative. This suggests that in this case the receptor silent population was derived from primitive B lymphocytes.

This patient also had the spleen removed at laparotomy; the organ was not involved, and showed normal cellularity, and normal weight, and the B cells were of B3.1 class. The patient died 2 months after diagnosis.

Robertson A. Spleen weight 105 gm. Total cells 6.89×10^{10}
cells per gm. 656.2×10^6 .

<u>Rosettes</u>	<u>Percent</u>	<u>Mass per gram</u>
E	44	287.6×10^6
Fc	28	180.9×10^6
C3/...		

<u>Rosettes</u>	<u>Percent</u>	<u>Mass per gram</u>
C3	42	276.2×10^6
Phagocytes	11	70.5×10^6
Capping	31	200×10^6
Non-Capping	16	104.7×10^6

The data shows that even in a group of histologically uniform tumours occasional individuals exhibit atypical features. The features suspected of association with poor prognosis in this case are (1) the primitive B2.C nature of the blood lymphocytes despite the mature lymph node population and (2) the presence of a receptor silent cell population in blood approximately one month before death.

The nodular lymphoma of McKenzie

In this macronodular tumour, the nodules were separated by dissection and the receptor profile of nodules and paranodular tissue assessed separately. The distinctive profiles of each are well shown, but contrary to expectation, the complement receptor bearing B cells were lower in the nodule than in the paranodular tissue, while in germinal centres complement rosettes are higher than Fc rosettes. In the nodules of this tumour, the Fc receptor cells substantially outnumbered the C3 receptor cells, and T cells were present. The substantial excess observed of non-capping cells to capping cells is a feature sometimes associated with follicular B lymphocytes of tonsil, but germinal centre cells are of/...

of B2.C rather than B2.Fc class. The Fc receptor excess shows clearly that the nodules in this case do not correspond to the profile of normal Germinal centre and the large numbers of non-capping cells support a classification B2 lymphocytes in a B lymphocyte predominant tumour.

The receptor profile of nodular lymphoma was expected, on the basis of the similarity between the germinal centre cells and the nodular lymphocytic lymphoma cells (which Lukes would classify as small cleaved), that follicular B3.2 patterns would predominate. Unexpectedly the profiles showed three B lymphocyte predominant B4 tumours, two predominant and one mixed B3.1 tumours, one B2 predominant, and one T predominant tumour with B3 lymphocytes. Only one patient with a follicular pattern (B3.2) emerged (Robertson A.) and this patient died rapidly with diffuse lymphoma at autopsy and a leukaemia of receptor silent cells.

Chapter 18

Receptor profiles in diffuse lymphocytic lymphomas

The Diffuse Lymphocytic Lymphomas

These tumours showed no evidence of nodularity, and were classified according to Rappaport as mixed cell (with equal numbers of lymphocytes and histiocytes), poorly differentiated lymphocytic, or well differentiated lymphocytic tumours. Diffuse histiocytic tumours, and diffuse undifferentiated tumours were classified together and are discussed in the section of histiocytic lymphoma.

Among this group of tumours, there was considerable heterogeneity of receptor profile, of histological appearance, and of survival.

Diffuse Lymphocytic LymphomaClinical Data, and Receptor Classification

<u>Name</u>	<u>Histology</u>	<u>A/D</u>	<u>Time</u> <u>(mo.)</u>	<u>Age</u>	<u>Sex</u>	<u>Clinical Stage</u>	<u>Receptor Profil</u>
Velzian	DWDL	A	2	56	M	IV	CLL B1
Moodie	DPDL	D	2	76	F	Extranodal	B2.Fc Predominant
Robertson J.	DPDL	D	6	68	M	IV	B2.C Predominant
Fraser	DWDL	A	12	60	M	IV	B3.? Predominant
Bathgate	DWDL	A	*	56	M	II	B3.1 Mixed
Swanson	DPDL	D	8	63	F	II	B3.? Mixed
McGrail	DPDL	A	14	68	M	IV	B3.3 Mixed
Cairns	DWDL	DQ	*	68	M	II	B5 Predominant
Wilson	DPDL	A	12	8	F	I	T Predominant
Kerr	DPDL	D	4	70	M	IV	Receptor Silent
Laverick	D.Mixed	A	14	14	F	II	Receptor Overla
Reid	DWDL	A	1	67	M	IV	CLL B2

*Long/...

* Long term survivors. Death (Cairns) due to myocardial infarction, patient had Waldenstrom's disease. The patients Velzian and Reid both had CLL associated with lymphadenopathy. They are described with this group, and considered later in detail in the text.

Of the twelve tumours examined, two were CLL associated at diagnosis, and two were tumours of B2 lymphocytes. Of the B3 tumours, two were unclassified, one was B3.1 and one was of B3.3 pattern. The only B5 tumour in the series occurred in this group. To emphasise the heterogeneity of the diffuse lymphocytic lymphoma, one T cell predominant, and one receptor silent case occurred in this group of lesions. Receptor overlap was present, in one case, and mixed E and Fc receptor bearing cells were found in the node and blood of the B2 lymphoma of Robertson.

Diffuse Lymphocytic Lymphoma

Receptor Profile

<u>Name</u>	<u>Viability</u>	<u>E Rosettes</u>	<u>Fc</u>	<u>G3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-Capping</u>
Velzian	90	59	2	1	4	-	99
Moodie	88	6	55	-	2	30	36
Robertson J.	91	28	58	49	14	15	28
Fraser	80	14	-	-	8	42	17
Bathgate	80	38	23	22	4	33	7
Swanson	90	50	23	-	-	43	7
McGrail	75	46	67	8	6	31	9
Cairns	93	14	4	4	1	53 + Plasma Cells	
Wilson	76	64	11	13	4	3	-
Kerr	88	14	17	21	3	1	2
Laverick	69	49	40	30	12	-	-
Reid	84	11	2	6	9	5	14

The/...

The interpretation of the diffuse lymphocytic lymphomas is rendered more complex because they are not a single disease, but on the basis of data obtained in this study include at least seven separate entities. These will be discussed in the following order (1) the receptor overlap tumour, (2) receptor silent tumour, (3) T cell predominant tumour, (4) B5 lymphoma, (5) B3 tumours, (6) the B2 tumours and (7) diffuse lymphoma associated with leukaemia.

(1) Cases of receptor overlap

The tumour in this case (Laverick) is illustrated (Figs. 11, 12, 13.) It is similar in receptor profile to a case of diffuse histiocytic lymphoma (Baird) discussed later. The biopsy consisted of a large node from the groin, which communicated with a sinus opening onto the skin surface. There was other lymphadenopathy at diagnosis. As the receptor profile shows, the bulk of the cells were showing E rosettes with high Fc and C3 rosettes. Surface Ig bearing cells were not estimated, for technical reasons, but Fc and C3 rosettes, which give reliable estimates of B cell numbers indicated 30-40% B lymphocytes. The viability in the node was low, but despite this, there was clear evidence of tumour cells exhibiting E, Fc and complement rosettes. On this basis the lesion was classified as "receptor overlap". Polymorph contamination was present and neutral red ingesting phagocytes were fairly numerous.

Receptor overlap was noted in the peripheral blood of one other case (Robertson) in this group. The receptor profile of the/...

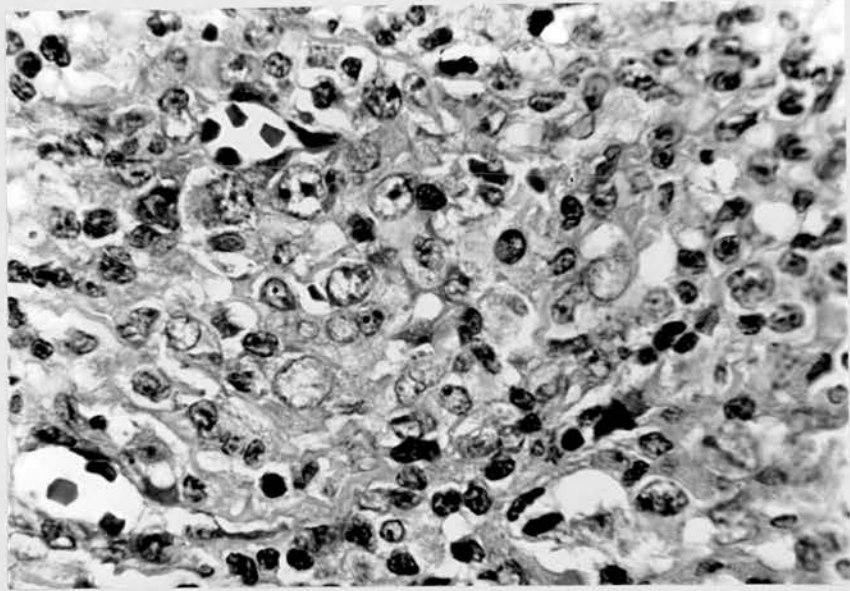


Figure 11: Histological Appearance of a Receptor Overlap Tumour

This node from patient LAVERICK was classified as a diffuse lymphocytic lymphoma of mixed histiocytic and lymphocytic pattern.

Haematoxylin and eosin x 600

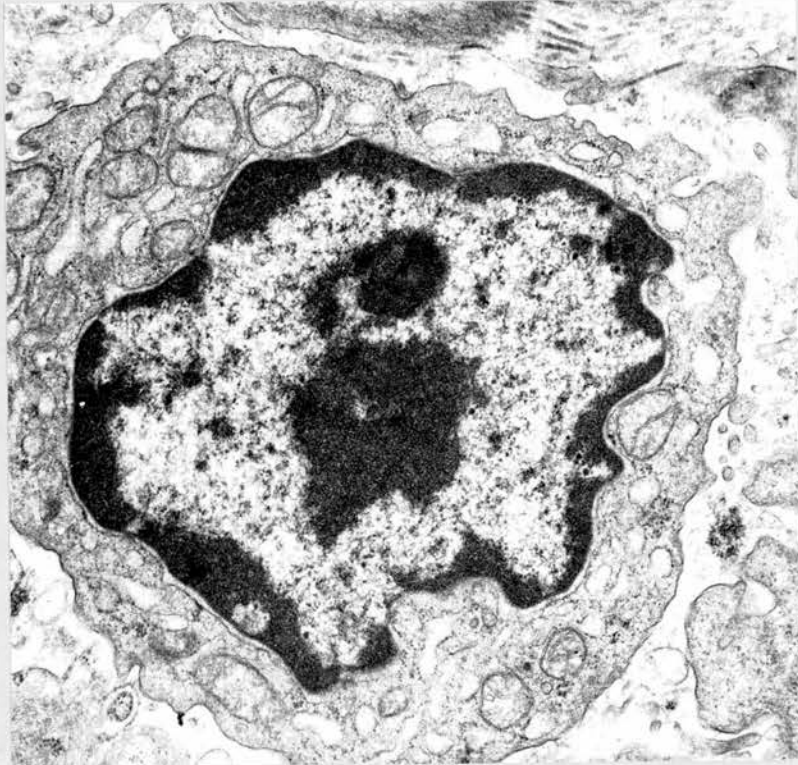


Figure 12: E.M. Appearance of Receptor Overlap Tumour

A typical cell shows a vacuolated cytoplasm with numerous mitochondria, large nucleus, and irregular nucleolus. These cells expressed receptor for both sheep red cells and complement.

E.M. x 2,500

the peripheral blood is shown below. Despite showing no overall receptor overlap in the node as in the first case, E and Fc receptor overlap was shown using mixed rosetting with human Fc and sheep cells in the blood and node. The glass adherent/non-adherent cells in this patient were also examined, proportions were essentially normal.

Patient Robertson (1-5 blood profiles; 6 lymph node.)

Figures are given as percentage of total cells.

	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>	<u>E/Fc Mixed</u>
1)	84	45	52	-	-	-	23.5%
2)	65	-	53	7	2 All cells showing faint fluorescence		
3)	27	42	10	10	21	10	6.7%
4)	38	29	30	18	29	7	11%
5)	48	19	28	-	20% showing E rosettes and fluorescence		
6)	28	58	49	14	15	28	21%

With a majority of 28% of non-capping cells present the profile is B2.C - 20%, B2.Fc - 8%, B3.1 - 15%, T.Fc - 21%, T - 8% and phagocytes - 15%. When the mixed E and Fc (T.Fc) rosettes are allowed for the profile is simplified into the classification B2.C, and the profile is B lymphocyte predominant.

Of the two patients showing E and Fc, E and fluorescence, and E and complement rosette overlap, one has done well despite "bad" histology (Laverick), and one with poorly differentiated diffuse lymphoma/...

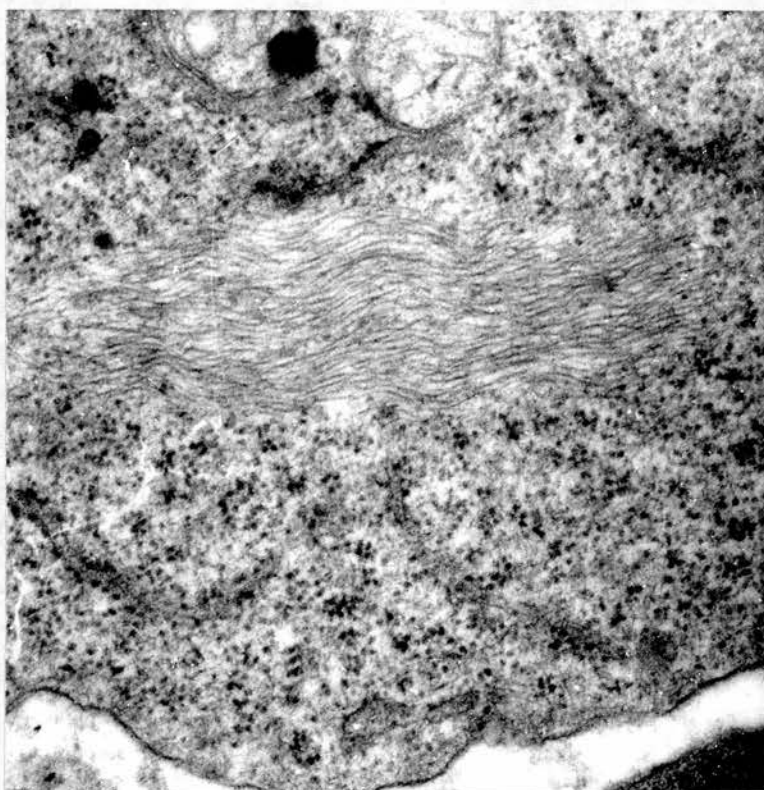


Figure 13: E.M. Appearance of Receptor Overlap Cell

This high power E.M. of a portion of the cytoplasmic of a single tumour cell from patient LAVERICK shows numerous cytoplasmic polyribosomes and a prominent leash of intracytoplasmic microfilaments.

E.M. x 31,250

lymphoma has done badly. If the overlap is taken account of, the receptor profile of B cells in the lymph node tumour of Robertson is B2, with B2.C cells predominating.

2) Receptor silent tumour (Kerr)

The patient with this tumour presented with small velvety slightly raised and pigmented plaques in the skin. He also had lymphadenopathy. A lymph node biopsy was performed, and peripheral blood studied at the time of biopsy. The data are shown below, and include data from a second blood sample removed after relapse, shortly before death.

Kerr. Receptor profile of blood (1) (2) and Lymph node (3).

Values are given as percentage of total mononuclear cells.

	<u>E Rosettes</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>	<u>Silent</u>
1)	42	32	28	1	3	3	23
2)	10	17	18	1	4	1	67
3)	14	17	21	3	1	2	63

Mononuclear cell count (1) $5.60 \times 10^6/\text{ml}$ (2) $27.9 \times 10^6/\text{ml}$ WBC per ml.

In both the first and second blood samples there are cells lacking surface Ig but expressing some C3 and Fc receptors. E.M. showed these to be rather granular in appearance; they probably represented myeloid cells:- a leukoerythroblastic blood picture due to replacement of marrow by lymphoma. There was an initial response/...

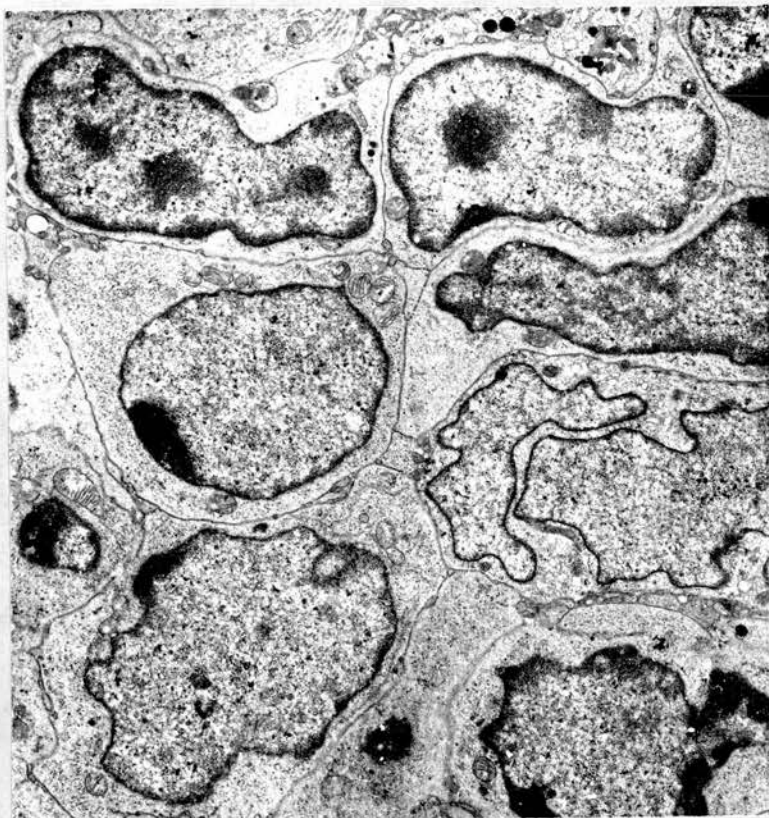


Figure 14: The E.M. Features of Receptor Silent Cells (Kerr)

As this low power E.M. picture shows, the receptor silent cells of patient Kerr, were of obvious "lymphoid" morphology. The closely packed cells in this section from lymph node show irregularity of nuclear outline and "empty" cytoplasm, with few mitochondria and little or no endoplasmic reticulum.

E.M. x 3,175



Figure 15: The E.M. Features of Receptor Silent Cells (Kerr)

At higher magnification the absence of intracytoplasmic fibrils and endoplasmic reticulum and the presence of clusters of polyribosomes is clearly seen in this cell from the lymph node of patient Kerr.

E.M. x 5,000

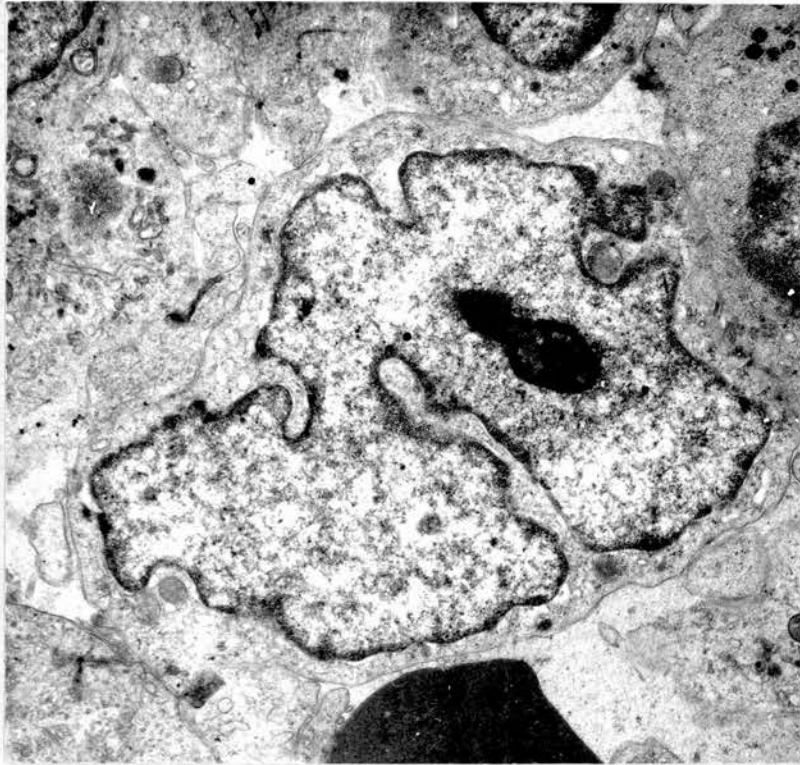


Figure 16: The E.M. Features of Receptor Silent Cells (Kerr)

This cell from the receptor silent tumour of patient Kerr illustrates the quite marked nuclear irregularity of cells in this tumour. There are several structureless dense bodies in the cytoplasm and some vacuolation of the cytoplasm.

E.M. x 3,175.

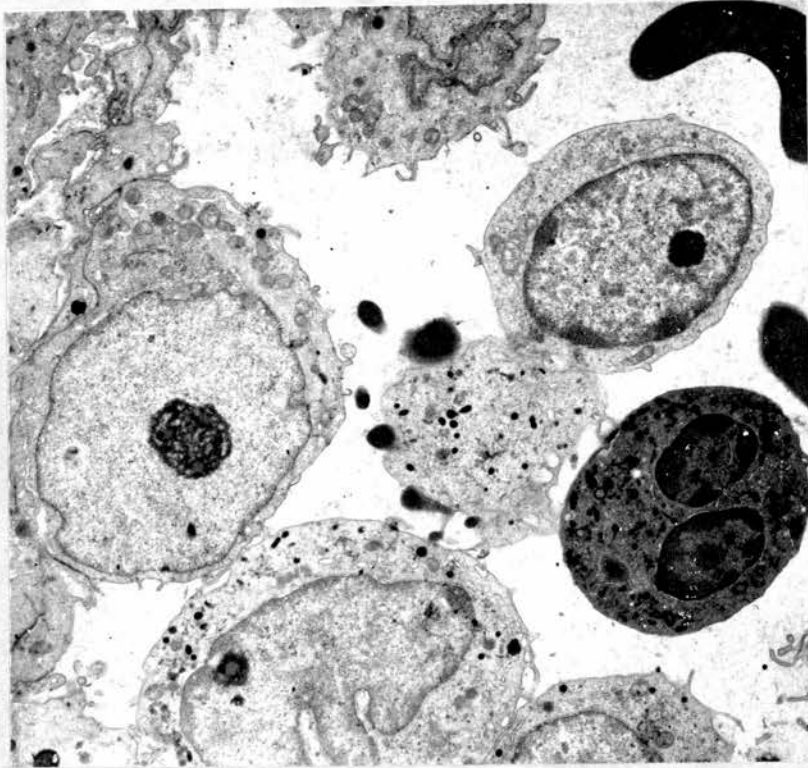


Figure 17: E.M. Features of Circulating Receptor Silent Cells (Kerr)

The appearance of tumour cells, isolated from the blood, is shown here. The same nuclear irregularity is seen in some cells, and electron dense bodies are bodies in the cytoplasm. The adjacent neutrophil (right) indicates the size of the leukaemic receptor silent cells.

E.M. x 3,000

response to chemotherapy, but bone marrow involvement became widespread with termination in a leukaemic state. Survival from diagnosis was five months. The histological appearances are illustrated (Figs. 14, 15, 16, 17.).

3) T cell predominant tumour

This patient, an 8 year old girl, presented with some mild constitutional symptoms and a single node in the neck. The histological appearances were those of a diffuse poorly differentiated lymphocytic lymphoma (Fig. 18). The receptor profile showed T cell predominance. T cell neoplasms are rare, and rapidly progressive and generally leukaemic; this patient did not have leukaemia. Reactive lymph nodes do show extensive T cell predominance but rarely to the extent shown in this patient. Despite these observations, the histological appearance is that of lymphoma. Subsequent investigations have revealed a toxoplasma titre of 1:8,000 (IgG antibody). The patient is well.

Wilson. Lymph Node.

<u>E Rosettes</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>	<u>Viability</u>
64%	11	13	4	3	Nil	76

4) B5 Lymphoma (Cairns)

This patient, had Waldenstrom's disease for about 18 years before/...

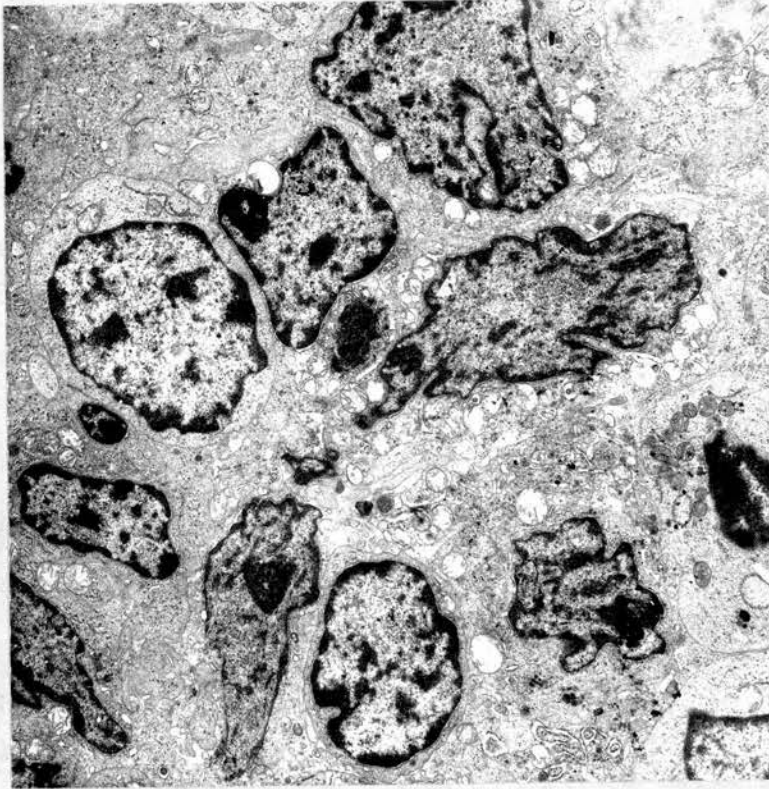


Figure 18: The E.M. Features of a T Cell Predominant Tumour (Wilson)

This tumour, showing the features of diffuse poorly differentiated lymphocytic lymphoma, was composed of E rosetting cells. The irregularity of nuclear outline and chromatin density are features said to be suggestive of T lymphocytes. The cell cytoplasm is ill defined, and widespread interdigitation of membranous processes is a characteristic of this tumour.

E.M. x 3,125

before a relapse necessitated repeat biopsy. The receptor profile is shown. A feature was the presence of IgM secreting lymphocytes, which were also PAS positive, in sections from the node. The patient responded to chlorambucil, but died some months later suddenly from a myocardial infarct. These tumours must be quite rare, since this is the only example so far studied. The appearances are illustrated in figures 19 and 20.

5) The B3 tumours

This class contains the longest survivor of the whole series (> 20 years; Bathgate) and one of the shortest survivals in the series (Swanson, < 8 months). Only 4 patients out of 12 had B3 tumours compared with 4 out of 9 of the nodular lymphocytic lymphoma group. The patient who did badly has very incomplete data, and was lost to follow up for some time so no account of the mode of progress of the disease can be given. The receptor profile of Bathgate is B3.1 (blood lymphocyte) and this corresponds to the profile of stable chronic lymphocytic leukaemias. The tumour of McGrail is interesting as one of the few B3.3 tumours in the series (parafollicular cells, capping with an Fc receptor).

6) The B2 tumours

The predominant tumour of Moodie was most unusual in being a diffuse lymphocytic lymphoma of the thyroid gland. Regional nodes were also involved. The predominant cells composing this/...

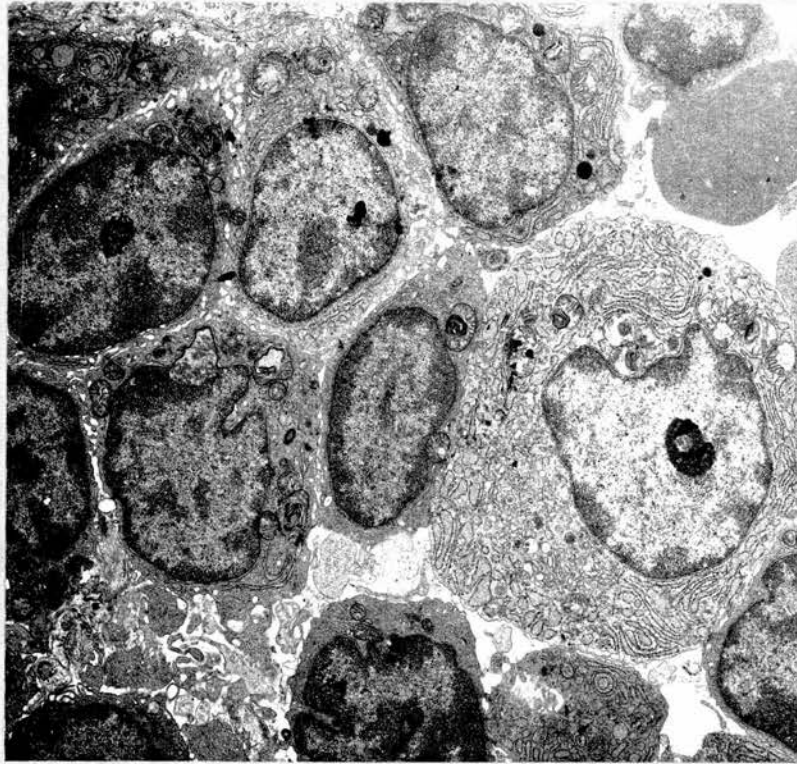


Figure 19: The E.M.Features of a B5 Lymphocyte Predominant Tumour (Cairns)

This low power E.M. picture clearly shows the characteristics of secretory B lymphocytes (B5) in Waldenstrom's disease. The cells show extensive development of rough E.R. and contained IgM by the Immunoperoxidase staining technique.

E.M. x 3,125

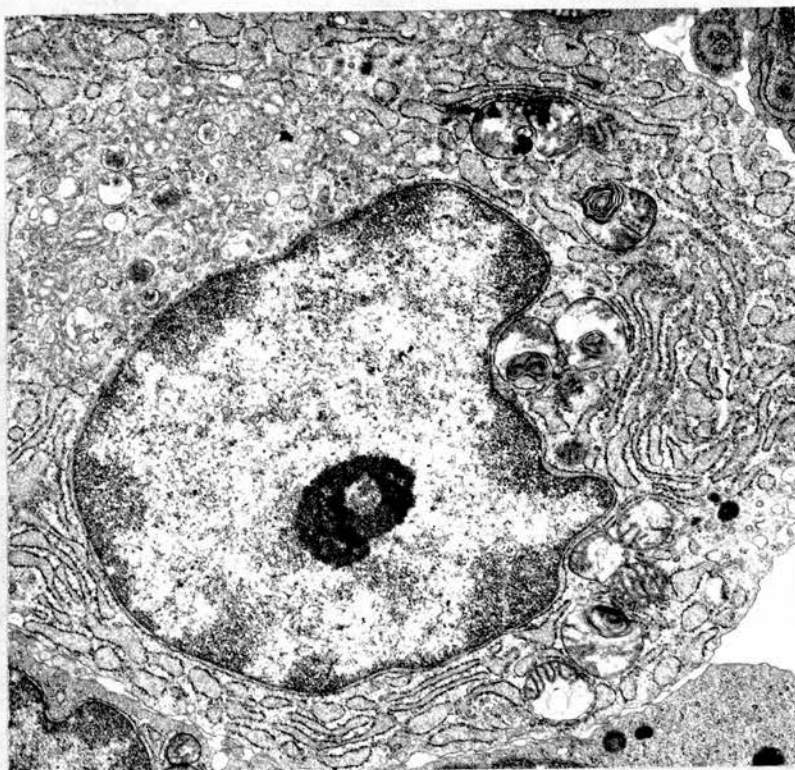


Figure 20: The E.M. Features of a Secretory B lymphocyte (B5)
(Cairns)

This cell, typical of those found in the B5 tumour of patient Cairns, shows well developed Golgi apparatus, and dilated endoplasmic sacs containing IgM antibody shown by the immunoperoxidase reaction. The mitochondria show degenerative changes and contain myelin figures derived from redundant mitochondrial membrane.

E.M. x 7,500

this tumour were large lymphocytes, expressing surface Ig in a non-capping form, but exhibiting Fc receptors (B2.Fc). As shown previously, if the receptor overlap (E and Fc) from the node of Robertson is taken into account, the B cells in this lesion are also B2, and the lesion a B2.C lymphoma.

Patient - Moodie (1) thyroid B2 lymphoma and Robertson (2) corrected for mixed E and Fc receptors.

	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
1)	6	55	Nil	2	30	36
2)	7	37	49	14	15	28

7) Diffuse lymphocytic lymphomas associated with leukaemias of CLL type.

The two cases Velzian and Reid are examples of a unique syndrome encountered in diffuse lymphocytic lymphoma with CLL. In both cases, there appears to be a "block" to the secretion of surface Ig by the tumour cells at diagnosis. These cells appear to be "receptor silent". On storage, or after trypsinisation it is discovered that the cells regain the capacity to secrete surface Ig, showing their B lymphocytic origins. The B cell subclass of these lesions is not clear. Velzian has been classified as B1, and Reid as a B2 tumour.

Patient Velzian.

This patient presented with enlarged nodes in the neck, and a/...

a high white cell count ($220 \times 10^6/\text{ml}$). The histological features were those of a diffuse lymphocytic lymphoma, of well differentiated type, and the peripheral blood showed the appearances of chronic lymphocytic leukaemia. The receptor profile of the lymph node and blood at diagnosis is shown below, and the histological appearances illustrated (Figs. 21, 22).

Receptor profile lymph node (1) and blood (2).

Patient Velzian (diffuse lymphocytic lymphoma, well differentiated, with CLL) Figures are given as a percentage of total cells.

	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
Node	59	2	1	4	*Diffuse Fluorescence	
Blood	33	3	1	2	13	0

* From these profiles, the lymph node cells were predominantly Ig bearing, but on repeated washing NO surface Ig could be detected. Following storage and trypsinisation the following profiles were obtained.

	<u>E</u>	<u>Receptor Silent</u>	<u>Capping</u>	<u>Non-capping</u>
Node (Storage)	27%	40%	10%	5%
Node (Trypsin)	17%	20%	4%	45%
Blood (Storage)	20%	10%	1%	62%
Blood (Trypsin)	6%	NIL	5%	60%

Further studies showed that when treated with anti-Kappa and anti-Lambda light chain serum 60% of cells showed staining, in contrast to/...

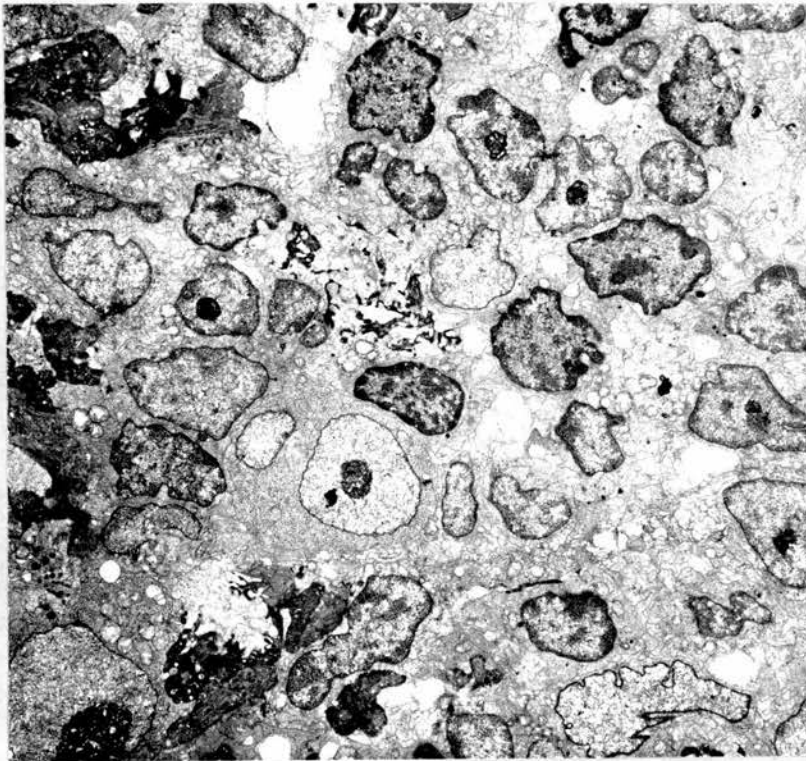


Figure 21: The E.M. Features of Well Differentiated Diffuse Lymphocytic Lymphoma (Velzian)

Although the surface phenotype in this patient was atypical, the features shown here are those of any well differentiated lymphocytic lymphoma. The small lymphocytes show regular nuclear outlines, and although some contain numerous ribosomes there is little rough E.R. in evidence. The cells showed no intracytoplasmic Ig, but expressed surface Kappa chain after trypsinisation and in vitro culture.

E.M. x 1,500

to the 13% obtained in the original sample. After treatment for 3 months, the white cell count in blood was 9.6×10^6 ml, and the following receptor profile was obtained.

	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
Blood	15	38	30	10	60% Kappa 3% Lambda	3% Kappa 4% Lambda

The interpretation of these findings are that in the initial blood and node samples, the cells expressed Kappa chain only which was not detected by polyvalent antiserum. Following trypsinisation, the cells expressed non-capping surface Ig detected by polyvalent antiserum. Furthermore with anti-Kappa chain antiserum, the cells showed capping behaviour. There is an improvement in cell maturity in the blood with treatment, with the cells in the last sample showing B3.1 receptor profile.

The initial diagnosis was of a B1 lymphoma, and this classification is adopted. The complexity of the receptor profile is thought to arise from "blocking" of lymphocyte differentiation in vivo.

Patient Reid

This patient presented with lymphadenopathy and chronic lymphocytic leukaemia. The biopsy showed well differentiated diffuse lymphocytic lymphoma. The receptor profile for the lymph node and diagnostic blood sample is shown below.

Receptor/...

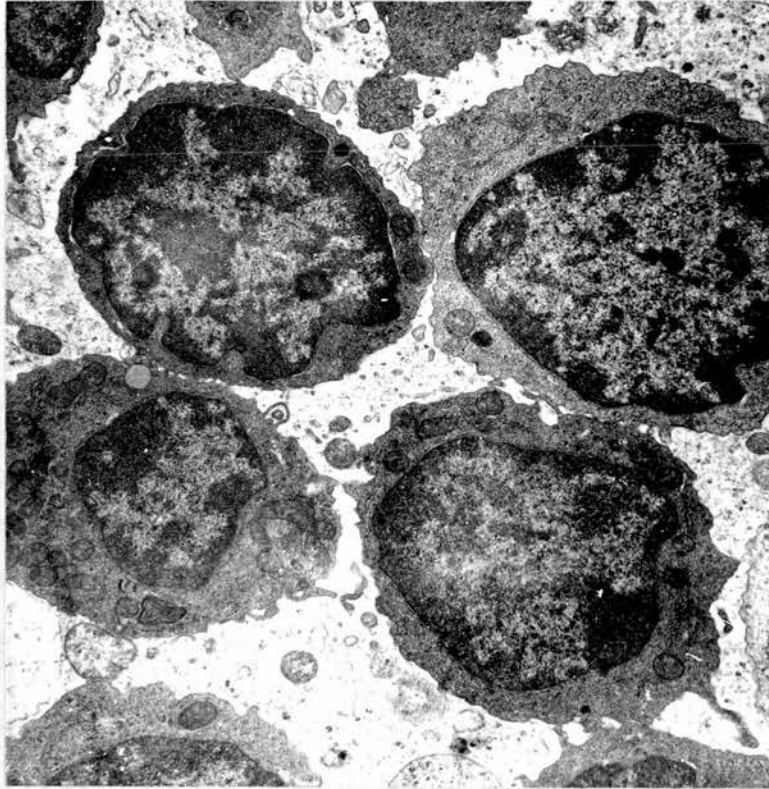


Figure 22: The E.M. Appearance of CLL Cells from the Blood of patient Velzian

The cells show typical features of CLL cells, with ribosome rich cytoplasm, and a few strands of rough E.R. In this Ficoll/Tricosil preparation the cells were initially receptor silent, but after washing expressed non-capping immunoglobulin reacting with polyvalent antiserum. The predominant surface Ig was Kappa chain. A slow response to treatment was associated with the reappearance in the blood of cells rich with the B3.1 phenotype.

E.M. x 7,000

Receptor profile of lymph node (1) and blood (2) of patient Reid (diffuse lymphocytic lymphoma with CLL).

	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
Node (1)	11	2	6	9	5	0
Blood (2)	1.5	15	0	7	0	2

A large population of receptor silent cells was present, but following trypsinisation or storage 50% of cells became positive on testing with anti-Kappa antiserum, and with polyvalent antiserum which did not stain the cells initially. Subsequently three receptor studies on the blood of this patient were done, on the two days subsequent to biopsy (1, 2), and three months later (3). The results are shown below.

Receptor Profile of Blood - Patient Reid.

Figures given are as percentages of total cells.

	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
Blood (1)	0	37	8	1	2	14
Blood (2)	0	20	0	4	0	0
Blood (3)	1	15	4	5	3.3	53 Kappa 28 Lambda

An interesting feature was the presence of the Fc receptor which developed on the blood cells of this patient on storage and in later blood samples. Anti-light chain antisera of both Kappa and Lambda specificity induced capping reactions, but the majority/...

majority of cells showed non-capping behaviour.

The extraordinary similarities between these two cases indicate a syndrome in which the maturation of cells in vivo is "blocked", and which can be reversed by trypsinisation of the cells and storage at 4°C in serum free medium.

Both patients showed light chain expression only, initially, but developed sufficient heavy chain synthesis to be detected by polyvalent antiserum following trypsinisation. Velzian was classified as a B1 lymphoma, Reid as a B2 lymphoma by reason of the Fc receptor present on his peripheral blood and lymph node cells (B2.Fc). In Reid, substantial numbers of cells with Lambda chain surface immunoglobulin were present in blood, compared with the monotonous Kappa chain monoclonality of Velzian. The B cells of Velzian capped well in the last sample giving a B3.1 receptor profile.

Both patients are currently alive, but response to therapy in both has been slow in comparison with the well differentiated chronic lymphocytic leukaemias.

Receptor Profile in Histiocytic Lymphoma

Chapter 19

Receptor Profiles in histiocytic lymphoma

Ten patients had diffuse lymphocytic lymphomas on the basis of Rappaport's classification. The receptor studies showed marked heterogeneity of this group, and survival data supported this view. Three of this group were extranodal, one a primary lymphoma of thyroid, one a lymphoma involving sacrum, and one a tumour of the orbit. Receptor data in one further case was obtained from the spleen.

Receptor silence was present in three of the ten cases. All the cases were diffuse, there were no nodular histiocytic lymphoma in the series.

The receptor profiles of this group and the clinical data are shown in the tables below.

Receptor profiles in diffuse histiocytic lymphoma

Figures given are percentage of total cells.

<u>Name</u>	<u>Viability</u>	<u>E Rosettes</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
Adams	82	19	4	10	1	13	51
Hall	84	10	1	4	10	14	59
Hogg	71	20	1	1	14	2	56
Smith	76	37	10	29	1	38	22
Ross	90	15	P	12	60	16	10
Oliver	85	30	6	8	4	6	0
Ritchie	70	50	4	-	-	4	0
Baird	85	58	43	68	22	15	17
Moyes	75	8	15	18	3	11	8
Johnstone	82	8	6	3	1	Nil	74

From/...

From the data shown, it is evident that four tumours were of undifferentiated B lymphocytes (B1) and three were receptor silent. Only one tumour of B3 lymphocytes was found and this was follicular (B3.2). One true histiocytic or macrophage tumour was noted, and there was one case of receptor overlap.

Diffuse Histiocytic Lymphoma

Clinical data and receptor classification

<u>Name</u>	<u>Histology</u>	<u>A/D</u>	<u>Time (Mo)</u>	<u>Age</u>	<u>Sex</u>	<u>Clinical Stage</u>	<u>Receptor Profile</u>
Adams	DHL	D	3	-	M	Extranodal	B1 Predominant
Hall	DHL	D	2	70	M	II	B1 Predominant
Hogg	DH/DPDL	D	4	76	F	Extranodal	B1 Predominant
Smith	DHL	D	5	44	M	II	B3.2 Predominant
Ross	DHL	D	5	70	M	III	Macrophage
Oliver	DHL	D	12	72	F	III	Silent
Ritchie	DHL	D	18	26	M	IV	Silent
Baird	DHL	A	18	64	F	II	Overlap
Moyes	DHL	A	2	56	F	Extranodal	Silent
Johnstone	DHL	D	3	66	M	IV	B1 Predominant

Analysis of the results in diffuse histiocytic lymphoma

B1 Tumours

Tumours of B1 lymphocytes do occur, but these cells are never encountered in normal lymph nodes, spleen, blood or tonsil except possibly as a very small minority population. One tumour classified as B1 but associated with "receptor block" was encountered in the diffuse lymphocytic lymphoma group. Two of the four B1 histiocytic lymphomas were extranodal in origin; in Adams the lesion was a mass overlying and involving the sacrum, and in Hogg it was a primary lymphoma of the thyroid gland. The tumour in Hall was associated with leukaemia and Johnstone became leukaemic before he died.

The receptor profiles of the B1 lymphomas show remarkable homogeneity, perhaps seen best in the data of Johnstone and Hogg. Figures given are percentage of total cells.

Name	<u>E Rosette</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
Johnstone	8	6	3	1	Nil	74
Adams	19	4	10	1	13	51
Hall	10	1	4	10	14	59
Hogg	20	1	1	14	2	56

The majority of surface Ig bearing cells are non-capping, and virtually no expression of C3 or Fc receptor occurs. Two cases show substantial macrophage populations. Non-capping B cells lacking Fc and C3 receptors occur in lymphoid cell lines, and these may/...

may be regarded as B1 prototypes.

In patient Hogg, cells from the original biopsy failed to show intracytoplasmic Ig, although marked pyroninophilia was observed in touch preparations. Following trypsinisation and culture, the cells showed the presence of intracytoplasmic IgG after 72 hours, and morphologically resembled plasma cells. The parallel between this tumour and the EB4 cell line is striking, and the resynthesis of cytoplasmic Ig suggests strongly a "receptor block" similar to that in Velzian or Reid of the diffuse lymphoma class. The longest survival in this group was four months, and no tumour responded satisfactorily to treatment.

Receptor Silent Tumours

These tumours, present in Oliver, Ritchie and Moyes behaved in the same way as that of Kerr (diffuse lymphocytic lymphoma). The tumour cells failed to exhibit surface Ig, even after trypsinisation (Moyes) and had no detectable receptor for sheep cells, Fc or complement. They failed to concentrate neutral red dye. The diagnostic biopsy in the case of Oliver was lymph node, and receptor studies were made at the time of diagnosis.

After initial response to treatment this patient relapsed and finally died with generalised lymphadenopathy, and undifferentiated tumour in kidney and lung at autopsy. Even at autopsy, the histological appearances cannot exclude anaplastic carcinoma arising in lung or kidney, although the clinical presentation was that of lymphoma. The morphology of these cells is shown in Figure 23.

Ritchie/...

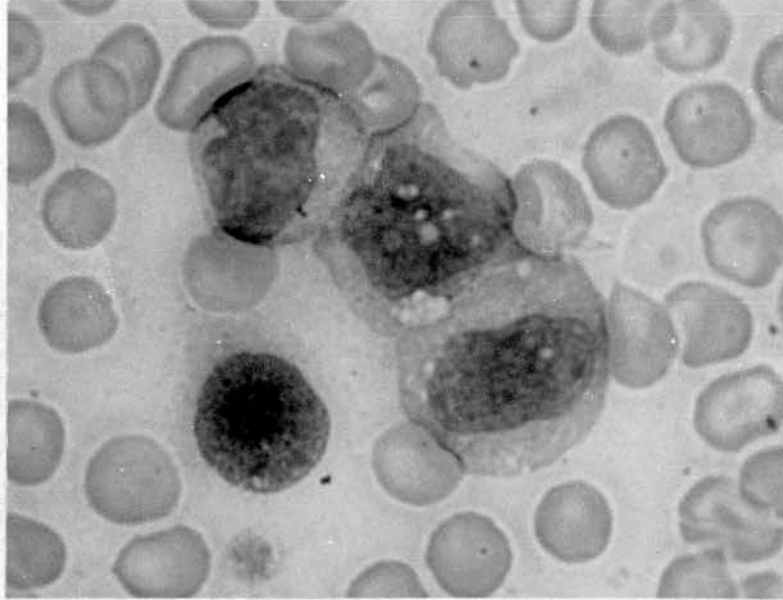


Figure 23: The Appearance of Receptor Silent Cells in Peripheral Blood (Oliver)

This patient had a receptor silent tumour, histologically classified as diffuse histiocytic lymphoma, terminating fatally after 12 months. Abnormal circulating tumour cells were present, and the appearance of these cells in a blood smear is illustrated. They are cytologically primitive cells with abundant cytoplasm, and exhibit cytoplasmic and nuclear vacuolation.

Oil immersion photomicrograph, Giemsa stained blood film

x 1,000

Ritchie presented initially with an enlarged node in the neck, and the initial diagnosis was that of lymphocyte depleted Hodgkin's disease. There was a temporary response to radiotherapy, but relapse soon followed with the development of splenomegaly and involvement of the lymph nodes in the coeliac axis leading to duodenal obstruction. The spleen was massively enlarged at laparotomy and proved to be extensively involved with lymphoma. Shortly before death, receptor silent cells appeared in the peripheral blood. The E.M. appearances in the spleen of this patient is illustrated (Fig. 24). Receptor studies were done on the spleen.

Moyes presented with an orbital tumour, which on removal was reported as a poorly differentiated plasmacytoma of the orbit. This was treated with radiotherapy, but shortly after neck gland enlargement was noted, and a diagnostic lymph node biopsy was reported as showing an undifferentiated tumour compatible with the diagnosis of histiocytic lymphoma.

These three cases illustrate the extreme difficulty of accurate histological diagnosis in undifferentiated tumours of this type. In these cases, the receptor silent nature of the cells does not support a firm diagnosis of lymphoma, although the presentation, histological features and clinical behaviour is that of lymphoma. This particularly applies to patients Oliver and Moyes, although there is little doubt that the tumour in Ritchie was a lymphoma.

The receptor profile of these patients is shown and the tumour cells of Ritchie and Oliver are illustrated (Figs. 23, 24).

Oliver/...

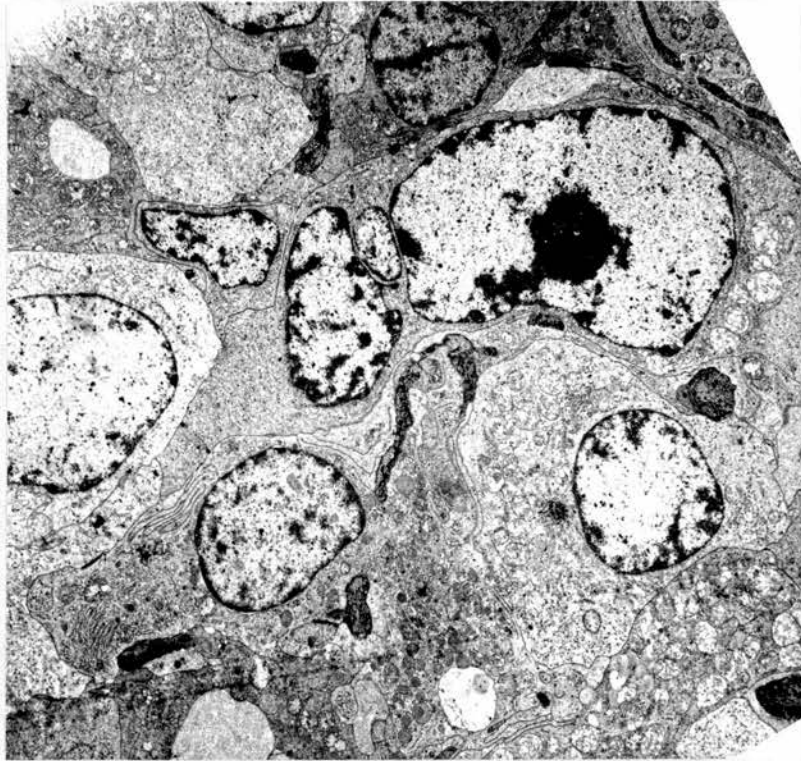


Figure 24: Electron Micrograph of the Receptor Silent Tumour of Ritchie

The receptor silent tumour of Ritchie, in contrast to that of Kerr, was composed of cells with irregular nuclear contour and electron dense cytoplasm. In the spleen they were closely applied to the reticulin fibre framework. Occasional examples showed well developed E.R. and Golgi apparatus and had abundant mitochondria. Similar cells were present in the circulation. The cells of this tumour clearly differ from those of patient Kerr (Figs 14-17) although both were characterised by the absence of surface phenotypic features (Receptor silence).

E.M. x 1,825.

Oliver

Smears from this lesion showed the large cells comprising the neoplastic population formed 70% of the lymph node population. The small lymphocytes formed the remaining 30%. All the small cells formed E rosettes, but failed to express Fc or C3 receptors. Many of the large cells showed weak diffuse surface staining with anti-Ig antisera, but this staining was readily eliminated by washing. The receptor profile is shown below:-

Figures given are percentages of total cells.

	<u>E Rosettes</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>	<u>Silent</u>
Node	30%	6%	7%	4%	6%	-	65%

As shown the contaminating population of small lymphocytes is overwhelmingly composed of T cells, with a few mature B cells present. The clinical stage at diagnosis was 1B, but was stage IIIB at 14 months and the patient died 16 months after diagnosis.

Ritchie

The first receptor studies of spleen, and blood, were carried out fifteen months after the initial diagnosis, and three months before death. From the spleen suspension, a pure population of neoplastic cells was obtained by density sedimentation through bovine serum albumin. These cells were completely receptor silent. Electron microscope studies were done on blood and/...

and spleen neoplastic cells (illustrated). The receptor studies are shown below. They include spleen (1) and blood (2) and a second blood sample obtained just before death (3)

Figures given are as percentages of total cells.

	<u>E Rosettes</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>	<u>Silent</u>
Spleen(1)	50	4	-	Nil	4	Nil	50%
Blood(2)	38	14	-	-	9	5	48%
Blood(3)	15	4	-	Nil	8	4	73%

The increase in receptor silent cells in blood, and the proportional decrease in T cells is well shown. Most of the splenic lymphocytes were T cells. The tumour cells were not phagocytic.

Moyes

The receptor profile of the neck lymph node is shown below. The cells showed marked cytoplasmic pyroninophililia, but exhibited no receptors, and were not phagocytic. Intracytoplasmic Ig was not present. Many of the contaminating normal lymphocytes were B cells and relatively few T cells were present. Figures given as percentage of total cells.

	<u>E Rosettes</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>	<u>Silent</u>
L.Node	8%	15%	18%	3%	11%	8%	67%

Many/...

Many of the Fc and C3 receptors detected were present on contaminating polymorphs, and not on the B lymphocytes. The histochemistry of the cells in this lesion is shown below.

Non Specific Esterase (ASD chloroacetate)	+++
Acid Phosphatase	+++
Sudan Stain occasional positive granules	+
Peroxidase	-
Alkaline Phosphatase	-
P.A.S. Stain granular	+
M.G.P. Stain	+++
Immunoperoxidase, negative for A, G and M chain, Kappa and Lambda.	

These results clearly exclude a myeloid, or histiocytic origin, but are compatible with some reported findings in anaplastic carcinomas. According to one pathological opinion the lesion is a carcinoma. This patient was still alive three months after diagnosis but died soon after.

All of the three patients with receptor silent tumours in this group have died. This fact taken in context with the findings of receptor silent populations in Robertson A. (Nodular lymphocytic lymphoma and Kerr (Diffuse lymphocytic lymphoma) where receptor silence again occurred during progress of the disease implies an ominous prognosis for patients with this class of neoplasm.

B3.2 lymphoma (Smith)

The receptor profile in this patient showed a predominance of B lymphocytes, of which the majority were capping and expressed/...

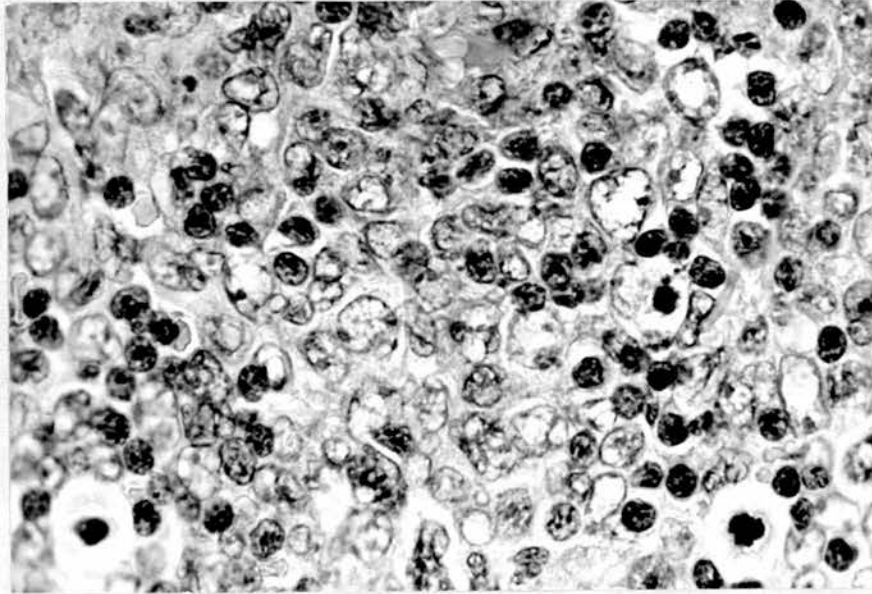


Figure 25: The Histiocytic Lymphoma of Smith

This tumour, which had the surface phenotype B3.2, is composed of large irregular cells admixed with small lymphocytes. The tumour is notably more "lymphoid" in character than other histiocytic lymphomas in this series.

H & E stain x 600

expressed the complement receptor. The profile looked similar to that of germinal centre, and was classified as B3.2. The profile is shown below.

Receptor profile of Smith. Figures given are percentages of total cells.

<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
37	10	29	1	38	22

The patient never gained complete remission and died 5 months after diagnosis. The histological appearances are illustrated (Fig. 25).

True Histiocytic Lymphoma

The lymph node in Ross showed a singular receptor profile. In a node where most cells were viable, the majority were macrophages as assessed by neutral red uptake. On tissue culture many of the cells were morphologically normal macrophages, and the majority were phagocytic for antibody coated cells. 26% of the cells were B lymphocytes with capping surface immunoglobulin on the majority, i.e. mature B lymphocytes. Tumour cells showed the capacity to ingest antibody coated erythrocytes. The receptor profile for blood is shown below. Phagocytes were numerous, although the white cell count did not reach leukaemic proportions. The complement receptor cells in node were B lymphocytes/...

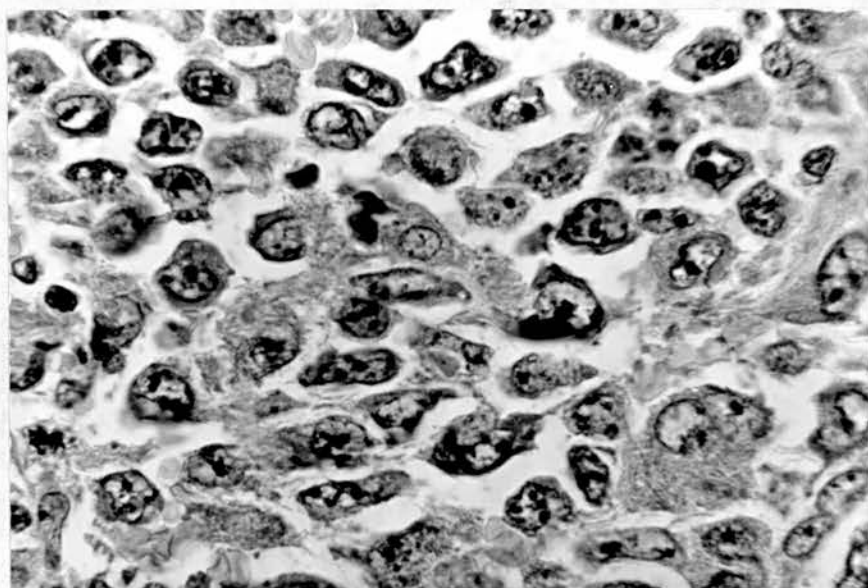


Figure 26: The Histological Features of True Histiocytic Lymphoma (Ross)

This tumour was the only example in this series of a lymph node tumour demonstrated to be of mononuclear phagocytes. As the histology shows the cells were separate, polygonal and pleomorphic with variable amounts of abundant cytoplasm.

H & E Stain x 600



**Figure 27: Surface Phenotype of True Histiocytic Lymphoma:
Absence of E Rosetting (Ross)**

This illustration shows a large tumour cell in contact with a small lymphocyte which has bound sheep erythrocytes: (E rosette). The absence of E rosetting by the tumour cell is clearly shown.

Phase contrast, living cells.

× 720

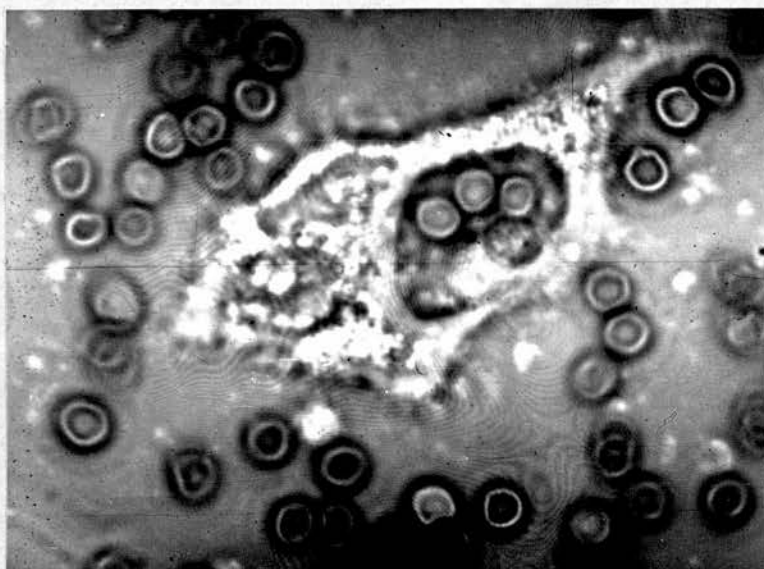


Figure 28: Surface Phenotype of True Histiocytic Lymphoma:
Phagocytosis of IgG Coated Red Cells (Ross)

Cultured cells from the lymph node were glass adherent, and as shown here exhibited phagocytic activity. The binucleate tumour cell has ingested a clump of erythrocytes and other debris into a single abnormally large phagocytic vacuole. The erythrocytes, added shortly before this photograph was taken, were coated with IgG antibody, and several are shown adhering to the cell processes on the right.

Phase contrast, 2 hour culture, IgG sensitised red cells added 30 minutes. Living cells.

x 1,000

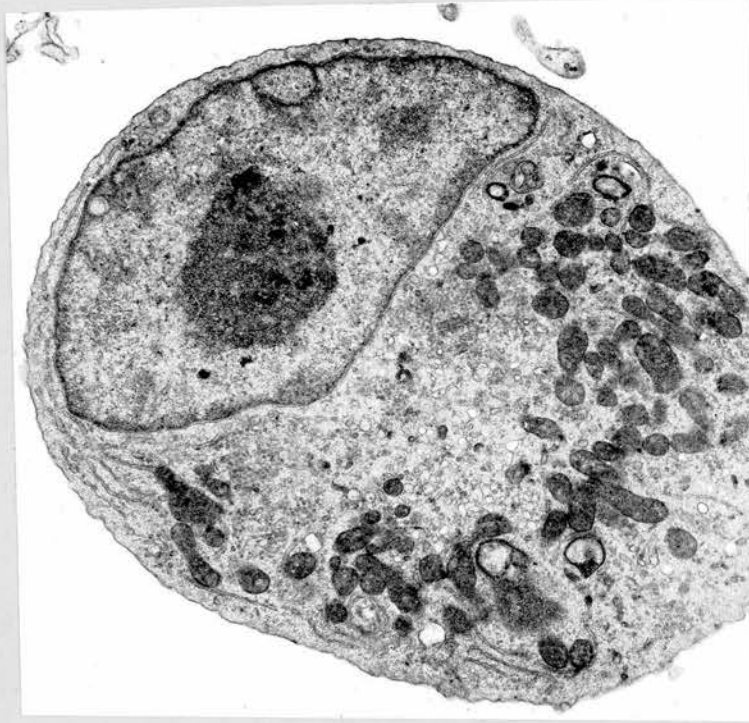


Figure 29: Electron Microscope Appearance of True Histiocytic Lymphoma (Ross)

A single cell from the tumour of Ross is illustrated. The appearances are of a mitochondria rich cell, with well developed Golgi apparatus and a few strands of smooth E.R. The cell surface lacks the characteristic ruffles of the normal macrophage, but occasional liposomes and phagoliposomes are seen within the cytoplasm.

E.M. x 7,500

lymphocytes, but the population in blood rosetted strongly with both complement coated and antibody coated red cells. The histology of the tumour is illustrated (Figs. 26, 27, 28, 29).

Receptor profile in Node (1) and blood (2) of patient Ross.

Receptors given as a percentage of total cells.

	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
Node(1)	15	Phagocytic	12	60	16	10
Blood(2)	38	58	67	46	18	1

This patient showed no response to treatment and died 5 months after diagnosis.

Receptor Overlap

The lesion of Baird, classified as diffuse histiocytic lymphoma, gave a receptor profile very similar to that of Laverick, classified as a diffuse mixed lymphocytic and histiocytic lymphoma. In a node where 85% of cells were viable, the sum of E rosetting cells, phagocytes, and surface Ig bearing cells was 112%. This means at least 27% of cells have dual markers for T cell and B cell, or T cell and macrophage. It was noted that a few tumour cells did not exhibit markers, which confirms the receptor overlap on the remaining cells. As with Laverick, the patient has done well despite "bad" histology, and these findings suggest that a subclass of lymphoma exists, of mixed histiocytic and/...

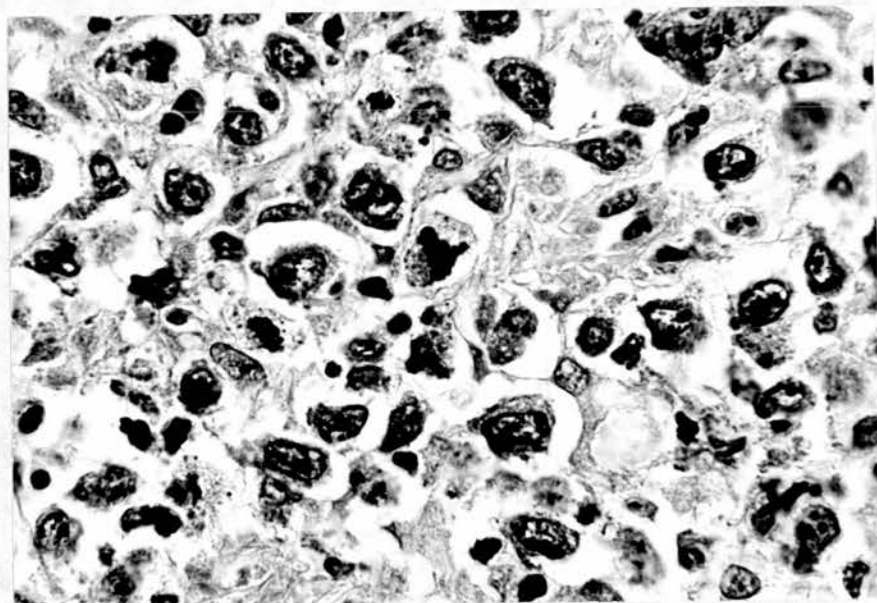


Figure 30: Histological Appearance of a Receptor Overlap Tumour (Baird)

Sections of this tumour show cells with marked nuclear hyperchromatism. Much shrinkage artefact is present. Compare these features with the tumour of Laverick (Figure 11) which was of different histological appearance, yet both tumours were of the same surface phenotype (Receptor Overlap) and both responded well to chemotherapy.

H & E Stain x 600

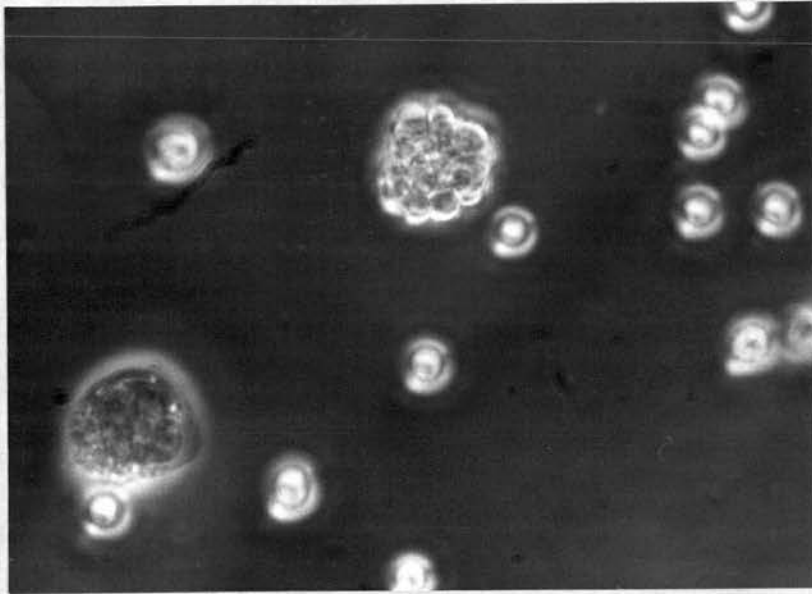


Figure 31: Surface Phenotype of a Receptor Overlap Tumour (Baird)

The appearances of two tumour cells in a complement rosette preparation are shown. One cell has bound a single complement sensitised red cell (and hence is not counted as a rosette) and the other forms the characteristic tight cluster with red cells. In this node 68% of the tumour cells formed complement rosettes, and 58% rosetted with sheep erythrocytes.

Phase contrast, complement rosette preparation living cells.

x 850

and lymphocytic or histiocytic histology, showing receptor overlap, which carries a better than average prognosis for tumours of these histological classes. The histological appearances are illustrated (Figs. 30, 31) and should be compared with those of Laverick (Fig. 11) who had a similar kind of tumour.

Receptor Profiles in Leukaemia

Chapter 20

Contents

- a) Acute lymphocytic leukaemia
- b) Chronic myeloid leukaemia
- c) Chronic lymphocytic leukaemia
- d) Monocytic leukaemia
- e) Receptor profile in hairy cell leukaemia
(leukaemic reticuloendotheliosis)
- f) Receptor silent leukaemia

Summary table of patients with leukemia either as a presenting feature or occurring with lymphoma.

Figures given as percentages of total cells.

<u>Patient</u>	<u>Type of Leukaemia</u>	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
Turnbull	ALL	3	3	5	1	27	Nil
Klimzcak	Monoblastic	5	56	44	50	Nil	Diffuse 100%
Plakowsky	CLL	9	17	1	4	5	78
Smith	"Hairy Cell"	3	-	14	Nil	Nil	70*
Russel	CML	12	55	8	3	12	8
Mowatt	CLL	28	38	22	18	18	1
Cooke	ALL	91	5	1	Nil	1	Nil
Blake	CLL	2	9	2	14	13	84
Velzian	CLL	33	1	1	2	1	66
Reid	CLL	2	15	0	7	0	53
Walkingshaw	Lymphocytosis	59	15	30	9	23	16
Coburn	CLL	11	4	2	Nil	2	1
Kerr	RSL	10	17	18	1	4	1

Note that in all cases here, the white cell count exceeded 10×10^6 ml with the exception of patients Walkingshaw, Russel, and Mowatt.

* The cells are here classed as non-capping, although NO evidence for synthesis of surface Ig was obtained, and they are not B cells.

Receptor Profiles in Leukaemia

Leukaemias were not a major part of this study but on occasions the author was asked to investigate leukaemias to discover the nature of the leukaemic cell. In some cases of lymphoma the disease was associated with leukaemia (as in Velzian and Reid) and in others a leukaemia of receptor silent cells was present (as in the patient Kerr). These cases have already been documented, but summaries of the data are included in this study. Each case or group of cases is discussed separately. The data shown in the summary table is based upon the first sample of blood from each patient.

Acute lymphocytic leukaemia

Two cases of acute lymphocytic leukaemia in children were studied. Only one blood sample was available from each, as both died within a short time of diagnosis. The receptor profiles of the two cases were very different, that of Turnbull being the more common "receptor silent" leukaemia, that of Cooke being the more rare T cell acute lymphocytic leukaemia.

ALL Receptor Profiles

Figures are given as percentages of total cells.

	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>	<u>Silent</u>
Turnbull	3	3	5	1	27	Nil	70%
Cooke	91	5	1	Nil	1	Nil	8%

The/...

The patient Cooke was a 12 year old boy with a mediastinal mass on X-ray, and a white cell count of 37×10^6 /ml. These cells were lymphoblastic and acid phosphatase positive. He died within 24 hours of admission to hospital. Turnbull was a female child of 8 years with recently diagnosed ALL. She was very ill on admission and died 2 days after the peripheral blood study. The white cell count was 80×10^6 /ml, and the cells were lymphoblastic, and acid phosphatase negative.

Chronic Myeloid Leukaemia

This single case of chronic myeloid leukaemia had been treated and was in apparently good health at the time of study. The patient had had a splenectomy, and the white cell count in the sample was 4.8×10^6 /ml. The study was undertaken at the haematologist's request to see if receptor studies could detect an abnormal population of "Myeloid" cells in a blood showing none of the cytological abnormalities of CML. The receptor profile is shown below.

CML Receptor Profile compared with normal blood values.

The figures given are as percentages of total cells.

<u>Patient</u>	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>	<u>Silent</u>
Russel	12	55	8	3	12	8	65
Normal	47	34	26	18	26	14	Nil

As indicated, a receptor silent population is present, which expresses/...

expresses, on some cells, Fc receptors. 65% of cells are of this type and 32% of these (allowing for Fc receptor on all B cells and macrophages) express the Fc receptor. This is an abnormal population, but it cannot be termed "myeloid" on the evidence given. The absolute values of each class of cell are shown below, together with the values and S.D. for normal blood. As seen, the total number of T cells is low, and that of Fc receptor bearing cells high. B cell, complement receptor and phagocyte levels are considered normal.

Patient Russel. Absolute numbers in millions of cells per ml of blood compared with normal values.

	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>	<u>Total</u>
Normal	1.01	0.74	0.55	0.38	0.55	0.31	2.25
	(+0.23)	(+0.23)	(+0.26)	(+0.12)	(+0.26)	(+0.11)	-
Russel	0.58	2.64	0.38	0.14	0.58	0.38	4.80

The mononuclear cell count is marginally higher than normal but the distortion in receptor profile is quite clearly seen. The conclusions are that even with adequate treatment, and apparent health, abnormal cells can persist in the circulation at least in this single case of CML.

Chronic Lymphocytic leukaemia

The cases of chronic lymphocytic leukaemia were Plakowsky, Mowatt, Blake, Velzian, Reid and Coburn.

The/...

The receptor profiles obtained in serial studies at monthly intervals of the patient Plakowsky from diagnosis to death, are shown below.

Receptor profile of Plakowsky. Blood samples (1) 20/12 (2) 24/3 (3) 14/4 (4) 1/6. The patient died 2 days after the last blood sample. Figures given are either in millions of cells ml (WBC count) or as percentage of total cells.

<u>WBC count</u>	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
1) -	9	17	1	4	5	78
2) 19.1	14	16	54	4	48	5
3) 23.5	5	9	18	8	58	Nil
4) 26.5	6	13	68	5	Nil	91

The cells composing the leukaemic population synthesised surface Ig. With treatment there was a rapid maturation of cells with an increase in complement receptor. The initial profile was that of a B1 lymphoma with weak expression of the Fc receptor. This rapidly changed, with treatment, to a cell type of B3.2 class. The cells subsequently matured to a B4 class (sample 3) but did not show cytoplasmic Ig. At this stage a receptor silent population was present, and by the next month (sample 4) the circulating cells had changed to a B2.C cell type. It is not possible to conclude that these changes were intrinsic differentiation steps or whether they were induced by treatment. The sequence of change in surface

phenotype is B1	B3.2	B4	
(1)	(2)	RS	B2.C
		(3)	(4)

The case confirms, once again, the ominous prognosis of neoplasms containing receptor silent cells, and non-capping lymphocytes of B class. T lymphocyte and phagocyte levels were increased (in absolute numbers) over normal values.

The patient Mowatt was an atypical CLL, with a low WBC count at presentation. The presence of receptor silent cells was noted on both occasions of study, but the recovery on Ficoll/triosil separation was only 10%. This highlights one of the difficulties of working with leukaemic blood. It is not possible to draw valid conclusions from a cell population representative only of 10% of the starting population. The differential count on both occasions was 15% polymorphs, 25% lymphocytes, 4% monocytes, 8% eosinophils and 48% atypical lymphocytes. There was evidence of cell death in the Ficoll/triosil separated smears. The patient died four months after diagnosis.

Receptor profile of blood of Mowatt, sample (2) one week after sample (1). Figures are given as a percentage of total cells.

<u>Sample</u>	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>	<u>Silent</u>
1)	28	38	22	18	18	1.1	30
2)	20	14	-	12	5	0	63

Another patient Blake had an acute relapse of chronic lymphocytic leukaemia, and she died within 24 hours of obtaining the first blood sample. The white cell count was 268×10^6 per ml and lymphoblasts were virtually the only cells present. The receptor/...

receptor profile is shown below. Figures given are as a percentage of total cells.

<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
2	9	2	14	13	84

Studies with anti-light chain antisera showed all the capping cells to express lambda chain, while all non-capping cells were of Kappa chain type. It is doubtful that all neutral red ingesting cells were of monocyte class, similar cells occur in lymphoid cell lines (e.g. the DAUDI cell line). On the basis of the receptor profile this lesion was classified as a B1 leukaemia.

The patient Coburn had a "classical" CLL lesion and blood samples were obtained at the first visit, and a repeat sample one week later. Because the differential count showed atypical lymphocytes (36%) he received treatment and the effect of this is shown by comparison of sample (1) with sample (2). The white cell count on the first occasion was 5.5×10^6 /ml, and on the second 2.75×10^6 /ml. Absolute values of each cell class are shown together with values from normal blood. He responded well to treatment and was still alive 2 years later.

Receptor profile patient Coburn, sample (1) and (2). The absolute values are given as millions of cells per ml. of blood, the other values are as percentages of total cells. Normal absolute values are shown in the last line.

E/...

	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>	<u>Silent</u>
Sample(1)	11	4	2	Nil	2	1	86
	0.57	0.21	0.09	-	0.09	0.03	-
Sample(2)	46	25	18	5	1	1	47
	1.26	0.69	0.51	0.14	0.02	0.02	-
Normal	1.01	0.74	0.55	0.38	0.55	0.31	
S.D.	(+0.23)	(+0.23)	(+0.26)	(+0.12)	(+0.26)	(+0.11)	

As shown, despite the reduction of cell counts, values for E rosettes (T cells) increase to normal levels, as do Fc and C3 receptor bearing cells. The receptor silent population does not express surface Ig detected by polyvalent antisera, but unhappily studies were not done using anti-light chain antisera in this case. These cells are probably B cells to judge by expression of Fc and C3 receptors, and like Reid and Velzian might be presumed to express Kappa or Lambda light chain only.

Receptor profiles of Reid and Velzian have already been discussed in the section on diffuse lymphoma. The blood data are repeated here for comparison with data from Coburn.

Velzian Receptor profiles of blood (1) at diagnosis (2) after storage, 24 hrs at 4°C (3) after trypsin (4) after 3 months treatment. Figures are given as percentage of total cells present.

E/...

	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
Sample(1)	33	3	1	2	13	0
	Non-capping cells with anti-light-chain 60 Kappa 13 Lambda					
Sample(2)	20	-	-	-	-	-
	The surface Ig was detected with polyvalent antiserum.					
Sample(3)	6	-	-	-	5	60
	The surface Ig was detected with polyvalent antiserum.					
Sample(4)	15	38	30	10	(60% Kappa) (3% Lambda)	(3% Kappa) (4% Lambda)
					63%	7%

Reid. Receptor profile of blood (1) at diagnosis (2) 24 hrs after diagnosis (3) 48 hrs after diagnosis (4) two months after diagnosis (5) three months after diagnosis. Figures shown are as a percentage of total cells present. Surface Ig (1) (2) and (3) with polyvalent antiserum.

	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
Sample(1)	1.5	15	0	7	0	2
Sample(2)	0	37	8	1	2	14
Sample(3)	10	20	0	4	0	0
Sample(4)	1	15	4	5	3	53 Kappa 28 Lambda
Sample(5)	1	1	6	4	1.8	79 Kappa 5 Lambda

Monocytic/...

Monocytic Leukaemias

Receptor profiles of the patient Klimczak are shown as an example of a typical leukaemia of monocytes and promonocytes. The receptor profile of blood was quantitated on 3 occasions on 9/4 (1) 12/4 (2) and 13/4 (3). The white cell count was 110×10^6 cells/ml, and most cells showed the cytological features of monocytes. The receptor profiles are shown below.

Patient Klimczak. (Peripheral blood) Figures given are as a percentage of total cells present.

	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
Sample(1)	8	64	56	61	50	50
Sample(2)	7	55	31	48	18	57*
Sample(3)	5	56	44	50	Nil	100*

*These two samples studied with polyvalent, and monospecific antisera.

Upon incubation of the cells for 2 hr. at 37°C , 14% rosetted with and 37% ingested ox C3 red cells. After 2 hrs. incubation at 37°C , 2.5% rosetted with, and 57% ingested ox Fc reagent. The cells were PAS positive, but Sudan black negative. An interesting feature was the high level of surface Ig positivity seen in this blood. On trypsinisation it proved impossible to remove surface staining without disrupting the cells, proving that Ig was mainly intracellular, although all capping Ig was removed with trypsin. The/...

The Ig present, by monospecific antiserum, proved to be IgD, and it was furthermore shown that overnight incubation in the patient's serum was necessary to preserve the IgD staining. If incubated in normal serum overnight, all staining of the cells disappeared. The reasons for this phenomenon are not clear, but the cells showed absolutely no evidence of Ig synthesis when cultured in normal serum. The interpretation of these results is that in the patient the monocytes exhibited cytophilia for IgD, which was ingested and became localised to phagocytic vacuoles, giving rise to a "dotty" fluorescent pattern which resisted mild trypsinisation. On culture in a neutral serum, no detectable immunoglobulin was synthesised by the cell, and neither was IgD detected on the surface or inside the tumour cells. The serum levels of IgD in the patient were normal. The patient has done well and is currently in remission. The receptor profile shown below is the latest available, the white cell count is now 5.7×10^6 /ml.

Receptor profile of patient Klimczak in remission. Figures given are as percentages of total cells.

<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
54	12	42	41	Nil	16

Notice that T cells are at above normal levels (2.9×10^6 /ml, normal values 1.01×10^6 /ml) and that cells exhibiting non-capping fluorescence are present.

Receptor/...

Receptor profile in Hairy Cell Leukaemia, (leukaemic reticulo-endotheliosis)

Examples of this condition are rare, and this patient was studied in an attempt to characterise the leukaemic cells. Blood was obtained on 3 occasions, (1) and (2) one week apart, and sample (3) two weeks later. The spleen was also examined. The surface Ig bearing cells were not "B" cells, as far as could be ascertained, since no resynthesis of surface Ig could be demonstrated following trypsinisation.

Receptor profile of Smith: Samples (1), (2) and (3) blood, sample (4) spleen. Figures given are as percentages of total cells present.

<u>Sample</u>	(1)	(2)	(3)	(4)
E	3	9	3	3
Fc	Aggr.	69	3	33
C3	14	7	-	2
IgM	18	54	-	1
Phagocytes	Nil	1	-	28
Capping	70	80	76	84
Non-capping	0	0	0	0
Fc (Hu)	Aggr.	-	-	17

The cells expressed IgM receptors, and were found to be the only IgM receptor positive cells in non-Hodgkin's lymphomata. The/...

The cells showed a weird pattern of staining with monospecific antiserum, 31% were Kappa chain staining and showed capping, with 66% Lambda chain positive and capping. 20% of cells expressed gamma heavy chain, but did not cap, and 41% capped with anti chain. Anti-delta and anti-alpha heavy chain staining was weak and non-capping in character, about 22% of cells staining with each. Immunoglobulin expression therefore was polyclonal in nature and when surface Ig was removed the hairy cells failed to resynthesise surface Ig (10% positive in 24 hrs.). Histochemically the leukaemia cells were esterase and acid phosphatase positive, and peroxidase negative.

The cells survived storage, and culture quite well. They were non-phagocytic and non-glass adherent. Anti-monocyte serum (courtesy of Dr. A.E. Stuart and Mrs. G. Young) failed to react with these cells. Marked coagglutination of the cells occurred when tested with heavily sensitised IgG coated ox or human erythrocytes. The conclusions show that the cell of leukaemic reticulo-endotheliosis is non-Ig-synthetic, has IgM receptors, and marked Fc receptor activity, but weak C3 receptor expression. It appears to be cytophilic for immunoglobulin of IgM, and IgG classes especially, but fails to restore surface Ig in 90% of the population following trypsinisation. It does not react with sheep erythrocytes, change its differentiation in culture, adhere to glass or exhibit phagocytic activity. It fails to express monocyte specific surface antigens. The patient has currently survived five months, and remains comparatively well. The serum IgM level is 1,250 international units (50-250 iu is normal) and the increase is polyclonal.

A/...

A case of lymphocytosis

Occasionally patients present with abnormalities which are not necessarily leukaemic, but which give rise to anxiety on examination of the blood film. Walkingshaw is such a patient, presenting with a febrile illness, abnormal liver function tests, and a markedly abnormal blood film characterised by a low number of polymorphonuclear leukocytes, and an absolute lymphocytosis. The mononuclear cell count was 4.67×10^6 /ml with a differential of 27% polymorphonuclears, 71% lymphocytes and 2% monocytes. The receptor profile and absolute cell counts are shown below. The receptor profile (1) is given as a percentage of total mononuclear cells, the quantitative data (2) as millions of cells per ml of blood. Normal values (3) are shown in the last line.

	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
(1)	59	15	30	9	23	16
(2)	2.81	0.70	1.38	0.42	1.07	0.75
(3)	1.01	0.74	0.55	0.38	0.55	0.31

As shown there are significant increases in T cells, in the complement receptor, and in capping and non-capping cells. Phagocytes are not significantly increased and Fc receptor levels are normal. This patient was suspected clinically of having Hodgkin's disease, but has recovered and remains well.

Receptor silent leukaemia

A/...

A number of receptor silent leukaemias have developed in patients with lymphoma (Kerr, Ritchie). Acute lymphocytic leukaemia is either receptor silent or T cell in type. The example given here is the blood of patient Kerr, studied on two occasions during the progress of the disease. Sample (1) is the receptor profile on diagnosis, with absolute values (2) and sample (3) was studied three months later, with absolute values (4). Figures given are as percentages of total cells present, or as millions of cells per ml. The white cell counts (72% (1,2), 43% (3,4) mononuclears) were $5.6 \times 10^6/\text{ml}$ (samples 1 and 2) or $27.9 \times 10^6/\text{ml}$ (samples 3 and 4). Normal values are given in the last line.

	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>	<u>Silent</u>
(1)	42	32	28	1	3	3	23
(2)	1.71	1.3	1.13	0.04	0.12	0.12	0.77
(3)	10	17	18	1	4	1	67
(4)	1.13	2.04	2.12	0.12	0.48	0.16	7.96
Normal	1.01	0.74	0.55	0.38	0.55	0.31	Nil

It is obvious that the values in (4) are normal, except for the excess of Fc and C3 receptor, which however, can be present on only a tiny minority of the receptor silent population. Here the normal blood lymphocytes co-exist in normal numbers with a large number of unidentifiable tumour cells, which, although lymphoid in morphology, are not functionally lymphocytic. In the last sample, a leuko-erythroblastic blood smear was obtained due to replacement of marrow by tumour. Many of the nucleated red cell series, and juvenile/...

juvenile polymorphs were lost on the ficoll triosil gradient, but early myeloid series cells were present in the tested sample, and probably account for the Fc and complement receptors detected. Examination of smears shows convincingly that most cells present were neoplastic cells of lymphoid morphology.

This study of leukaemic blood emphasises that the examination of the surface phenotype of mononuclear cells can (2) convincingly demonstrate the class of cell involved in most cases, (b) allows quantitative monitoring of the response to treatment, (c) confirms that the major classes of lymphocyte demonstrated in lymph node can with certainty be identified also in leukaemic blood. There is no obvious phenotypic difference between the cells in the node of patients with lymphoma and in the blood of patients with lymphocytic leukaemia.

Lymphocytic leukaemia was of cells classifiable as T cells, B cells or receptor silent cells, as found in the study of lymphoma lymph nodes. Apart from the single case of leukaemic reticulo-endotheliosis, no characteristic "leukaemia" profile was discovered in lymphocytic leukaemias of either acute or chronic type.

Predictive Value of Receptor Classification

Chapter 21

- a) Relationship of clinical stage, receptor classification and histology to prognosis in patients with non-Hodgkin lymphoma and leukaemia.

- b) Statistical appendix. Method of analysis of receptor and survival data.

The relationship of clinical stage, receptor classification, and histology to prognosis in patients with non-Hodgkin lymphoma, and leukaemia.

The predictive value of receptor studies in lymphoma is based on statistical evaluation of criteria such as the relationship of the class of tumour determined by histology, or by receptor studies to the survival of the patient. There is an appendix to this section which explains the methods used to test the difference between patients with B1 and B2 lymphomas, or with B3 and B4 lymphomas on the basis of their survival, and investigate whether the clinical staging of such tumours was an influence on the different survival characteristics observed. The calculations shown were due to Miss S.M. Gore (Department of Medical Computing and Statistics, University of Edinburgh). They show that in the lymphomas examined, the survival data correlates strongly with receptor classification, the less well differentiated B1 and B2 tumour patients having a poorer prognosis than the patients with the better differentiated B3 and B4 tumours. There appears to be no correlation with the clinical staging of the disease at diagnosis.

The table which follows summarises the nodular, diffuse and histiocytic lymphoma and the leukaemia data to date relating survival and histological class, to surface receptor characteristics. The experimental survival curve (illustrated Fig. 32) calculated for all patients with B1 and B2 lymphomas (group B), and with B3 and B4 lymphomas (group A), shows a very marked difference in survival/...

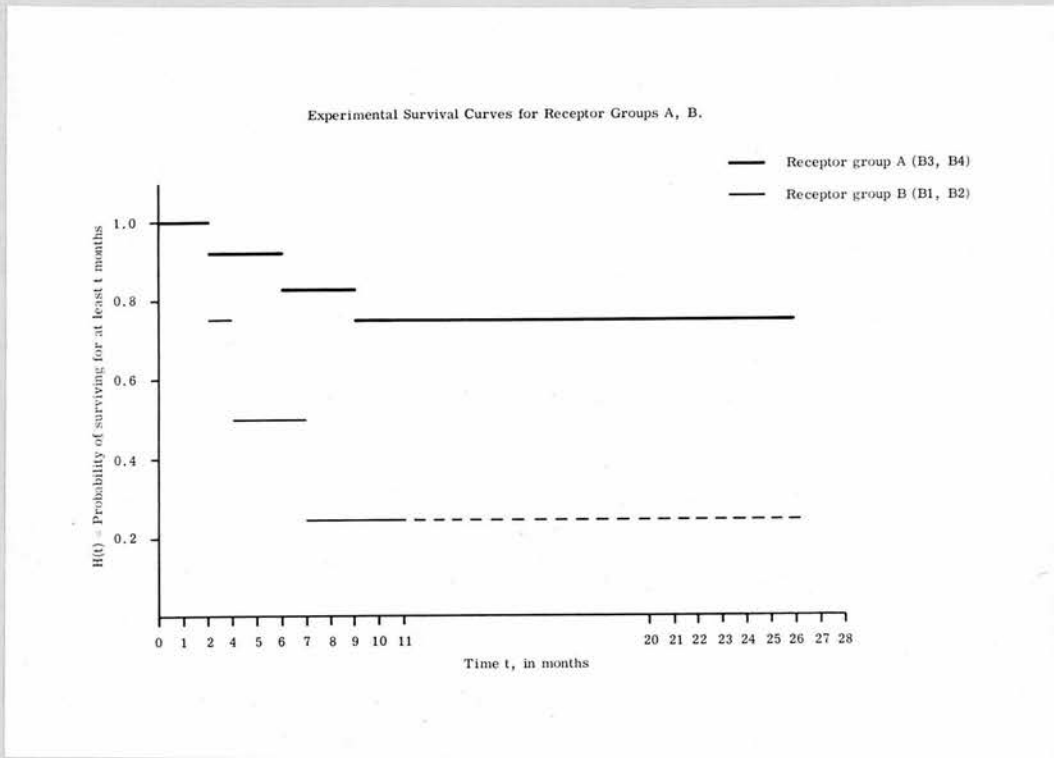


Figure 32: Relationship of B Cell Subclass in Lymphoma to the Survival Characteristics of B1 and B2 , and B3 and B4 Lymphoma Patients.

The plot is of probability of survival for each patient in group A (B3 and B4 lymphomas) and for Group B (B1 and B2 lymphomas) against survival time in months. The graph clearly illustrates that populations A and B are significantly different in terms of survival. It also shows the better prognosis for patients in group A as against group B. In group A, patients have approximately 8 out of 10 chances of surviving 10 months, while in group B patients have roughly 2 out of 10 chances of surviving the same period. As shown in the statistical appendix, the significance of the differences between population A and population B increases with increasing time of study.

survival between these two groups (P 0.02). Insufficient numbers of the other classes of lymphoma does not allow significant testing. It is pertinent to note that all patients with receptor silent tumours or leukaemia have died (six patients in all), only one patient out of six with B1 lymphoma or leukaemia remains alive, two out of four patients with B2 lymphoma have died, yet in the B3, B4 and B5 subgroups of lymphoma only 4 deaths are recorded in fifteen patients. Of the deaths in the B3, B4 and B5 group, in the patient A. Robertson a B2.C and receptor silent population was present in blood before death, the patient Cairns died from myocardial infarction, the patient A. Smith had a diffuse histiocytic lymphoma of B3.2 profile, and the death of Swanson remains unexplained due to inadequate data. Both patients Smith Robertson had B3.2 predominant "follicular" tumours, and these were the only two tumours showing the B3.2 follicular profile in the entire series. The laboratory impression that patients with capping B cell tumours do better than patients with non-capping B cell tumours is confirmed statistically. The relationship between subclass of B lymphocyte and survival is not as well established in the small series here. It is noteworthy that (1) no patient with a B4 tumour had died (b) no patient classified as having a B3.1 tumour had died (c) both patients with B3.2 tumours have died. The major problem in relating these subclasses to survival, is the gathering of sufficient numbers of patients to test each subclass, statistically, for survival value against each other. This would require a minimum of ten patients within each subclass, probably eighty to one hundred patients in all, and would require an/...

an additional 2-3 years of clinical follow-up.

The relationship of the receptor profiles to the histopathology of the lesions shows that, using Rappaport's classifications, there is good correlation between the nodular lesions, and the B3 and B4 lymphomas, with rare exceptions (e.g. McKenzie). There is also good correlation between the diffuse histiocytic lymphoma category and poor prognosis, because of the large number of receptor silent and primitive B1 lymphocyte tumours in this group. In the diffuse lymphocytic lymphoma class, in several cases there is no obvious correlation between the histological subtype, the receptor profile and survival.

<u>Patient</u>	<u>Subtype Rappaport</u>	<u>Subtype Lukes</u>	<u>Receptor Class</u>	<u>A/D(months)</u>
Velzian	DWDL	SL	B1	A(2)
Swanson	DPDL	LC	B3	D(8)
McGrail	DPDL	SC	B3.3	A(14)
Wilson	DPDL	T cell	T	A(8)
Laverick	DM(H+L)	LC	Overlap	A(14)
Reid	DWDL	SL	B2	A(3)

In the cases illustrated here, the histological evaluation is a better guide to prognosis in Swanson (by both Rappaport and Luke's classification) and offers a worse guide to prognosis in McGrail, (poorly differentiated by Rappaport's classification, B3.3 by receptor classification), Wilson, (poorly differentiated by Rappaport's classification, T lymphocyte predominant by receptor classification/...

classification) and Laverick, (mixed histiocytic and lymphocytic tumour by Rappaport, large cleaved cell by Luke's, receptor overlap by receptor classification). The patient Reid has responded poorly to treatment; despite "good" histology by both Rappaport (diffuse well differentiated) and Luke's (small lymphocyte) the clinical response to date* is like that of a poor prognosis B2 lymphoma as classified on the basis of the receptor profile.

A weakness of both Luke's and Rappaport's or indeed any histological classification is the failure to identify probable good prognosis tumours (for example Laverick and Baird - receptor overlap) in a class (histiocytic lymphoma) which includes the very bad prognosis B1 and receptor silent tumours identified by receptor studies. The role of surface phenotype assessment in evaluating the prognosis of lymphoid tumours is that of an aid to conventional histological interpretation. It is importantly not claimed as a diagnostic test in lymphoma, a role more suited to conventional histopathology. In particular the value of receptor studies lies in their ability to identify the class of tumour cell, and to indicate likely clinical behaviour of tumours in the diffuse lymphocytic and diffuse histiocytic (small lymphocyte, small cleaved and large cleaved groups of Luke's) groups where the techniques offer a real advantage over conventional histopathology.

* Since this statement the patient has made a good clinical recovery although an abnormal circulating lymphocyte population still persists.

Receptor Class	Histology Rappaport	Histology Lukes	Alive	Time from diag. mths.	Dead	Clinical Stage	Name	Sex	Age	Additional Comment
B1	DH	LNC	-	3	D	-	HA	M	-	Extranodal (Sacrum)
B1	DU	LNC	-	3	D	IV	GJ	M	66	Leukaemic
B1	DH	LNC	-	2	D	II	DH	M	70	
B1	DU	LNC	-	4	D	-	IH	F	66	Extranodal (thyroid)
B1	DWDL	SL	A	4	-	IV	RV	M	56	Leukaemic
B2Fc/B2.C	NPDL	SC	A	12	-	III	JMcK	M	35	
B2.Fc	DPDL	LC	-	2	D		SM	F	76	Extranodal (thyroid)
B2.C	DPDL	LC	-	6	D	IV	JR	M	68	
B3.2	DH	LC	-	5	D	II	AS	M	44	
B3.1	NPDL	SC	A	26	-	III	MM	F	71	Involved Sple
B3.?	DNDL	SL	A	14	-	IV	JF	M	60	Leukaemic
B3.1	NPDL	SC	A	16	-	III	ES	F	72	
B3.2	NPDL	SC	-	2	D	IV	AR	F	-	Developed Receptor Silence
B3.1	NPDL	SC	A	18	-	IV	HR	F	54	
B3.?	NPDL	SC	A	14	-	II	JT	M	63	
B3.1	DWDL	SL	A	*	-	II	JB	M	56	*Long term survivor 20y+
B3.?	DPDL	SC	-	8	D	II	AS	F	63	
B4	NPDL	SC	A	26	-	III	AM	F	73	
B4	NPDL	SC	A	15	-	IV	AD	M	71	
B4	NPDL	SC	A	17	-	III	DG	F	43	
B3.3	DPDL	SC	A	16	-	IV	TMcG	M	68	
B5	DNDL	SL	-	*	D	II	WC	M	68	*Long term survivor deat. due to M.I. Waldenstrom's macroglobuli-naemia.
T Cell	DPDL	T	A	14	-	I	JW	F	8	Toxoplasma titre positiv 1:8000(T)
Macro- phage Silent/...	DH	H	-	5	D	III	DR	M	70	

Receptor Class	Histology Rappaport	Histology Lukes	Alive	Time from diag. mths.	Dead	Clinical Stage	Name	Sex	Age	Additional Comment
Silent	DH	LNC	-	12	D	III	JO	F	72	
Silent	DH	LNC	-	18	D	IV	JR	M	26	Involved Spleen
Silent	DPDL	LC	-	4	D	IV	JK	M	70	With leukaemia
Overlap	DM	LC	A	16	-	II	LL	F	14	
Overlap	DH	LNC	A	20	-	II	RB	F	64	
Silent	DH	LNC	-	3	D	II	CM	F	56	Orbital tumour

Relationship between Survival, Histological type and Receptor class

<u>Patient</u>	<u>Sex</u>	<u>Age</u>	<u>Histology</u>	<u>A/alive D/dead</u>	<u>Survival (months)</u>	<u>Cell class</u>
Velzian	M	56	DWDL	A	4	B1
Adams	M	-	DHL	D	3	B1
Hall	M	70	DHL	D	22	B1
Hogg	F	76	DPDL	D	4	B1
Johnstone	M	66	DHL	D	3	B1
Reid	M	67	DWDL	A	3	B2.Fc
Moodie	F	76	DPDL	D	2	B2.Fc
Robertson	M	68	DPDL	D	6	B2.C
McKenzie	M	35	NPDL	A	12	B2.Fc/B2.C
Robertson	F	54	NPDL	A	12	B3.1
Smith E.	F	72	NPDL	A	16	B3.1
Muir	F	71	NPDL	A	26	B3.1
Bathgate	M	56	DWDL	A	8	B3.1
Robertson A.	F	54	NPDL	D	2	B3.2
Smith A.	M	44	DHL	D	5	B3.2
McGrail	M	68	DPDL	A	16	B3.3
Fraser	M	60	DWDL	A	14	B3(unclassified)
Swanson	F	63	DPDL	D	8	B3(unclassified)
Thompson	M	63	NPDL	A	14	B3(unclassified)
Munroe	F	73	NPDL	A	28	B4
Dobie	M	71	NPDL	A	15	B4
Gaffney	F	43	NPDL	A	17	B4
Kerr	M	70	DPDL	D	4	Silent
Oliver	F	72	DHL	D	12	Silent
Ritchie	M	26	DHL	D	18	Silent
Moyes	F	56	DHL	D	3	Silent

Baird/...

<u>Patient</u>	<u>Sex</u>	<u>Age</u>	<u>Histology</u>	<u>A/alive</u> <u>D/dead</u>	<u>Survival</u> <u>(months)</u>	<u>Cell class</u>
Baird	F	64	DHL	A	20	Overlap
Laverick	F	14	DMLL	A	16	Overlap
Ross	M	70	DHL	D	5	Macrophage
Cairns	M	68	DWDL	D	8	B5 predominant
Turnbull	F	8	ALL	D	2 days	Silent
Cooke	M	12	ALL	D	1 day	T cell
Plakowsky	M	56	CLL	D	6	B1
Mowatt	M	69	CLL	D	4	Silent
Blake	F	-	ALL	D	1 day	B1
Coburn	M	-	CLL	A	24	B3?
Klimczak	F	55	ML/FML	A	2	Monocytic
Smith	M	62	LRE	A	2	Unknown

Statistical Appendix. Method of Analysis of Receptor and Survival Data.

Survival data on 19 lymphoma patients are analysed by the regression methods due to Cox (1972). The explanatory information on each patient is B cell receptor classification, and clinical staging.

Clinical stages 1-4 have been condensed to a binary observation "coded staging" on each patient. Stages 1, 2 are coded 0, while tumours staged as 3, 4 are coded 1. Five of the nineteen patients have "coded staging" 0. The coding scheme adopted follows the clinical significance of clinical staging although a more powerful explanator may result from coding stages 1, 2 and 3 as 0.

Lymphomas are assigned to groups A, B according to B cell receptor classification. Group A is the set of 12 lymphomas which are classified as B3, B4. The remaining 7 lymphomas are B1, B2 and constituted group B.

The composition of groups A, B by age and sex is shown in table 1(a), 1(b). Neither factor is associated with group, although the proportion of males is higher in group B than in group A.

TABLE 1(a)

Group	Age*		Total	
	60	60		
A	4	7	11	*Age is unknown for 2 patients
B	2	4	6	
Total	6	11	17*	

TABLE 1 (b)

Group	Sex		Total
	<u>Male</u>	<u>Female</u>	
A	5	7	12
B	5	2	7
Total	10	9	19

Preliminary analysis of the survival since biopsy of group A, B patients (Table 2) involves the estimation and plot of experimental survival curves for the two groups, shown in Figure 32. For each group, to calculate the likelihood of a patient in that group surviving for at least t months after biopsy, denoted by $H(t)$. The plot of $H(t)$ against one T is the experimental survival curve. The values of $H(t)$ for groups A, B are presented in Table 3.

Table 2/...

TABLE 2 **Survival Data and Explanatory Variables**

<u>Patient Number</u>	<u>Coded clinical Stage</u>	<u>Receptor Group</u>	<u>Death = 1 Censored = 0</u>	<u>Survival in Months</u>
1	1	B	0	1
2	0	B	0	1
3	0	B	0	1
4	1	A	1	2
5	1	B	1	2
6	1	B	1	3
7	0	A	1	5
8	1	B	1	6
9	0	A	1	8
10	1	B	0	9
11	1	A	0	10
12	1	A	0	12
13	1	A	0	13
14	1	A	0	14
15	1	A	0	14
16	0	A	0	14
17	1	A	0	15
18	1	A	0	24
19	1	A	0	26

TABLE 3/...

TABLE 3 Experimental Survival Curves

Time in Months	Probability (group A patient survives $\geq t$ months)	Probability (group B patient survives $\geq t$ months)
t	$H_A(t)$	$H_B(t)$
1	1.00	1.00
2	0.92	0.75
3	0.92	0.50
5	0.83	0.50
6	0.83	0.25
8	0.75	0.25
9	0.75	0.25

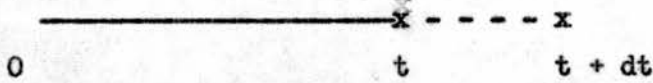
We shall now consider whether the increased survival in group A patients is indeed attributable to receptor classification or whether the apparently better prognosis for group A patients is accounted for by differing staging within groups A, B. The problem is formulated neatly in the following model.

If a patient has survived for t months after biopsy, the risk or hazard to that patient of dying within a short interval after t is called the time - specific failure rate, denoted by $a(t)$.

Figure 2/...

Figure 2

BIOPSY



$a(t)$ describes the risk of dying in the interval (---) t to $t + dt$, given that the patient survived (—) from 0 to t .

The notation and nomenclature suggest the definition : "time - specific", since dependent on the time t already survived, "failure rate" indicates the risk or rate of failures or deaths among patients who have survived for a specific time, t months after biopsy.

Cox suggests a model in which the time-specific failure rate for patient i , $\lambda_i(t)$, is the product of two components one of which is the time-specific failure rate for a "standard" patient denoted by $\lambda_0(t)$, while the second is a function of the explanatory information for patient i , which I shall denote as $f(i)$.

An example is helpful.

Mac Standard has time-specific failure rate $\lambda_0(t)$.

Pat Aye is another patient in a fictitious series. Information explaining her survival prognosis is available. We estimate that the function f evaluated for Pat is 3, and hence, according to Cox's model

$$\lambda_{\text{Pat}}(t) = \lambda_0(t) * 3$$

Warning that if both Mac and Pat survive for t months after biopsy then Pat has risk three times greater than Mac of dying very/...

very shortly after t .

Honey Bee is a further patient for whom the value of the function f is $\frac{1}{2}$. Honey is lucky. Given that both she and Mac have survived for t months the risk that Honey shall die in a short interval after t is half the corresponding risk for Mac. Thus

$$\begin{aligned} \text{Honey} \quad f(t) &= f(t) * \frac{1}{2} \end{aligned}$$

In the present study the information which may be important in predicting patient survival is coded clinical stage, receptor group.

Define for patient i the following variables

$$\begin{aligned} Z_{1i} &= 0 \text{ if patient } i \text{ has coded stage } 0 \\ &1 \text{ if patient } i \text{ has coded stage } 1 \end{aligned}$$

$$\begin{aligned} Z_{2i} &= 0 \text{ if patient } i \text{ belongs to receptor group A} \\ &\quad \text{(i.e. B3, B4 lymphoma)} \\ &1 \text{ if patient } i \text{ belongs to receptor group B} \\ &\quad \text{(i.e. B1, B2 lymphoma)} \end{aligned}$$

Following Cox we shall consider the function

$$f(i) = \exp (P_1 Z_{1i} + B_2 Z_{2i})$$

that the hazard or time-specific failure rate for patient i at time t is

$$\begin{aligned} f_i(t) &= f(t) f(i) \\ &= f(t) \exp (B_1 Z_{1i} + B_2 Z_{2i}) \dots \dots \dots (1) \end{aligned}$$

where $B_1, B_2, f(t)$ are unknown constants to be estimated from the data.

Refer/...

Refer to Table 2.

$$\begin{aligned}
 \lambda_9(t) &= \text{time specific failure rate for patient 9 who has } Z_1 = 0, Z_2 = 0 \\
 &= \lambda_0(t) \exp(B_1 \cdot 0 + B_2 \cdot 0) \quad \text{from } \dots (1) \\
 &= \lambda_0(t) \exp 0 \\
 &= \lambda_0(t) \cdot 1 \\
 &= \lambda_0(t).
 \end{aligned}$$

while

$$\begin{aligned}
 \lambda_{10}(t) &= \text{time-specific failure rate for patient 10 who has} \\
 &\quad Z_1 = 1, Z_2 = 1. \\
 &= \lambda_0(t) \exp(B_1 \cdot 1 + B_2 \cdot 1) \\
 &= \lambda_0(t) \exp(B_1 + B_2)
 \end{aligned}$$

The method of analysis is by comparison of models, searching for a model which adequately fits the data and involves a minimum number of explanatory variables. The explanatory variables "coded staging", "receptor group" are of interest in the present study. The search for an optimal model is as follows.

Include both explanatory variables

$$\lambda(t) = \lambda_0(t) \exp(B_1 Z_1 + B_2 Z_2) \dots \dots \dots \text{Model 1}$$

and derive our estimate for goodness of fit of Model 1.

Omit the variable "coded staging", fitting

$$\lambda(t) = \lambda_0(t) \exp(B_2 Z_2) \dots \dots \dots \text{Model 2.}$$

Compare the goodness of fit estimates for models 1 and 2. Does model 2 fit the data significantly less well than the full model, model 1? If so, the variable omitted, "coded staging", is our important predictor of survival and should be included in the model.

Consider/...

Consider a third model, discounting the variable "receptor group"

$$(t) = {}_0(t) \exp B_1 Z_1 \dots \dots \dots \text{Model 3}$$

Again compare the goodness of fit estimates from models 1 and 3, in order to decide how seriously the fit has been impaired by disregarding the explanatory information "receptor class".

Further investigation of models 1, 2, 3 involves comparison of each in turn with the null model

$$(t) = {}_0(t) \dots \dots \dots \text{Model 0}$$

which claims that neither explanatory variable is necessary for the prediction of survival.

Results Section

The analysis of changes in the log-likelihood when different models are assumed is presented in the statistical appendix.

When both "coded" staging and receptor class are included as explanatory information the improvement in fit of model - for the time-specific failure rate or risk - to data is not significantly greater than the fit when no concomitant information is considered in the prediction of survival. It is noted from this model that "coded staging" and "receptor class" are negatively correlated in the present data set, so that one or other may prove explanatory.

Indeed it is established that regression on "receptor group" as the sole explanatory variable results in a markedly improved fit of model to data ($0.05 < p < 0.10$, test statistic = $3.39 X^2$) than/...

than is established using the null model in which no concomitant information is accounted for. Equally the importance of "receptor class" is assessed and confirmed by measuring the impairment to goodness of fit which results when the full model ("coded staging", "receptor class") is reduced by discounting the information "receptor class". The impairment is significant (test statistics = 4.978. The test statistic is distributed as χ^2 ; $0.01 > p < 0.05$) and hence indicates that receptor class is an important explanatory variable.

The analysis leads to an estimated 90% confidence interval for the regression coefficient on receptor class: 1.53 ± 1.37 (0.17, 2.90). We deduce that the hazard or time-specific failure rate in receptor class B (B1, B2 lymphomas) is 4.6 times that for group A patients. The corresponding 90% confidence interval for the multiplying factor is (1.2, 18.2).

That "coded staging" is not our informative explanatory variable is similarly established by the two methods described with reference to receptor class. The present series of 19 patients however includes only 5 lymphomas coded 0 for staging. Further study is necessary before clinical staging is confidently described as a valid predictor for survival prognosis.

Statistical Appendix

Model 1	$(t) = {}_o(t) \exp (B_1 Z_1 + B_2 Z_2)$
Model 2	$(t) = {}_o(t) \exp (\quad + B_2 Z_2)$
Model 3	$(t) = {}_o(t) \exp (B_1 Z_1 \quad)$
Model 0	$(t) = {}_o(t).$

where/...

where $Z_1 = 0$ if coded staging is 0 (stages 1, 2)
 $= 1$ if coded staging is 1 (stages 3, 4)

$Z_2 = 0$ if receptor class for patient is A (B3, B4 lymphoma)
 $= 1$ if receptor class for patient is B (B1, B2 lymphoma)

and $B_1, B_2, \theta(t)$ are estimated from the data for each model.

Analysis

Model 1 Test statistic with 2 degrees of freedom (distributed as χ^2_2) = 4.13.

$$\chi^2_{2, 0.10} = 4.61$$

Hence model 1 does not fit the data significantly better than the null model, model 0.

Model 2 Test statistic with 1 degree of freedom (distributed as χ^2_1) = 3.39

$$\chi^2_{1, 0.10} = 2.71, \quad \chi^2_{1, 0.05} = 3.84$$

Hence (0.05 p 0.10) receptor class is an important predictor of survival

$$B_2 = 1.53 \quad \text{Var}(B_2) = 0.695.$$

Model 3 Test statistic with 1 degree of freedom (distributed as χ^2_1) = 0.68. Hence "coded staging" does not appear to be an important predictor of survival. However, only 5 of the 19 patients have clinical stage coded 0 and this model is not powerful due to small sample size. Further data/...

data are necessary to draw valid conclusions.

Change in Maximum - Log Likelihood

Model (regressor variables)	Maximum Log Likelihood L_1	Change in Maximum Log Likelihood (distribution interpretation) $2(L_1 - L_2)$	Significance
Coded Staging (Receptor class)	- 12.076		
(Receptor Class)	- 13.309	$2(L_1 - L_2) = 2.466 \chi^2_1$ Interpretation: disregarding the explanatory information "coded staging" does not markedly impair the goodness of fit. Prefer model 2 to model 1.	NS
(Coded Staging)	- 14.565	$2(L_1 - L_2) = 4.978 \chi^2_1$ Interpretation: omitting the information on receptor class has seriously impaired goodness of fit. Prefer model 1 to model 3.	$0.01 < p < 0.05$ $0.01 < p < 0.05$

CONCLUSION: Receptor class is the
minimum necessary explanatory infor-
mation useful in predicting survival:
Accept Model 2.

In paragraph 2 of this report it is noted that the coding of
clinical staging follows the American interpretation of staging,
namely that stages 1 and 2 are considered alike, and separated from
the more advanced lymphoma in stages 3 or 4. In the present series
of 19 patients, however, only 5 have stages 1, 2 lymphoma, so that
coded/...

coded stage 0 is represented by only 5 patients. Model 3 in the above discussion is correspondingly weakly founded.

An alternative coding scheme would be to group stages 1, 2, 3 separating off the most advanced stage 4. Eight patients in the present series have stage 4 lymphoma. Allowing this scheme an analysis was made which led to the same conclusions as those reported above.

The patients in this series were retested three months after the analysis presented here. The experimental survival curves clearly show that B3, B4 patients have a better prognosis in terms of survival than B1, B2 patients. Paralleling the previous analysis the data were inspected using

1) Cox's regression model $B_3 = 1.85$, $SE = 0.746$ $p < 0.025$.

2) Peto & Peto's logrank test, with comparison when the

underlying distribution is lognormal or normal $p < 0.02$.

This shows that with increasing time of study the differences in survival pattern between patients with B1 and B2 lymphomas and patients with B3 and B4 lymphomas becomes more highly significant ($p < 0.05$ $p < 0.02$).

REFERENCES - CHAPTER 21

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RESULTS

Chapter 22

Hodgkin's Disease

Contents

- a) Changes in the spleen in Hodgkin's disease.
- b) Changes in the lymph node and blood in Hodgkin's disease.
- c) The nature of the Reed Sternberg cell, and abnormalities of lymphocytes in Hodgkin's disease.

Hodgkin's Disease

The study of Hodgkin's disease involved three related but independent investigations; firstly the study of the spleen in which quantitative data were sought in order to monitor the changes in cell populations during the disease, secondly, typical Hodgkin's disease receptor profiles were looked for in involved lymph nodes, and in blood, and thirdly the biology of the Reed-Sternberg cell was studied from its surface phenotype as detected by receptor studies, and for presence or absence of cytoplasmic immunoglobulin. On the basis of the findings a theory of the pathogenesis of Hodgkin's disease is proposed.

Studies of Hodgkin's Disease Spleen

Spleens from patients with Hodgkin's disease undergoing laparotomy for staging, were collected. The total cell count, cell concentrations, receptor profiles and histology were all determined as in "methods". Blocks were also stained by the immunoperoxidase technique to detect Ig bearing cells.

Some precautions need to be taken to ensure that involved tissue is not missed, as involvement of spleen is an important matter in staging and managing Hodgkin's disease. To this end, each 2-4 mm slice (after taking material for receptors, cell counts and special histology) was fixed for 24 hours in neutral 10% formalin solution and then again sectioned at 2 mm thickness with a skin graft knife blade. All significant nodular white areas were removed and blocked for diagnostic evaluation. If no involvement/...

involvement was apparent, 9 standard micro blocks were taken from each spleen. Fourteen male, and six female spleens are incorporated into this study. Two male and two female spleens showed involvement with Hodgkin's disease, the others showed either a reactive pattern with germinal centres, eosinophil and neutrophil infiltration of red pulp, non-Hodgkin's granulomata, or a "normal" pattern without these features.

In some later spleens (Bull, Reynolds and Kowbel) full quantitative data was not obtained, but receptor profiles were studied, especially in respect of IgM receptors.

Hodgkin's Diseased Spleens

<u>Patient</u>	<u>Age</u>	<u>Sex</u>	<u>Spleen Weight</u>	<u>Cell Concentration</u>	<u>Diagnosis</u>	<u>Histology</u>
H1	27	M	460 gms	$341.3 \times 10^6/\text{gm}$	HDNS	Reactive
H2	15	M	160	493.8	HDLF	Normal
H3	24	M	265	158.5	HDLF	Normal
H4	14	M	312	155.1	HDLF	Reactive
H5	14	M	100	800.0	HDNS	Normal
H6	18	M	138	310.2	HDNS	Reactive
H7	34	M	200	1235.5	HDNS	Normal
H8	12	M	109	449.5	HDLF	Normal
H9	33	M	165	381.9	HDNS	Reactive
H10	66	M	253	276.7	HDLF	Diffuse Involvement
H12		M	310	419.4	HDMC	Nodular Involvement
H13	35	M	270	372.2	HDMC	Normal
H14	8	M	142	516.2	HDMC	Normal
H15	42	M	90	218.9	HDMC	Normal
H16	14	F	102	752.0	HDLF	Reactive
H17	19	F	92	510.9	HDNS	Reactive
H18	20	F	189	391.5	HDNS	Reactive
H19	24	F	335	656.1	HDNS	Nodular Involvement
H20	74	F	782	181.6	HDLF	Nodular Involvement
H21	21	F	117	327.4	HDLF	Normal

Mean spleen wt. males 198 gm. females 269.5 gm.

Receptor Profile of Hodgkin's Disease Spleens

Values given are percentage of total cells. Ficoll triosil separated mononuclear cells, mean viability 90%

<u>Patient</u>	<u>Sex</u>	<u>E Rosettes</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
H1	M	55	29	61	19	10	21
H2	M	42	31	41	45	34	27
H3	M	28	2	-	-	7	1
H4	M	63	38	30	6	32	20
H5	M	44	26	30	5	28	13
H6	M	42	68	63	25	26	11
H7	M	50	39	21	4	39	8
H8	M	34	16	35	24	21	13
H9	M	53	38	50	9	18	46
H10	M	48	6	-	-	18	15
H12	M	52	27	52	5	44	9
H13	M	52	41	49	6	39	17
H14	M	41	20	21	9	59	10
H15	M	46	33	38	24	27	12
H16	F	35	25	28	11	18	6
H17	F	25	31	35	5	8	47
H18	F	34	45	29	32	29	15
H19	F	48	4	-	-	24	Nil
H20	F	53	39	54	31	25	19
H21	F	23	-	1	14	35	7
Bull	F	27	7	55	7	46	8
Reynolds	M	46	26	40	5	33	8
Kowbel	M	62	24	5	19	17	Nil

Involved Spleens

In involved spleens, the receptor profiles of involved tissue differed from uninvolved tissue. In the example shown below the receptor profiles of involved and uninvolved tissue are/...

are shown, for a case with nodular involvement of the spleen.

Patient	Uninvolved Tissue		Involved Tissue	
	<u>Percentage</u>	<u>Cells per gm</u>	<u>Percentage</u>	<u>Cells per gm</u>
<u>DW (H7)</u>				
E Rosettes	34	143×10^6	50	212×10^6
Fc	27	112×10^6	39	165×10^6
C3	46	193×10^6	38	158×10^6
Phagocytes	12	50×10^6	12	51×10^6
Capping	17	71×10^6	19	82×10^6
Non capping	1	6×10^6	8	32×10^6

In this case, as in most cases examined, involved tissue shows increased numbers of T lymphocytes and increased proportions of these cells. The uninvolved tissue shows increased numbers of complement rosetting cells which exceed the totals of B cells and macrophages combined. This singular feature, which occurs in some but not all cases of Hodgkin's disease, is not present in involved tissue. As shown in the receptor profiles of Hodgkin's spleen, an excess of complement receptor cells was found in the cases H1 and H6 designated below.

<u>Case</u>	<u>Sex</u>	<u>Status of Spleen</u>	<u>Excess C3 receptors over B cells + Macrophages</u>
H1	M	Reactive not involved	11%
H6	M	Reactive not involved	7%

From the receptor data it appears that in these two cases T cells in Hodgkin's disease possess C3 receptors. This phenomena was/...

was later shown to be due to IgM receptors on Hodgkin's disease cells.

Abnormalities of B cells in Hodgkin's disease

In three cases of Hodgkin's disease, an excess of non-capping B cells over capping B cells was shown. The receptor profiles for these cases is shown below.

<u>Case</u>	<u>Histology</u>	<u>Sex</u>	<u>Spleen Status</u>	<u>Capping</u>	<u>Non-capping</u>
H1	HDNS	M	Reactive not involved	10	21
H9	DHNS	M	Reactive not involved	18	46
H17	HDNS	F	Reactive not involved	8	47

This is an unusual feature of Hodgkin's disease, and occurred only in uninvolved spleens in Hodgkin's disease of nodular sclerosing subtype. Despite the findings in non-Hodgkin's lymphoma and normal spleen, that non-capping cells do not express C3 receptors, but are more often of the B2.Fc class, in these cases C3 rosette levels were not low despite the predominance of non-capping B cells. Rather low levels of phagocytes were present and cannot be the source of the anomaly. The conclusion is that in some cases of Hodgkin's disease the B cell population can be non-capping, yet still express complement receptors, that is the B cells are of the B2.C class.

Phagocytes

The/...

The greatest proportion of phagocytes encountered in normal spleens was 26%, and the highest concentration was $125 \times 10^6/\text{gm}$. In Hodgkin's disease spleens these levels of phagocytes were equalled or exceeded on six occasions. The patients in which this occurred are shown below.

<u>Case Number</u>	<u>Histology</u>	<u>Sex</u>	<u>Spleen Status</u>	<u>Phagocytes</u>	<u>Phagocytes/gm</u>
H2	HDLP	M	Normal	45%	230×10^6
H6	HDNS	M	Reactive	25%	32×10^6
H8	HDLP	M	Normal	24%	108×10^6
H15	HDMC	M	Normal	24%	52×10^6
H18	HDNS	F	Reactive	32%	32×10^6
H20	HDL D	F	Nodular Involvement	31%	56×10^6

As can be seen in only one patient (H2) was the concentration of phagocytes greater than that encountered in normal spleen. This evidence shows that abnormalities of spleen phagocyte populations are not a feature of Hodgkin's disease, despite the histological evidence of granuloma formation in reactive Hodgkin's disease spleens.

Sex Differences in Hodgkin's Disease Spleen

The most surprising feature to emerge from this study was the obvious sex differences between normal male and female, and Hodgkin's disease male and female spleens. This finding requires that/...

that the appropriate control for male Hodgkin's disease spleen is the normal male spleen, and for female Hodgkin's disease spleen, the normal female spleen. The tables of comparison for male, and for female spleens, are shown. The principal differences are shown in the summary table, for male and female spleens from patients with Hodgkin's disease, compared with normal male and female controls.

Mean Values for Hodgkin's Disease Spleen. Male compared with Female.

Rosettes	Percentage		Cells/gm x 10 ⁶		Total cells x 10 ¹⁰	
	Male	Female	Male	Female	Male	Female
E	46	36	201	169	3.98	4.56
Fc	30	29	131	136	2.59	3.67
G3	40	39	175	183	3.47	4.90
Neutral Red Phagocytes	15	19	66	89	1.30	2.40
Capping	29	23	127	108	2.51	2.90
Non-capping	16	19	70	89	1.39	2.40
TOTAL CELLS	106	97	437.8	469.9	8.67	12.69
Spleen Weight	198	270				

Note: mean total B cell concentration Male HD spleens (197×10^6) are equal to mean total B cell concentration for female HD spleens (197×10^6). Female HD spleens are significantly larger than male HD spleens ($p < 0.05$) and in comparison with normal spleens the increase in spleen weight in Female, but not in the male, is significant. There is a significant decrease in cell concentration in male HD spleen compared with normal male spleen ($p < 0.05$) and a significant/...

significant increase in cell concentration in female HD spleen compared with normal female spleen (p 0.02).

Comparison of Normal Male Spleen, with Hodgkin's Disease Male Spleen.

Percentage of rosette forming cells, with absolute values of cell concentration (No. of rosetting cells per gm spleen wt.)

Rosettes	Percentage Values		Cell concentration (cells x 10 ⁶ gm)	
	Normal	HD	Normal	HD
E	40	46	234	201
Fc	34	30	201	131
C3	32	40	189	175
Neutral Red Phagocytes	10	15	60	66
Capping	33	29	193	127
Non-capping	12	16	73	70
Spleen weight	166	198	-	-
TOTAL CELLS	95%	106%	591.5*	437.8*

Note: the tendency for the cell concentrations of all cells except phagocytes and non-capping B cells to show a decrease in male HD spleen. The only significant difference (**) is between the total cell concentration of the normal male spleen which is higher than that of the male HD spleen (p 0.05).

Comparison/...

Comparison of Normal Female spleen with Hodgkin's Disease Female

spleen. Percentage of rosette forming cells, and absolute values of cell concentration (No. of rosetting cells per gm spleen wt.)

Rosettes	Percentage Values		Cell concentration (cells x 10 ⁶ gm)	
	Normal	HD	Normal	HD
E	40	36	187	169
Fc	30	29	111	136
C3	28	39	106	183
Neutral Red Phagocytes	17	19	62	89
Capping	29	23	108	108
Non-capping	12	19	44.6	89
Spleen Weight	156	270	-	-
TOTAL CELLS	-	-	367.6	437.8

Note: that female HD spleen is enlarged compared with normal female spleen (p 0.05). There are more phagocytes, and non-capping cells than in normal female spleen, and T cell concentrations are decreased. These changes are not in fact significant, probably because of the small numbers of spleens in the female series. Cell concentration is significantly increased in female HD spleen (p 0.05).

Comparison/...

Comparison of total cell counts (No. of cells $\times 10^{10}$) of Normal Male and Hodgkin's Disease Male Spleen, and Normal Female and Hodgkin's Disease Female Spleen.

Rosettes	Total Cells, Male Spleens ($\times 10^{10}$)		Total Cells Female Spleens ($\times 10^{10}$)	
	<u>Normal</u>	<u>HD</u>	<u>Normal</u>	<u>HD</u>
E Rosettes	3.40	3.98	2.99	4.56
Fc	2.95	2.59	1.73	3.67
C3	2.77	3.47	1.66	4.90
Neutral Red Phagocytes	0.88	1.30	0.97	2.40
Capping	2.83	2.51	1.69	2.90
Non-capping	1.06	1.39	0.68	2.40
TOTAL	8.37	8.67	4.97	12.69

Males: spleen weight in HD patients is increased, but cell concentration is lower than in normal male spleen. The only changes are minor variations with absolute increases in complement receptor cells, and in phagocytes.

Females: in Hodgkin's disease patients total cell numbers, T cell, B cells (especially non-capping) and phagocytes are raised. These values are probably significant, bearing in mind that the number of available spleens in the female series is too low for rigorous statistical analysis. In both male and female, T cells, complement receptor cells, phagocytes and non-capping B cells are increased.

Summary Table: Sex differences in Hodgkin's Disease

<u>Features</u>	<u>Difference from Normal</u>	<u>Statistically Significant</u>
Spleen weight Male	Increased	No
Spleen weight Female	Increased	$P < 0.05$
Cell concentration Male	Decreased	$P < 0.05$
Cell concentration Female	Increased	$P < 0.02$
T cell concentration Male	Increased	No
T cell concentration Female	Decreased	No
Phagocyte concentration Male	Increased	No
Phagocyte concentration Female	Increased	No
B cell concentration Male	No change	No
B cell concentration Female	Increased	No*
Total cell count Male	Decreased	$P < 0.05$
Total cell count Female	Increased	$P < 0.02$
Total T cells Male	Increased	No
Total T cells Female	Increased	$P < 0.05$
Total B cells Male	No change	No
Total B cells Female	Increased	$P < 0.05$

* Significance not attained in the small number of cases available.

Comparison for all other receptors showed no constant relationship to sex or to Hodgkin's disease, although changes in capping and non-capping B cells and complement receptors have been already commented upon. The B cell concentration (*) would probably/...

probably be significant if more cases had been available, as this result just failed to reach significance in females.

In male Hodgkin's disease spleen the spleen weight is increased but the total cell counts remain low, and cell concentration is decreased. Therefore despite a slight increase in proportion of T cells in male Hodgkin's spleens, the actual T cell numbers and concentration fail to reach a significantly increased level. In female spleens, spleen weight cell concentration, total cell count, the total numbers of T cells, the concentration of B cells and the total numbers of B cells are increased. When compared with diseased control spleens, the cell concentration, and the total numbers and concentration of B cells remain significantly raised in the female.

These findings show that Hodgkin's disease produces profound alterations in the cellularity of both male and female spleens. In males, there is a tendency for the cell counts to fall and, despite increased proportions of T cells in the male spleen their total number or concentration is not increased. Thus Hodgkin's disease in the male results in decreased lymphocyte concentrations in the spleen, without profound changes in the T cell, B cell or macrophage proportions. In the female, the most marked increase is in total cell number and in the B cell number and concentration.

The total numbers of B cells in female spleens do not exceed the total numbers in male spleens. In this series involved and uninvolved spleen data are pooled, since the numbers of involved spleens are too small for separate statistical evaluation, but as shown T cell levels in involved spleen tissue were higher than in uninvolved/...

uninvolved spleen. The evidence indicates (1) a clear sex difference in the response of spleen cell populations in Hodgkin's disease (2) abnormalities of B cell populations in the female but not in the male (3) an increase in the proportion and concentration of T cells in Hodgkin's tissue and (4) an excess of complement receptor bearing cells in some uninvolved Hodgkin's disease spleens of nodular sclerosing subtype.

The findings in spleen led to further studies of Hodgkin's disease, using samples from lymph node and blood. Unlike lymph nodes from non-Hodgkin's lymphomas it was not possible to classify Hodgkin's disease nodes for the following reasons: (1) most involved nodes were T cell rather than B cell predominant, (2) B cells in Hodgkin's tissue may express complement receptor while failing to cap surface Ig (B2.C class), (3) there was a distinct overlap between T cells and complement receptor bearing cells in at least two cases. These features compromised attempts to classify B lymphocytes in Hodgkin's disease lymph nodes. In addition plasma cells were frequently present in Hodgkin's disease lymph nodes where they sometimes formed a substantial "receptor silent" population. They were detected by the immunoperoxidase technique. Few involved lymph nodes were available for study, and in most instances blood receptor profiles were within normal limits. The relevant data are examined in the next section.

Lymph Nodes in Hodgkin's Disease

An examination of quantitative data in the Hodgkin's disease spleen led to the discovery of several features which appear to be peculiar to Hodgkin's disease. These were:-

- (a) The T lymphocyte predominance of involved areas of spleen compared with uninvolved areas.
- (b) An excess of complement receptor bearing cells over the sum of B cells and macrophages in uninvolved Hodgkin's spleen.
- (c) The presence of B2 cells of B2.C class (complement receptor bearing) which are postulated to occur in germinal centre. In other lymphomas and in blood B2 cells usually express the Fc receptor (B2.Fc).
- (d) The sex differences between male and female Hodgkin's disease spleen.

Only eight involved Hodgkin's disease lymph nodes were available. No general conclusions could be reached on such limited numbers, and quantitative investigation of these parameters are omitted.

The receptor profiles of involved Hodgkin's disease lymph nodes, and of these nodes when the subtype of Hodgkin's disease is considered a variable, are given in the following two tables.

Receptor/...

Receptor Profiles in involved Lymph Nodes in Hodgkin's Disease

Figures as percentage of Lymph Node Cells.

<u>Patient</u>	<u>Viability</u>	<u>E Rosettes</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
Muir	60	31	29	49	1	17	10
Cochran	78	69	34	88	2	18	6
Dunmore	66	41	6	5	12	25	-
Henderson	70	36	19	35	4	13	17
Jacob	80	66	26	25	2	19	9
Stewart	70	65	56	52	11	12	4
Rae	74	54	7	1	4	9	11
McIvor	70	33	6	20	4	10	12

<u>Histological subtype</u>	<u>Patients</u>	<u>Sex</u>
HDMC	Rae, Cochran, Jacob, McIvor	M.M.M.F.
HDNS	Dunmore, Henderson	M.F.
HDLF	Stewart, Muir	M.M.
HDLD	None.	

Receptor Profile for Subtypes of Hodgkin's Disease

<u>Subtype</u>	<u>Viability</u>	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
HDMC	76	56	18	34	3	14	10
HDNS	68	39	13	20	8	19	9
HDLF	65	48	43	51	6	15	7

There/...

There are no significant differences because of the small numbers of nodes in the series. There are some interesting features, firstly that T cells do appear to be raised in HDMC subtype, secondly that complement rosettes are much increased in HDLP subtype. One of the mixed cellularity group (Cochran) showed increased complement rosettes. In the cases Cochran, Stewart and Muir complement rosettes exceeded the sum of capping cells, non-capping cells and macrophages as shown for two spleens in the series. This apparent overlap of complement rosetting cells and T cells is probably due to IgM receptors on T cells in Hodgkin's disease.

The specific features of Hodgkin's disease in the individual cases mentioned are now considered.

T cell predominance

T cells exceeded the sum of B cells and macrophages in all the lymph nodes examined. In cases Muir, Dunmore, Henderson, the predominance of T cells can scarcely be regarded as significant. Moreover the normal or reactive lymph node can also show T cell predominance so the validity of this finding is difficult to assess. In cases Cochran, Jacoby, Stewart and Rae the excess of T cells over other cells is very marked, being 43%, 37%, 38% and 30% respectively. All these patients are male, three with Hodgkin's disease of mixed cellularity (HDMC) and one with lymphocyte predominance (HDLP).

The pattern of T lymphocyte predominance found in the Hodgkin's disease node stands in marked contrast to the findings of B lymphocyte predominance in non-Hodgkin's lymphoma. Several alternative explanations of these locally elevated tissue levels of T cells are possible:☺

- 1) T cells may be neoplastic
- 2) T cells may be functionally immature, failing to "peripheralise" or mature in Hodgkin tissue.
- 3) The T cells could be co-operating with abnormal B cells in an ineffectual immune response.
- 4) The T cells could be reacting against a neoplastic B cell population.

It is important to know whether Hodgkin tissue invariably contains high proportions of T lymphocytes, and to know whether such/...

such T lymphocytes are normal or abnormal. If the T cells are reactive, their presence in Hodgkin tissue could be accounted for by the sequestration of reactive cells into involved tissue, with local elevation of T cell levels, and corresponding depletion of T cells in blood or spleen. If this is the case, involved spleen should show greater T cell proportions, and numerical increases in T cells with corresponding depletion of blood or node T lymphocytes. In uninvolved spleen T cell levels should be decreased, while levels in the involved node should be increased beyond normal values. Finally if T cells are functionally immature or neoplastic the "sequestered" population might show surface features indicating abnormality. In the following three examples, the first (Jacob) shows evidence of sequestration of T cells from uninvolved spleen into lymph node, the second (McKillop) fails to show sequestration of T cells into involved spleen, and the third (Cochran) shows evidence of sequestration of abnormal T cells from blood into lymph node.

In Jacob the receptor profile of uninvolved spleen showed a normal percentage of T cells (46%) but a reduction in T cell concentration (101×10^6 : normal male 233×10^6) implying that there could be sequestration of T cells from the uninvolved spleen into the involved lymph node. The T cell predominance in the lymph node was very marked (37%). If high T cell levels do signify sequestration of T lymphocytes around foci of active disease, the phenomenon does not occur in every case.

In McKillop, a male with HDMC and involvement of the spleen, T cell levels were 53.2%, 147×10^6 despite the presence of Hodgkin's/...

Hodgkin's disease in the spleen.

In other cases (case DW/H7) involved spleen tissue showed increased T cell numbers and proportion compared with uninvolved spleen. In Cochran, the blood was studied at the same time as the lymph node biopsy, and with the same test particles. The data for lymph node (1) and blood (2) in terms of percentage values and quantitative data (3) are shown below. Figures given are either as percentages of total cells, or as millions of cells per ml. Normal blood values are shown in the last line(4).

	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
(1)	69	34	88	2	18	6
(2)	49	36	1%	2	8	28
(3)	2.48	1.80	0.036	0.11	0.42	1.38
(4)	1.01	0.74	0.55	0.38	0.55	0.31

In this example, the mononuclear cell count was 5.02×10^6 /ml. with 61% mononuclears on differential count. There is no evidence of T cell depletion, in fact circulating T cells are more than twice the normal number. The most striking evidence is of sequestration of complement receptor cells from blood into lymph node. These cells must be T cells, since a very marked T cell C3 receptor overlap is present in node with C3 depletion in blood and leads to the conclusion in this example that most of the apparent C3 receptors are on T cells and not B cells. The overlap of E and C3 rosetting will be examined later. The conclusions reached in examining evidence of the T cell predominance in Hodgkin's disease are/...

are that:-

- (1) It does not occur in every case.
- (2) There is no firm evidence that it is due to overall sequestration of normal T cells with T cell depletion of other compartments (e.g. blood, spleen).
- (3) It has been shown in one case to be associated with sequestration of an abnormal T cell population apparently bearing complement receptors.
- (4) In this series T cell predominance occurred only in male patients, a point of little significance since only two involved nodes from females have been examined.

The Excess of complement receptors over the sum of B cells and macrophages.

In the spleen series, 2 cases, both with uninvolved spleen tissue showed an excess of complement receptor bearing cells over B cells and macrophages. This fact shows that T cells apparently express a complement receptor. The lymph node of Cochran also showed this phenomenon. The lymph nodes of Muir, and Stewart also showed complement receptor excess. The lymph node data showing the magnitude of the excess is illustrated below. The data from the two spleens (H1 and H6) is included.

Patient/...

<u>Patient</u>	<u>C3</u>	<u>Excess C3 receptors over B cells and macrophages</u>	<u>E rosettes</u>
H1	61%	11%	55%
H6	63%	7%	42%
Cochran	88%	42%	69%
Muir	49%	21%	31%
Stewart	52%	35%	65%

In the involved nodes showing the phenomenon, quite marked C3 receptors excess is noted, amounting to almost half the lymph node population in the case of Cochran. Only 3 out of 8 nodes show the effect, one from Hodgkin's disease of mixed cellularity, and two from cases of lymphocyte predominant Hodgkin's disease. The entire series shows T cell and C3 receptor overlap on 5 occasions out of 31 cases. The discovery of this phenomenon, never observed in normal nodes and rarely in non-Hodgkin lymphoma appeared to be specific for Hodgkin's disease. Since T cells are known under certain circumstances to express receptors for IgM, and such cells never normally exhibit receptors for complement (C3), it was decided to review cases for evidence of IgM receptors, since this ligand could account for these gross overlaps between E and C3 rosettes.

IgM Receptors

Although frequently employed as a negative control for the complement rosette with the sheep erythrocyte, very infrequent IgM/...

IgM rosettes were seen in normal tissue. Using ox red cells and IgM antibody a search for IgM receptor bearing cells was carried out in blood, lymph node, and spleen of Hodgkin's disease patients.

The results obtained are shown below. Figures given are as percentage of total cells.

<u>Patient</u>	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>IgM</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
Smith (Blood)	25	-	-	12	32	37	
Walker (Spleen)	34	27	46	17	12	17	1
Walker (Node)	50	39	38	6	12	19	8
Reynolds (Spleen)	46	26	40	4	5	33	8
Reynolds (Blood)	46	28	34	2	7	26	5
Kowbel (Spleen)	62	24	5	Nil	19	17	Nil
Dewar (Node)	22	11	27	4	2	32	4
Rae (Node)	54	7	1	2	4	9	11
Simm (Blood)	34	26	22	29	15	30	2

As this table shows, on 3 occasions (Smith - blood, Walker - spleen, and Simm - blood) significant numbers of IgM receptors were observed on cells in cases of Hodgkin's disease. In each case a large proportion of phagocytes were present (32%, 12%, 15%) and in one case (Walker) there was an excess of C3 receptor over B cells and macrophages (excess of 16%). The only constant feature is presence/...

presence of Hodgkin's disease in the patient, the tissue does not have to show involvement.

The other cases show suggestive increases in IgM rosetting cells - the upper limits of normal being 8%, the mean value being 2.8%. The mean value of IgM rosettes in the cases shown (excluding Smith, Walker and Simm) was 3.6% and including all cases 9.5% .

Subclass B2 lymphocytes in Hodgkin's disease

In 2 reactive lymph nodes from cases of Hodgkin's disease, a follicular receptor profile was obtained, in which B2 lymphocytes predominated. Similar findings occurred in some spleens in the series (H1, H9, H17), but only in the nodular sclerosing subtype of Hodgkin's disease. The receptor profiles of tissue in which a predominance of B2 lymphocytes over B3 cells was seen are shown below. Figures given are as percentages of total cells present.

<u>Patient</u>	<u>Tissue</u>	<u>E</u>	<u>Fc</u>	<u>C3</u>	<u>Phagocytes</u>	<u>Capping</u>	<u>Non-capping</u>
H1	Spleen	55	29	61	19	10	21
H9	Spleen	53	38	50	9	18	46
H17	Spleen	25	31	35	5	8	47
Blair	Node	56	29	-	-	12	17
Rae	Node	35	5	33	1	15	15

In 5 patients with Hodgkin's disease, in uninvolved tissue, the receptor profile shows non-capping B cells to be the predominant B cell type. This is unusual in comparison to normal nodes/...

nodes, where excess of non-capping cells is never seen.

In the series of Hodgkin's involved lymph nodes, the pattern of B2 lymphocyte excess was observed once only (Henderson). In this node complement receptor bearing cells were in excess, and the B cells could be classified as B2.C as in the spleens of cases H1, H9, H17 and the node of Rae. Insufficient data is available for the lymph node of Blair.

There is thus evidence that in uninvolved tissue of the nodular sclerosing subtype of Hodgkin's disease (HDNS - 3 cases) or mixed cellularity subtype of Hodgkin's disease (HDMC - 1 case) or in a minority of involved nodes containing Hodgkin's tissue of HDNS type (1 case) the class of B lymphocyte present is B2.C (rather than B3 subclass which are the most common normally).

This feature can, on histological grounds, be linked to the follicular reactivity seen in uninvolved lymphoid tissues in the course of Hodgkin's disease. Such changes are clearly reactive, and not "neoplastic" and correlate with the descriptive histogenesis of nodular sclerosing Hodgkin's disease. In this subgroup there is evidence of a hypersensitivity reaction in the tissue, with necrosis, heavy neutrophil and eosinophil polymorph infiltration, and plasma cell accumulation. These events certainly represent antibody mediated hypersensitivity of type II or III, which result in necrosis, complement fixation and neutrophil and eosinophil chemotaxis. The antibody may be produced locally, and is probably IgG in type as can be concluded from study of these hypersensitivity reactions. It is suggested that the excess of B2.C cells seen in Hodgkin's disease is due to marked follicular reactivity as a consequence/...

consequence of a secondary humoral immune response.

In this brief survey of Hodgkin's disease several facts clearly emerge.

- 1) The disease in males and females shows a different pattern. From the spleen, data, in males the cellularity of the spleen is less than in normal males, while in females the reverse is the case.
- 2) There is evidence of abnormality of the T cell population in that
 - a) overlap between E rosettes and C3 receptors is noted
 - b) T cells probably express IgM receptors in some cases of Hodgkin's disease.
- 3) Attention is drawn to the follicular reactivity in Hodgkin's disease as a consequence of which B cell populations of B2.C subclass are occasionally encountered.
- 4) In at least two cases there is clear evidence of the sequestration of T lymphocytes from the blood into an involved lymph node.

These changes reflect an abnormal immune response. The T cells encountered may be immature T cells or T helper cells which, by virtue of IgM receptors, localise in Hodgkin tissue in an attempt to initiate a secondary humoral immune response by B lymphocytes resulting in germinal follicle formation and plasma cell accumulation. There is evidence that macrophages may be increased in number in areas where IgM receptor bearing T cells are found, and if substantiated this would imply the release of lymphokines (migration inhibition factor) by activated T cells on contact/...

contact with antigen. (In other words could provide evidence for a delayed type of hypersensitivity reaction). The further studies are based on this interpretation of Hodgkin's disease, and in particular sought to elucidate the following points.

- a) Is there morphological evidence which supports the concept of secondary stimulation of B cells with their transformation into plasma cells?
- b) Do lesions occur in regions where T cells are likely to be found?
- c) What relationship is there between the Reed Sternberg cell and the presence of activated T cells?

Evidence for the presence of plasma cells in Hodgkin's disease

Using the immunoperoxidase technique it is immediately obvious that in spleen, lymph node and in Hodgkin's tissue, plasma cells secreting IgG antibody, of either Kappa or Lambda light chain type are much more frequent than in normal lymphoid tissue. This is particularly striking when Hodgkin's disease is compared with the other lymphomas in which intracellular immunoglobulin is but rarely found. Moreover, lymphoblasts containing IgG or IgM antibody are relatively frequent in Hodgkin tissue, though rare in all except extremely reactive lymph nodes. Very marked plasma cell accumulations around non-Hodgkin's granulomata are noted in some cases. However, in common with reactive nodes, intracellular immunoglobulin is never present in germinal centre cells, despite the often prominent follicular hyperplasia. The appearances of sections/...

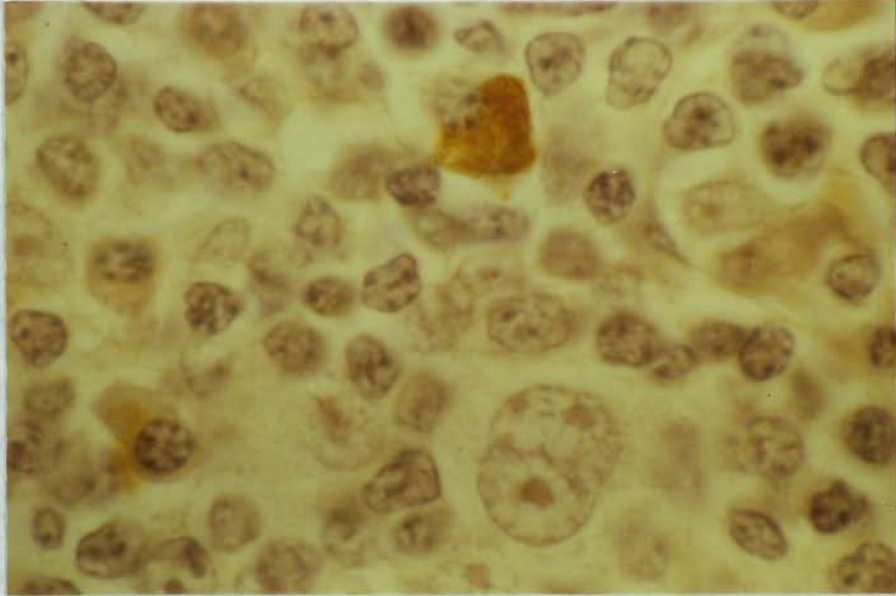


Figure 33: Immunoperoxidase Reaction in Hodgkin's Disease

In this section from a Hodgkin's disease lymph node, the upper centre field shows a cell staining intensely for IgG antibody (brown). Another plasma cell occurs to the left of the field, and to the lower right is an "Owl's eye", lacunar cell, typical of nodular sclerosing Hodgkin's disease. Note the absence of cytoplasmic staining of this cell.

P.A.P. immunoperoxidase for IgG Counterstain
Haematoxylin Oil Immersion x 1,200

sections stained for intracytoplasmic immunoglobulin are illustrated (Figs. 5, 33).

In sections stained conjointly for iron (PBR) and immunoglobulin a striking feature was the association between iron containing macrophages and antibody forming plasma cells. This was most marked in the red pulp, where it frequently involved the sheathed arterioles (Schweigger Seidal sheaths). As shown macrophages in this region often contained iron, and plasma cells in close association were invariably found (Figs. 9, 10, 34). The principal interest of this observation lies in the findings of Order, Porter and Hellman (1971) of an antigen associated with Hodgkin's disease which later proved to be ferritin. Banding in the B2 region of the electrophoretic strip has been noted to occur with regularity in electrophoresis of Hodgkin's serum, and the material responsible for this banding is a complex of transferrin and IgG antibody (Habeshaw - personal observations). These observations strongly support the argument that abnormal immune responses occur in Hodgkin's tissue, even to antigens which are of widespread distribution. It furthermore seems to imply a lack of T cell suppression of B lymphocyte responses in this disease.

Intracellular immunoglobulin does occasionally occur in cells of Reed Sternberg type, and in "reticular lymphoblasts" and lacunar cells. These features (Fig. 35) show that abnormal morphological forms of "plasma cells" could be part of the neoplastic component of Hodgkin tissue.

Areas in which Hodgkin's disease lesions are found

In/...

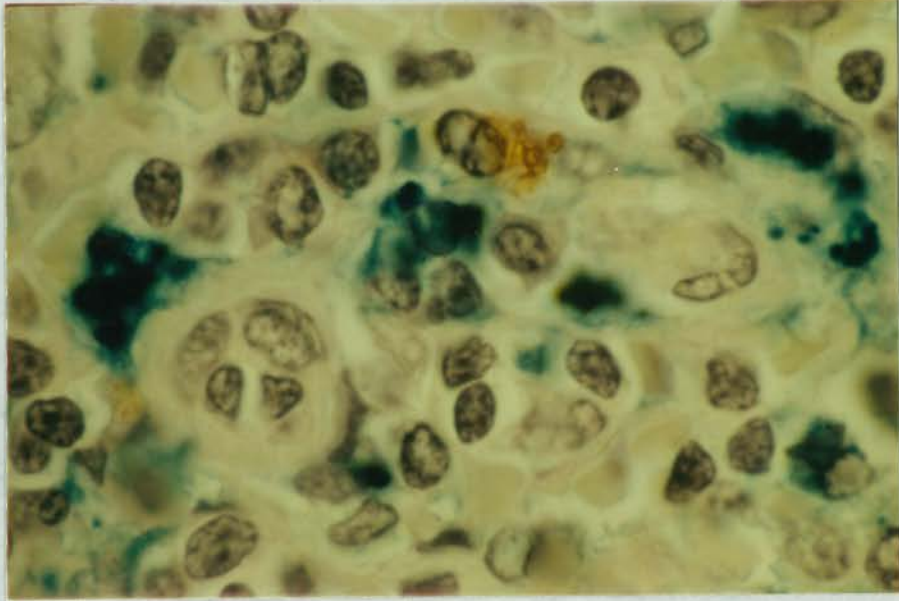


Figure 34: Immunoperoxidase Reaction in Hodgkin's Disease

A section stained for IgG antibody (brown) and iron (blue) reveals a close association between several iron containing macrophages and a single antibody forming cell. It is suggested that this association accounts for the ferritinemia and the presence of ferritin/antibody complexes known to be a feature of Hodgkin's disease.

P.A.P. immunoperoxidase for IgG/PER reaction

x 600

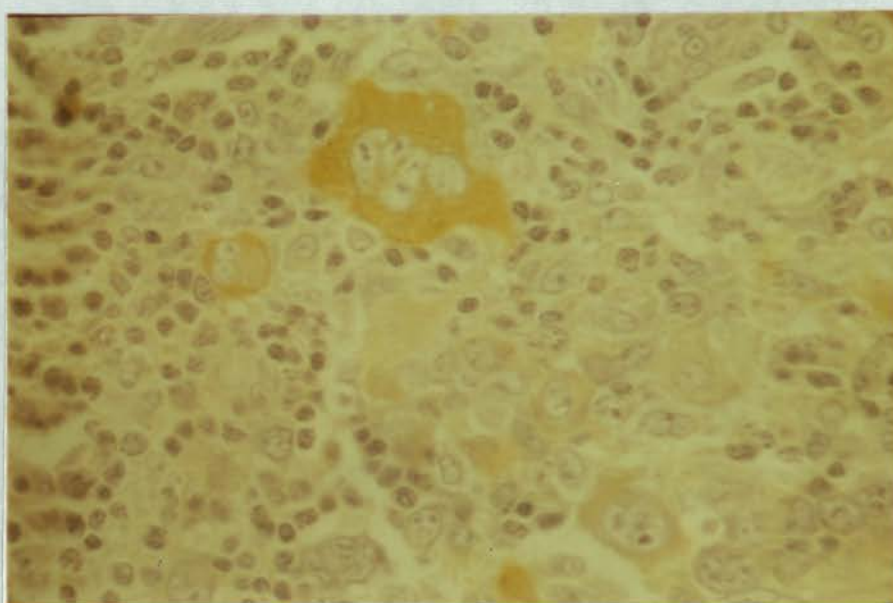


Figure 35: Immunoperoxidase Reaction in Hodgkin's Disease

In this section from the lymph node of RAE, stained for Kappa chain by the P.A.P. technique several Reed Sternberg cells exhibit definite cytoplasmic staining above background levels. These cells may be morphologically bizarre equivalents of the plasma cell.

P.A.P. Immunoperoxidase, Kappa chain
Counterstained Haematoxylin

x 400

In the spleen early involvement can be found to occur in regions of thymic dependency. These are in the periarteriolar sheath, and at the margins of follicles where the peripheral white pulp (a B cell area) lies adjacent to the marginal sinus (through which T cells migrate to reach the periarteriolar lymphoid sheath). In the lymph node no good examples of paracortical involvement were seen, but in lymphocyte predominant forms of the disease, the paracortex may be substantially increased. The impression from histology is that Hodgkin's lesions occur first at the meeting points of T and B cells areas, and that there is morphological evidence of hyperactivity of both B and T cell components in node and spleen.

Relationship between the Reed Sternberg cells and the activated T lymphocyte .

In this study numerous examples of Reed Sternberg cells were seen. Their surface features were variable, but some characteristic abnormalities were observed.

In suspensions of Hodgkin's disease lymph node, Reed Sternberg cells, and the other abnormal cell types were constantly found closely associated with T lymphocytes. The co-rosettes are illustrated (Figs. 36, 37) and it is clearly seen that convoluted small lymphocytes are the major cell class. These T lymphocytes can also be shown, in many instances to express Fc receptors and this is known to indicate an "activated" T lymphocyte. Occasional abnormal forms of lymphocytes occur:- the hypersegmented lymphocyte, but what these represent is unknown. They frequently play no part in the interaction with the Reed Sternberg cell, but often show E rosetting/...

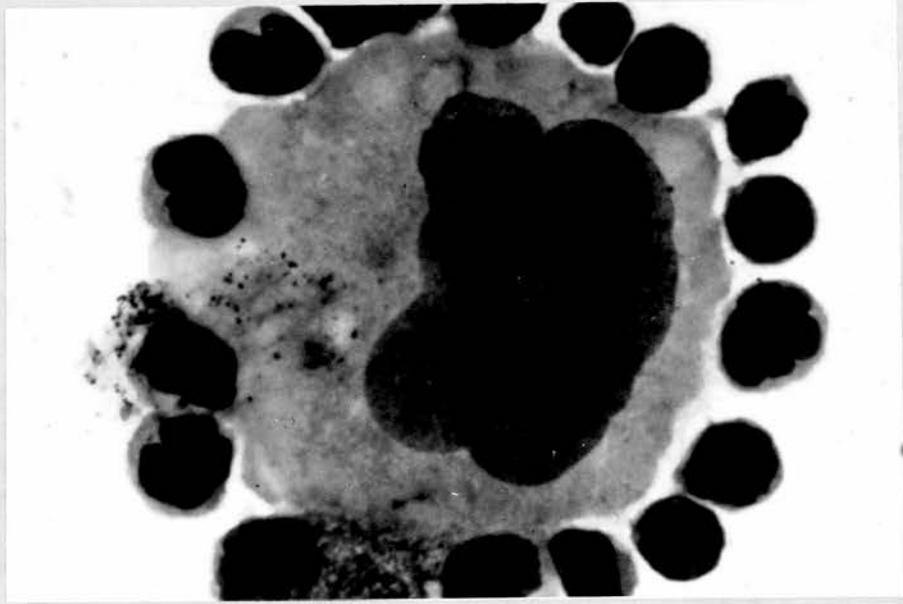


Figure 36: The Reed Sternberg Cell in Hodgkin's Disease

A binucleate Reed Sternberg cell is shown, surrounded by small lymphocytes, some with convoluted nuclei. This cell was isolated from an involved lymph node (Rae).

Cytocentrifuge preparation, Giemsa Stain

x 1,000

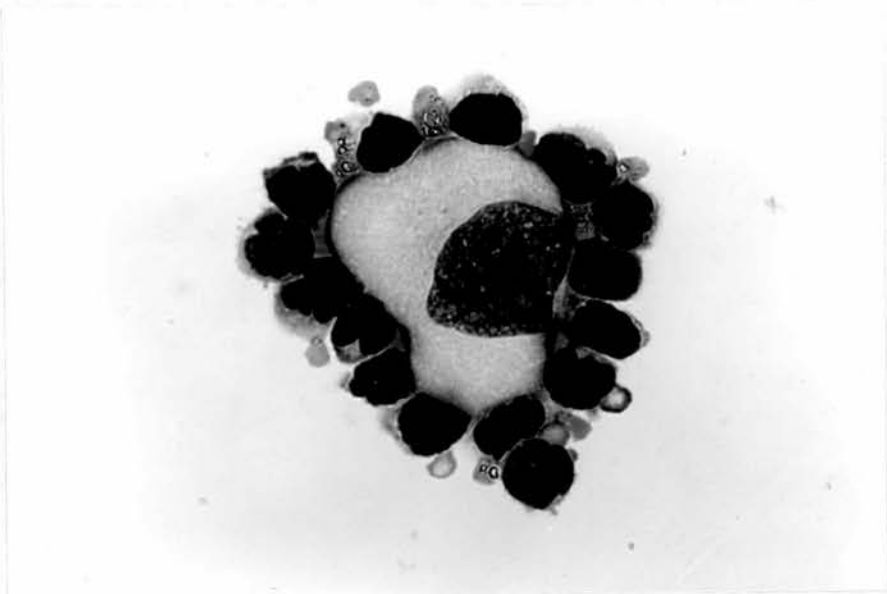


Figure 37: The Reed Sternberg Cell in Hodgkin's Disease

This uninucleate Reed Sternberg Cell is surrounded by small lymphocytes and some adherent IgG sensitised ox erythrocytes. The appearances suggest weak Fc receptor expression by both the Reed Sternberg cell and the surrounding lymphocytes, and this is confirmed by studies in which the clusters are dispersed with the enzyme pronase.

Cytocentrifuge preparation, Fc rosettes,
Giemsa Stain.

x 600

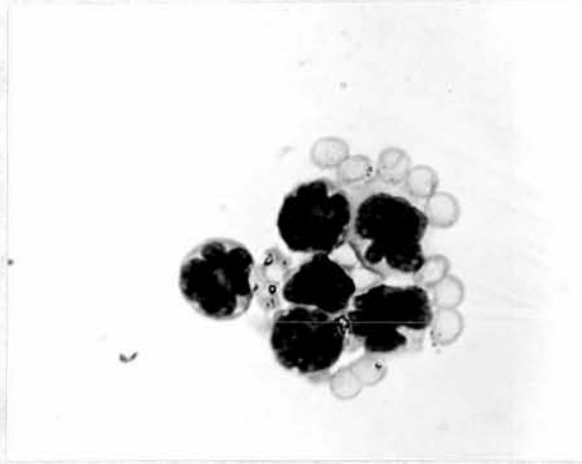


Figure 38: Convoluted Lymphocytes in Hodgkin's Disease

A cluster of convoluted lymphocytes showing rosette formation with sheep erythrocytes.

E rosettes, cytocentrifuge preparation, Giemsa stain
x 900

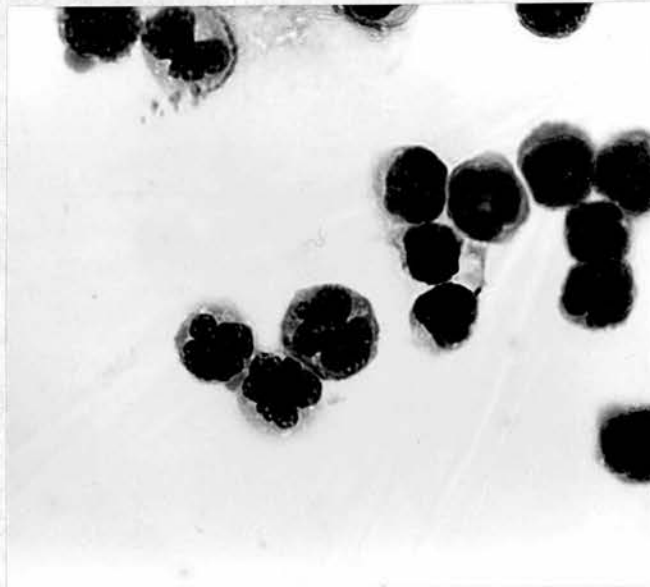


Figure 39: Convoluted Lymphocytes in Hodgkin's Disease

This cytocentrifuge preparation clearly shows that a substantial part of the small lymphocyte population in involved Hodgkin's disease nodes may be of convoluted lymphocyte type.

Lymph node cell suspension, cytocentrifuge preparations, Giemsa stain.

x 1,000

rosetting capability and may express the Fc receptor. These cells are illustrated (Figs. 38, 39).

Reed Sternberg cells themselves expressed the Fc receptor, but not the complement receptor, and occasionally showed the presence of non-capping surface Ig. This would be evidence for the Reed Sternberg cell being a B2.Fc lymphocyte. These features were not constant; some showed the presence of weak intracytoplasmic staining for IgG or more rarely IgM, which would suggest B cells of B5 differentiation subclass. Yet others (the majority) expressed no surface features other than clustering with T cells, and the presence of the Fc receptor. Reed Sternberg cells did not react with sheep erythrocytes (and are thus non-T) or with anti-macrophage serum (A.E. Stuart - personal communication) and are not macrophages. The conclusions are that the Reed Sternberg cell is, in a minority of cases, demonstrably a B lymphocyte, either of B2.Fc subclass or of B5 subclass. It appears to be associated with activated or committed T cells in an attitude of co-operation. (There was no evidence of cytotoxicity by the T cells against the Reed Sternberg cell. Indeed, only "healthy" Reed Sternberg cells associated with T cells). The implications are strong that the abnormalities observed in Hodgkin's disease centre on T cell induced B cell stimulation as seen in the production of a secondary humoral immune response. This failure may be due to some abnormality of the B lymphocyte, since bizarre morphological forms of this cell are encountered in this disease. There are abnormally large accumulations of actively secreting plasma cells. In Hodgkin's disease the lesions seen represent an abnormal secondary immune/...

immune response in which T helper cell function is intact, but in which suppression of B cell function is lacking. There are two aetiological possibilities

- a) A defect in T cell suppression, and T cell cytotoxicity is present, but the T helper functions are preserved. Any antigen could then initiate a Hodgkin-type disease, which because of stimulation of B lymphocytes coupled with lack of immune surveillance eventually results in a neoplasms of B lymphocytes or associated cells.
- b) A virus in B cells (e.g. EB virus) is not eliminated in the usual way (by DTH reaction) because of a T cell defect (cytotoxic T cells not present). The virus causes T helper stimulation because of its antigenicity, to which the target B cell is partially refractory because of its virus burden.

REFERENCES - CHAPTER 22

ORDER, S.E., PORTER, M. and HELLMAN, S. (1971) Hodgkin's disease: evidence for a tumour associated antigen. *New. Eng. J. Med.* 285, 471-474.

Chapter 23

- a) Receptor classification: its validity and limitations
- b) Incidence of different types of non-Hodgkin lymphoma in this series.
- c) Relationship of receptor studies to morphological and clinical behaviour
- d) The findings in Hodgkin's disease.

Receptor classification, its validity and its limitations

The data presented represent the first systematic study of lymphomata in which the tumours have been classified by the surface characteristics of the cells in the affected lymphoid tissue. The classification scheme employed was based on a thorough survey of the known facts about the behaviour of normal lymphoid cells during their differentiation from the stem cell to the functional end cell in both humoral and cellular immune responses. The survey included a detailed assessment of the known surface characteristics of each cell class, and a tentative scheme of normal differentiation markers for B lymphocytes was proposed. A detailed investigation of the characteristics of lymphoid cells from normal tissues showed the necessity of including in such a scheme methods of comparing the normal "receptor profile" with the receptor profile of neoplastic tissues. It became clear that in some cases the receptor profile of the tumour closely resembled the receptor profile of blood (B3.1) or tonsil (B3.2) lymphoid tissue or was indistinguishable from profiles obtained from normal or reactive lymph node (B3.3, B4). This fact precludes the use of surface phenotypic studies, such as those described, to diagnose lymphoma. The diagnosis of lymphoma depends upon the histological appearance of the biopsied tissue, and not upon its receptor profile.

In comparing normal with neoplastic tissues, it was apparent that neoplastic tissues showed B lymphocyte predominance more commonly than normal lymph node or spleen, and that they also showed the presence of three unique receptor profiles not seen in normal lymphoid tissue. These were called receptor silence, receptor/...

receptor overlap and B1 lymphocyte predominance.

As a result of these observations, it was proposed that the neoplasms be classified

- 1) according to the predominant cell class
- 2) if no class was predominant, according to the subclass of B lymphocyte.

The possibility that subclasses of B lymphocyte were present in lymphoid tumours was indicated by the following observations. Differences in capping of surface Ig anti-Ig complexes between immature and mature B lymphocytes have been shown (Sidman and Unanue, 1975a,b), and differences in expression of the Fc and C3 receptor on immature (Metcalf et al., 1975) blast transformed (Moller, 1974) or mature secretory (Corte et al., 1976) B lymphocytes have been reported.

Studies of normal blood showed that two subclasses of B lymphocyte were present (B3.1 and B2.Fc). Studies of tonsil showed that an additional two subclasses of B lymphocyte, B3.2, B2.C, were present and studies of lymphoid cell lines showed the relationship between capping and non-capping behaviour, and the presence of Fc and C3 receptors. The receptor profile of normal or reactive nodes, showed two further classes of B lymphocyte to be present (B3.3, B4), and the presence of B4 phenotype by the cell line EB4 was demonstrated.

The scheme eventually used in the classification of lymphomata depended upon determination of the proportions of each cell class (T/...

(T lymphocyte, B lymphocyte or macrophage), and the subsequent determination of the major B lymphocyte subclasses in each tumour. In order to achieve this, a minimum of 6 observations must be made on the cell suspension from each tumour - the numbers of E, Fc and complement rosettes, the numbers of mononuclear phagocytes, the distribution of the surface Ig anti-Ig complex and the numbers of surface Ig bearing cells. When this classification scheme was applied to a series of 30 non-Hodgkin lymphomas and 9 leukaemias in only two cases (myeloid leukaemia and leukaemic reticulo-endotheliosis) was there failure to classify the tumour by this scheme.

The incidence of the different types of non-Hodgkin Lymphoma in this series.

The receptor profile showed that of the 31 tumours studied the different cell types occurred as follows:

B1 - 5 times, B2 - 4 times, B3 - 10 times, B4 - 3 times, B5 - once, Receptor overlap - twice, macrophage - once, T cell - once and receptor silent tumours - 4 times. The most common profiles identified in the lymphomas were of B3 sub-class, which formed approximately one third of all lymph node tumours. The next most common B subclass found was B1, forming 15% of solid lymphoid tumours. Receptor silent tumours, and B2 tumours were found on 4 occasions (13%) and B3 on 3 occasions (10%) in non-Hodgkin lymphomas. The incidence of B5, receptor overlap, macrophage and T lymphocyte tumours cannot be reliably assessed in this small series/...

series, but probably amounts to no more than 3-6% of non-Hodgkin lymphomata in each case. These frequencies are summarised below.

<u>Receptor Profile</u>	<u>Percentage of Non-Hodgkin Lymphomas</u>	<u>Frequency in normal Control Nodes</u>
B3	30	79%
B1	15	Nil
B2	13	7%
Receptor Silent	13	Nil
B4	10	14%
B5	5	Nil
Overlap	5	Nil
Macrophage	5	Nil
T cell	5	Nil

The receptor profile of the lymphoma lymph node resembles the normal lymph node profile in about half the cases (B3 + B2 + B4 = 53%), but in the remainder is clearly different, usually because the B lymphocytes are more primitive (B1) or the cells lack surface markers altogether. In all the B1 and B2 lymphomas, the profile is of B lymphocyte predominance, while in the B3, B4 lymphomas the profile is B predominant in only 85% of the cases, the remaining 15% showing a mixed pattern. This again differs from the normal lymph node, where 19% only show B lymphocyte predominance, 46% are of mixed pattern and 36% show T cell predominance. The non-Hodgkin lymphoma lymph node can be said to differ from the normal or reactive lymph node by (1) the more primitive nature of the cells composing the tumour/...

tumour, and (2) the tendency of the node to show B lymphocyte predominance, rather than a mixed or T cell predominant pattern. B lymphocyte predominant tumours form between 60 and 70% of all non-Hodgkin lymphomas, the remaining 30-40% being receptor silent, mixed, T cell, receptor overlap or macrophage tumours.

The relationship of receptor studies to morphology and clinical behaviour.

Most of the tumours classed as B3 or B4 were nodular poorly differentiated, nodular small cleaved cell, or diffuse small lymphocyte in type, and there was good agreement in this group between the histological assessment, the receptor profile and survival. Most of these did not progress appreciably over the time they were studied.

The tumours classified as histiocytic lymphoma, showed marked heterogeneity of surface phenotype including receptor silence, receptor overlap and B1 lymphocyte tumours. Only one example of true "histiocytic lymphoma" - a macrophage tumour was seen. Patients in this group did uniformly badly, with the exception of "receptor overlap" tumours which responded readily to treatment and did not recur. In diffuse lymphocytic lymphoma, there was relatively poor agreement between the cytological assessment of differentiation and the receptor profile.

There is a strong correlation between the receptor profile of B1 lymphocyte tumours and the presence of receptor silent cell populations and prognosis. It has been statistically shown that (1) B1 and B2 lymphocytic lymphomas are distinct from B3/...

B3 and B4 lymphocytic lymphomas when the survival of patient is considered. B1 and B2 tumour patients have a significantly lower probability of survival than B3 and B4 tumour patients. (2) clinical staging of the lymphoma is of less importance in predicting survival than determining the receptor class to which that tumour belongs.

The findings in Hodgkin's disease

A study of Hodgkin's disease spleen showed that in males the effect of the disease was to decrease the cell count per gram of spleen and the total cell count, while in females the cell count per gram, and the total cell count was higher than in normal female spleen. In the female the concentration of T cells is decreased, while there are suggestive but not significant increases in the concentrations of non-capping B cells and phagocytes. The total numbers of T cells and B cells in the female spleen in Hodgkin's disease are significantly increased, to the levels found in normal male spleen.

In involved spleens there was some evidence of increased T cell proportions, and concentration in the involved area. In the nodular sclerosing subtype of Hodgkin's disease the uninvolved spleen showed an excess of complement receptor cells over the combined sum of B cells and macrophages.

Investigations of seven involved lymph nodes showed clearly that T cell proportions were raised in some, but not all cases of Hodgkin's disease. The same excess of the proportion of complement receptor bearing cells over B cells and macrophages was/...

was found in lymphocyte predominant Hodgkin's disease. IgM receptors on T cells in Hodgkin's disease were probably present in 5 out of 31 cases, and were directly demonstrated in blood (2) and spleen (1) of a further 3 cases.

From the results presented the following features, unique to Hodgkin's disease as far as is known, have been demonstrated:

- a) that Hodgkin's disease affects the cellularity of male and female spleen differently.
- b) That abnormal T cells, bearing IgM receptors and cross reacting with IgM and complement coated red cells are present in a minority of Hodgkin's disease patients.
- c) That increased numbers of T cells in Hodgkin tissue, although present in some patients, are not found in every case, and in one patient the T cells described were abnormal T cells reacting with IgM and complement coated red cells.
- d) That uninvolved nodes, and spleen contained in some cases B cells exhibiting non-capping behaviour and bearing complement receptors (B2.C class). Their presence correlated with follicular hyperplasia in the tissue studied. The nature of the Reed Sternberg cell was investigated, and it was shown that in some cases it resembled a B2.Fc lymphocyte, occasionally showed intracytoplasmic immunoglobulin, and was frequently surrounded by activated T cells expressing Fc and receptors and rosetting with sheep erythrocytes.

It/...

It was hypothesised that in Hodgkin's disease, T cells were immature, exhibiting helper cell rather than suppressor cell, or cytotoxic activity, and that the B cell was both the target for helper activity, and the source of antigenic stimulation of the T cell.

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Chapter 24

DISCUSSION

DISCUSSION

Of the three quotations preceding the introduction to this thesis, two emphasise the role of the pathologist as a "pathological physiologist" and the third summarises the difficulties which would have faced any pathologist approaching the subject of lymphomas two decades ago. The fact that in the interim a vast expansion of knowledge about the life cycle of the lymphocytes, and the origins and life history of the mononuclear phagocyte occurred, allows a survey of lymphoma from the standpoint of pathological physiology for the first time. The price paid for an increased understanding of the nature of lymphomas is the inevitable increase in the complexity of the classification schemes employed in their diagnosis. For example Rappaport (1966) lists 6 major classes of lymphoma (Hodgkin's disease classified separately). Lukes (1975) currently lists 11 types. The receptor studies presented in this thesis show clearly the existence of 12 distinct receptor profiles in lymphoma/leukaemia and when the predominant and mixed primary classification is also employed an additional 4 categories at least can be distinguished. The indications are that in lesions diagnosed histologically as non-Hodgkin lymphoma, as many as 17 separate subclasses of this disease can be demonstrated by surface phenotype as shown in this series. The techniques employed all detected normal phenotypic features of lymphoid cells, no attempt has been made to detect abnormal or neoplastic features such as tumour associated surface antigens, although these are known to exist (Billing, Rafizadeh and Terasaki, 1975; Pendergrass et al., 1975; Baker/...

Baker, Ramachander and Taub, 1974). If both surface antigen class, and surface phenotype were used together to classify lymphoid tumours it is probable that many more subclasses might be found. In addition, the cases in the present study are few, since lymphoma of non-Hodgkin type remains an uncommon disease. More extensive studies could reveal further phenotypic profiles again increasing the potential pool of subclasses of lymphoma. At present it is not possible to reliably estimate the potential numbers of subclasses of lymphoma detectable by methods in use today, but they will almost certainly exceed 20. When it is considered that each subclass may exhibit different clinical behaviour, response to therapy and so on it is evident that the classical pattern of assessing survival in each subgroup and empirically developing appropriate forms of therapy for each subgroup would be an enormous task, and probably beyond the capabilities of current medical practice, even allowing that sufficient standardisation of techniques between individual centres enabled valid comparisons to be made.

There is an easier road to take. If the true nature of proliferations of lymphoid tissue could be understood, and if the lymphoma could be interpreted as a perversion of the normal functions of lymphoid tissue, then the identification of the reactive state of the cells in the lymphoma could serve as the basis for rational therapy. This approach is far from being a fully developed diagnostic procedure but it is presented here in the hope that it may influence future approaches to the classification and therapy of lymphoma.

Identification/...

Identification of lymphoid tumours according to their cellular constituents

In the introduction, the definition of lymphoma adopted included in this group of diseases tumours of the constituent cells of central (thymus, bone marrow) or peripheral lymphoid tissue. The constituent cells concerned are derivations of the lymphoid stem cell, the monocyte precursor cell (colony forming unit) or derivatives of coelemic epithelium (the reticular cell). This group of tumours includes all tumours of the cells shown in the table below.

Constituent Cells of Lymphomas

- (a) Derivatives of lymphocyte system
 - 1) Bone marrow precursor (stem cell)
 - 2) Thymocyte
 - 3) Peripheral T lymphocyte
 - 4) B lymphocyte
 - 5) Plasma cell

- (b) Derivatives of the mononuclear phagocyte system
 - 1) Colony forming unit
 - 2) Promonocyte
 - 3) Monocyte
 - 4) Macrophage

- (c) /...

(c) Derivatives of coelemic epithelium and mesenchymal derivatives.

- 1) Reticulin fibre forming cell (mesenchymal)
- 2) Dendritic cell of germinal centre
- 3) Reticular cell (coelemic epithelium)

These cells generally show surface features which allow their detection in lymphomas, and these are shown below:

Constituent Cells of Lymphoma

Surface features of each class

(a) Derivatives of the lymphoid system

- | | |
|----------------------------|---|
| 1) Stem cells | No surface Ig
No Fc or C3 receptor
No receptors for E |
| 2) Thymocyte | E Rosette positive
Surface Ig negative
No Fc or C3 receptor
May have IgM receptor |
| 3) Peripheral T lymphocyte | E Rosette positive
Surface Ig negative
No C3 receptor
No IgM receptor
May express Fc receptor if activated. |

4)/...

4) B lymphocyte

E receptor negative
 Fc and/or C3 receptor
 positive
 Surface Ig positive
 No IgM receptor

(b) Derivatives of the Mononuclear phagocyte system

1) Colony forming unit

Surface Ig negative
 May have Fc receptor
 No C3 receptor
 Non phagocytic

2) Promonocyte

Has monocyte specific
 antigen
 Fc receptor positive
 Ingests neutral red
 Non-adherent to
 glass/plastic

3) Monocyte

Has monocyte specific
 surface antigen
 Fc and C3 receptor
 positive
 Ingests neutral red dye
 Glass and plastic
 adherent
 Phagocytic
 Surface Ig negative

4)/...

4) Macrophage

Has monocyte specific
surface antigen

Fc and C3 receptor positive

Surface Ig negative

Ingests neutral red dye

Phagocytic

Adherent to glass and
plastic

(c) Derivatives of coelomic epithelium and
mesenchymal derivatives

1) Reticulin fibre forming cell

Surface Ig negative

Non phagocytic

No Fc or C3 receptors

Extracellular "coat"

Adherent to glass and
plastic

2) Dendritic cell of germinal
centre

Surface Ig negative

Non phagocytic

Has Fc but no C3
receptor

? Adherent

3) Reticular cell

Surface Ig negative

Adherent to glass and
plastic

Selectively phagocytic
(Ingests lymphocytes)

Has IgM receptors

Miscellaneous/...

Miscellaneous**1) K cell**

Has Fc and C3 receptors

Surface Ig negative

Non-adherent to glass

Non phagocytic

2) Endothelium

Not known

There are thought to be 12 different classes of cell of different histogenetic derivation which could give rise to tumours diagnosed as lymphoma. The receptor profiles obtained through the use of simple surface receptor and tissue culture techniques correspond to the known phenotypic characteristics of some of these cell classes. Superficially the value of these studies lies in the identification of the class of cell by the surface characteristics, and in assessing the survival of patients with each class of tumour.

Receptor silent tumours

In the case of receptor silent tumours, the detection of cell class appears to indicate the prognosis, since all patients with these tumours have died despite therapy. This is not a satisfying conclusion for two reasons:- firstly the receptor silent tumours are of different cell types and histological pattern (compare patients Kerr and Ritchie) indicating heterogeneity within this group, and secondly the lymphoid stem cell, the colony forming unit, and the reticular cell classes would all be classified as receptor silent according to current techniques, as summarised below.

Possible/...

Possible origins of receptor silent tumours

- a) Lymphocyte (? tumour of Kerr)
- b) Bone marrow lymphoid stem cell (? receptor silent ALL)
- c) Mononuclear phagocyte stem cell (? none in this series)
- d) Reticular cell tumours (? tumour of Ritchie)
- e) Reticulin fibre forming cell (? none in this series)
- f) Undifferentiated tumours of epithelial derivation (? tumour of Moyes)

The fact that receptor silent tumours occur tends to obscure the more important question why should cells lacking surface phenotypic markers give rise to tumours with a poor prognosis? There are two possibilities, firstly that such cells are by nature undifferentiated and so have a greater potential for growth, and secondly that the surface phenotype dictates the behaviour of the cell as it interacts with the other cell classes in lymphoid tissue. In the absence of any receptor by which control over its growth and differentiation could be exerted by surrounding cells, the tumour cell exhibits a constant and uninhabitable tendency to proliferate without differentiation. This conclusion may apply not only to lymphoma but to all classes of malignant neoplasm, and brings into question the concept of biological control of growth of cells through modifications of the cell membrane (reviewed by Wallach, 1975). Further discussion of this topic is beyond the scope of this thesis, but if the cell surface is the eyes of the cell, the receptor silent tumour is a tumour of "blindly" proliferating cells. The intrinsic capacity to differentiation is lost, and growth can be controlled only/...

only by the recognition of and reaction to the surface antigens of the neoplastic cells (immune surveillance) (Burnet, 1972).

Macrophage tumours

Tumours of the mononuclear phagocyte system pose similar problems. In this case tumours of promonocytes or monocytes are likely to prove leukaemic (monocytic and promonocytic leukaemia) since these cells arise in bone marrow, and exist only in the circulation. Their surface receptor characteristics differ but slightly from the mature tissue fixed macrophage (Fc and C3 receptors) but their phagocytic capacity (except for vital dyes) is less pronounced. The mononuclear phagocyte system is phylogenetically very primitive preceeding the evolution of the B lymphocyte and T lymphocyte systems. Control over mononuclear phagocyte proliferation is therefore likely to be intrinsic rather than influenced by T or B lymphocyte products. There is evidence that proliferation of the promonocyte (colony forming unit) might be non specifically suppressed by activation of T lymphocytes (McNeil and Killen, 1971b), but in the interactions between lymphocytes and macrophages, stimulation and suppression appears to operate one way (macrophages lymphocytes) with little evidence of control of macrophage production and differentiation directly by the lymphocytes. There is a form of feedback on the macrophage by T and B lymphocyte products, lymphokines producing non-specific local immobilisation (M.I.F.) or proliferation (Mitogenic factor) of macrophages, and B lymphocytes producing chemotactic and phagocytic behaviour through antibody and the interaction of that antibody and complement/...

complement with the Fc and C3 receptors on the macrophage. This feedback affects macrophage behaviour rather than the kinetics of mononuclear phagocyte production. The presence of the Fc receptor on the monocyte and macrophage appears to determine the capacity of lymphoid cells to interact with the macrophage rather than the rate of macrophage production or the differentiation of tissue macrophages from monocytes. What does control the rate of production of mononuclear phagocytes? It was shown by Smith and Stuart (1975) that simple lipid esters such as cholesterol oleate and ethyl palmitate affect macrophages directly producing effects upon macrophage motility and spreading upon glass. Cells treated with ethyl palmitate appeared to lack receptors for Fc portion of IgG, which may imply interference with Fc receptor synthesis by the macrophage. The intravenous injection of lipids, particularly cholesterol oleate, produced an initial depression of phagocytosis of foreign red cells, and changes in the distribution of red cells between liver (normal controls) and spleen (treated animals). Electron microscope studies revealed the appearance, in the livers of lipid injected animals, of numerous immature bone marrow derived monocyte precursors, which were observed to change in situ to Kupffer cells (Stuart and Smith, 1975). In treated animals there was profound depression of the immune response, due to impaired macrophage function. The lymphocytes were unaffected. This series of experiments shows that the monocyte precursor can be generated from bone marrow 48 hours after reticuloendothelial blockade with lipid. The mechanisms of control of this production are not known, but increase in the numbers of colony forming units from the bone marrow/...

marrow can be detected following blockade. This argues strongly for a control mechanism acting through the monocyte precursor for maintaining the numbers of circulating and tissue macrophages (Dewar and Ramage, personal communication). The differentiation of mononuclear phagocytes is little understood. Preliminary work shows that human monocyte specific antigen can be detected on bone marrow precursors, and on tissue macrophages, but the mechanism by which the circulating monocyte matures to the tissue fixed macrophage is still obscure. In this thesis data from monocytic leukaemia and the solitary true histiocytic lymphoma show no significant differences in surface phenotype excepting the presence of IgD in the cells from the patient with monocytic leukaemia. It is noteworthy that circulating monocytes in the case of histiocytic lymphoma (Ross) were markedly elevated. The interpretation favoured is that macrophage numbers in tissue are determined by the rate of production of monocyte precursors in bone marrow from the stem cell. There is continuous maturation of the cells so produced from circulating monocyte to tissue macrophage. The presentation of mononuclear phagocyte tumours therefore depends upon the rate of cell production, and the eventual tissue localisation of the tumour cells. There is no evidence for specific control of production by lymphocytes, although tissue localisation could be influenced by either antibody production or lymphokine secretion. Tumours of macrophages can therefore for practical purposes be regarded as tumour of the series stem cell, the nature of the tumour (histiocytic lymphoma, monocytic or promonocytic leukaemia) being determined by the kinetics of cell production and cell/...

cell death rather than a maturation sequence of individual differentiation steps as proposed for B and T lymphocytes.

In the two examples given (receptor silent tumours, macrophage tumours) assessment of differentiation is not possible, in the first case because differentiation markers are absent from the cell, and in the second because the whole life cycle of the cell is encompassed by a single differentiation step (from stem cell promonocyte) as shown by the presence of Fc and C3 receptors on both "mature" and "primitive" cell forms, and the occurrence of a single class of surface antigen on all cells of this lineage.

B lymphocyte tumours

In the hypothesis of Salmon and Seligmann (1974) the B cell neoplasm was considered as a tumour evoked by (a) a primary, or intrinsic neoplastic event, coupled with (b) antigen stimulation. This resulted in the development of neoplastic B cells, at different stages of their maturation cycle. This very useful method of assessing B cell tumours has been adopted here. It must be emphasised that since the methods used assess only normal phenotypic features of cells, no opinion as to their "malignancy" can be offered. In order to show that the cells in a lymphoma are truly "malignant", it is necessary to demonstrate a tumour specific or neoantigen on or in the cells, by the use of a monospecific antiserum. The data presented apply, then, only to the variations in normal phenotype which occur as B cells differentiate. The fact that such phenotypic profiles occur at all in B lymphocyte tumours is strong evidence in favour of the mode of development of these/...

these tumours as proposed by Salmon and Seligmann. On this basis, in the following section the application of the phenotypic staging of B cell to the lymphoma is examined.

Differentiation of B lymphocytes and its relationship to tumours of these cells

The T and B lymphoid populations take origin from a bone marrow stem cell. The different classes of T cell and B cell were purported to arise through the inductive influence of the Bursa equivalent tissue (in the case of B lymphocytes) or the thymic epithelium (T lymphocytes). It is now known that in both B and T lymphocyte systems, the T or B lymphocyte is committed before entering bursa or thymus. Pre-bursal B lymphocytes in the chicken are shown to express surface IgM antibody (Jankovic et al., 1975) and in thymus deprived, and congenitally thymusless mice, T cells expressing theta (thy 1) antigen do occur (Raff, 1973; Loor and Roelants, 1974). In mammals, evidence for the inductive role of bursal equivalent tissue for B lymphocyte maturation was never conclusive, and it is likely that immature "pre-bursal" B lymphocytes are formed directly from a stem cell precursor. These uncommitted cells, seed to the peripheral lymphoid tissues, (Nossal and Pike, 1973) encounter antigen, especially in sites such as the lamina propria of the gut and become committed to eventual antibody formation by antigen. This process is termed "antigen drive" and is probably necessary for the expansion of the B cell population in normal animals (Nieuwenhuis et al., 1974a,b). The immature B lymphocyte can be arrested in its development by continuous treatment of the intact animal with anti μ chain antisera, which reacts with the surface μ chain. Such treatment prevents the further development of the immature B cell, whilst not affecting the immature B lymphocyte. Neonatal mice so treated fail to develop/...

develop normal B cell populations (Raff et al., 1975). The immune response of B cells from neonatal animals can be abolished by the contact in vitro between specific antigen and the B cell reactive to it. Adult B cells were not affected (Metcalf and Klinman, 1976). The immature B cell fails to develop into an antibody forming cell within 2 days of antigen challenge, and using this criterion it was shown that foetal mice contain no mature B cells, but that these develop very rapidly between 3 and 5 days after birth (Rosenberg and Cunningham, 1975). Sidman and Unanue (1975b) showed inhibition of early (immature) B lymphocytes by anti-immunoglobulin antisera, and related this inhibition to failure of the immature B lymphocyte to cap and hence shed the blocking complex on its surface. The relationship between the capping of cells and the ability to escape from antigen induced or antibody produced blockade shows clearly that inhibition of capping by drugs prolonged the effect of blockade and prevented resynthesis of surface Ig. Inhibitors of protein synthesis were ineffective in preventing blockade. The complete recovery from blockade occurred in about 10 minutes at 37°C consistent with the capping and shedding of Ig anti-Ig complex (Ivanyi, Fuensalida and Lydyard, 1976). Confirmation of the existence of mature, immature and secretory IgM bearing lymphocytes has been shown. Type I cells (immature antigen inexperienced or virgin B cells) synthesised and released 7S IgM rapidly, Type II cells (small resting memory B cells) synthesised and released 7S IgM slowly, and type III cells (secretory "plasma cells") released 19S IgM rapidly, mainly from stored cytoplasmic IgM (Melchers, Cone, Bon Boehmer and Sprent, 1975). The ability of anti-allotype or anti-immunoglobulin antisera to blockade the immature B lymphocyte can persist for longer periods in vivo.

Anti-/...

Anti-allotype antisera administered to neonatal rabbits within 2-5 days of birth brought about complete suppression of secretion of that immunoglobulin allotype. Delay beyond day 6 produced only partial suppression of shorter duration (Lowe and Catty, 1976). These findings all suggest the existence in the neonate of a surface Ig bearing, u chain expressing, non-capping B lymphocyte, which is easily inhibited from further maturation by the presence on its surface of complexes of surface Ig and antigen, or anti-Ig surface Ig immune complexes. This population is quite distinct from that appearing in adult life, capable of capping, not inhibited by complexes, and showing the ability to shed capped surface Ig and regenerate this molecule.

The existence of these two major classes of B lymphocyte was grasped as the central theme for the classification of B lymphomas, non-capping lymphomas exhibiting the surface characteristics of immature or transformed B cells, capping lymphomas corresponding to the more mature adult B lymphocyte.

The B1 category of tumour, expressing surface Ig, non-capping, and lacking receptors for complement and IgG corresponds very closely to the descriptions of B lymphocyte characteristics in the neonate, and also the cell grown in vitro from splenic colonies in the mouse (Metcalf et al., 1975). Importantly most of the B1 tumours in this series were either leukaemic or extranodal as would be expected in a cell corresponding to the neonatal B lymphocyte.

The B lymphocyte is unique in the ability of cells of this class to undergo transformation following antigenic stimulus, and to show thereafter markedly different behaviour from similar B cells not/...

not exposed to antigen. In particular a substantial proportion develop into antibody synthesising plasma cells, and a smaller number persist as memory lymphocytes. During these phases of development changes in the surface phenotype of the cell occur. It has been shown that B lymphocytes acting as precursors of 7S and 19S antibody forming cells express the C3 receptor. Depleting populations of complement rosette forming cells following sensitisation subsequently prevented an adoptive immune response on transfer of complement receptor depleted cell suspensions (Mason, 1976a). The same author has also shown that, in rats, the IgG memory cells has two subclasses, one of which has the complement receptor, the other not. These did not appear to represent different stages in the maturation of memory cells. A small number of IgG secreting precursors were found to show surface IgM and would give rise to an adoptive IgG response when transfused with T lymphocytes. Complement receptor was found on memory B lymphocytes secreting either IgM or IgG antibody, but most complement receptor expressing cells were IgM positive (Mason, 1976b). Moller, (1975) showed that non-specific excitation of the B cell surface by occupation of the C3 receptor was sufficient to trigger transformation and antibody production. The antibody secreted, and its specificity was determined by the presence of antigen. In other words antigen selected the cell to respond, and the response was triggered by complement acting through the complement receptor. This "two signal hypothesis" was explored further by the demonstration that binding of antigen alone to the surface Ig receptors for antigen was not sufficient to trigger blast/...

blast transformation and antibody secretion, unless a second signal was delivered via the C3 receptor, or unless complexing and extensive cross linking of surface receptors by antigen was achieved (Moller, Coutinho and Persson, 1975). The conclusions reached also showed that there was no synergistic response to thymus independent antigens (LPS) and polyclonal B cell activators and that occupation of the complement receptor is not necessarily the only second signal. Antibody against surface Ig, and T cells can also act as triggers for B cell transformation and antibody synthesis in capping B lymphocytes. Stimulation by complement alone does not initiate antibody synthesis (Moller and Coutinho, 1975). What happens to B lymphocytes engaged by antigen and triggered by a second signal? As shown by Zauderer and Askonas (1976) antibody secreting cells will spontaneously develop, and from IgM bearing B lymphocytes will differentiate IgM, IgG and IgA secreting mature lymphocytes. Antibody forming B lymphocytes can themselves be "blocked", by the continued presence of immunogenic antigen (Schrader, 1975). This blockade can be reversed by the removal of antigen from the surface of the cell by washing or trypsinisation. Another form of blockade of antibody forming cells can be produced by a subclass of T lymphocytes - the suppressor T cell (Baker, 1975). The suppressor T cell acts principally by limiting the degree to which B lymphocytes proliferate in response to antigen. Following contact with antigen, especially one causing blast transformation, B lymphocytes are observed to develop into one of two forms. The majority are small lymphocytes, with high density capping surface Ig but without intracellular Ig. A minority are infrequent large cells with intracellular Ig and the ability/...

ability to secrete free Ig into the medium (Askonas et al., 1976). It has been demonstrated that the Fc receptor was present upon cells secreting Ig, but not upon non-secretory cells in the MPC 11 mouse myeloma (Corte et al., 1976). It is often assumed that the specificity of the antibody produced does not vary during growth and development of the B lymphocyte. There is no a priori reason why this need be so, and it has been shown that daughter clones isolated from single antibody forming cells can subsequently synthesise 2 or 3 different and distinct antibodies. The suggestion is made that antibody diversity, that is the range of different specificities of antibody produced in the whole animal may only develop after the stimulus which produces proliferation in the B lymphocytes (Cunningham and Fordham, 1974). The data presented here together with that in the introduction (Problems of B cell differentiation Chapter 10), allows a fairly full construction of the life history of the B lymphocyte including points at which T cell and macrophage co-operation, and at which blockade can occur. In addition it is possible to indicate the likely surface phenotype of the cells at any stage.

The separate forms of B lymphocyte developing from the stem cells are clearly:-

- 1) Non-capping, IgM bearing, Fc and C3 receptor negative, virgin B cells blocked by surface complexes of antigen and surface Ig, or anti u chain, anti-allotype or anti-idiotypic antisera. They rapidly **synthesise** monomeric surface IgM.
- 2) The memory B lymphocyte of the IgG response (IgG memory cell), which possesses the Fc receptor (Ramasaamy et al) and/...

and the C3 receptor.

- 3) The C3 receptor bearing, IgM bearing, cell which is not a precursor of the IgG secreting B lymphocyte. This cell is probably the one triggered by LPS and complement, giving a thymus independent IgM antibody response.
- 4) IgM bearing cells which differentiate into IgM, IgA, or IgG secreting lymphocytes.
- 5) The prosecretory committed lymphocytes which exhibit inhibition by free antigen, or are prevented from proliferation by suppressor T cells.
- 6) Small lymphocytes without intracellular Ig.
- 7) Larger lymphocytes with intracellular Ig.

In normal human blood, tonsil, and lymph node, B lymphocytes exhibited several receptor profiles which are held to correspond to the classes of cell listed above. The most common B cell was a small, capping, brightly fluorescent cell, expressing C3 and Fc receptors (B3-1 cell). In lymph nodes showing follicular hyperplasia, and in tonsil, two classes of cell were described; the non-capping B lymphocyte with C3 but no Fc receptor, and the capping B lymphocyte with C3 but no Fc receptor (B3.C, B3.2 cells). It is thought likely that the B2.C cell is a blast transformed B3.2 cell; neither show any trace of intracytoplasmic Ig. A capping lymphocyte expressing only the Fc receptor, with no C3 receptor or cytoplasmic Ig was the predominant cell type (B3.3) on some lymph nodes. Finally a capping cell, small, and found in constant association with plasma cells was also present in some samples (B4). Plasma cells were termed B5, and all showed intracytoplasmic Ig. The category of/...

of non-capping B lymphocyte with no Fc or C3 receptor has been referred to as analogous with the immature B lymphocyte of the mouse, and a class of similar cell bearing Fc receptors was found in both lymph node and in blood (B2.Fc).

The B1 lymphocyte, corresponding to the immature B cell must transform eventually into the antibody secreting plasma cell. The data from Sachs, Lieberman and Paul (1974) indicates clearly that the B1 lymphocyte in mice lacks the C3 receptor. The data of Metcalf, Warner, Nossal, Miller, Shortman and Rabellino (1975) show that the Fc receptor is acquired before the C3 receptor. Activation of Fc and C3 receptor bearing B cells causes blast transformation and loss of C3 receptor (Moller, 1974). Loss of Fc receptor probably occurs only when the cells bearing it secrete IgG onto their membrane (Ramasamy et al, 1974) or have that receptor occupied by extraneous IgG aggregates or immune complexes. Precursor cells of IgG secreting cells have IgM antibody and C3 receptors on their membranes. Cells present in the mouse spleen will differentiate into antibody secreting plasma cells over a period of 17 days (Zauderer and Askonas, 1976). It takes 4 days for the B1 lymphocyte to become refractory to inhibition by anti-allotype antisera (Lowe and Catty, 1976), and mature B cells require 3-5 days to become the major B cell population in the neonatal mouse (Rosenberg and Cunningham, 1975). Thus in a minimum period of 4-5 days, and possibly as long as 17 days the B1 lymphocyte should gain an Fc receptor (B2.Fc) gain C3 receptor (B3.1) undergo blast transformation losing C3 receptor (B2.Fc) differentiate into a memory cell population possessing Fc and C3 receptors (B3.1), into a secretory B/...

differentiation either into the B2.Fc cell or into the B4 cell and plasma cell. The B1 cell is derived either from the lymphoid stem cell, or from blast transformed B3.2 cells which have lost C3 receptor, as may occur if transformed via the Fc rather than the C3 receptor.

The B2.Fc lymphoma is either derived from the B2 cell (by maturation) or is derived from the B3.1 cell by transformation and loss of C3 receptor in the presence of complement. The B3.1 cell is the most common class of B lymphocyte. It is either an IgG memory cell derived from follicular B3.2 cells, or is a non-transformed committed B cell destined to secrete IgM resulting from antigen drive acting on the B1 cell.

The B3.3, B4 and B5 lymphomas are the result of maturation arrest after secondary antigen stimulation in the sequence leading up to the IgG or IgM antibody secreting plasma cell.

The B3.2 cell is strongly associated with the follicle in tonsil and hyperplastic nodes, and must derive from an IgM bearing, committed B3.1 cell, the precursor of the IgG secreting plasma cell. It may have a transformed equivalent - the B2.C cell.

The possible sites and mechanisms of maturation block or arrest in lymphoma

Lymphomas of B1 cells are equivalent to leukaemias of these cells, and both may arise from an intrinsic defect of the immature B lymphocyte resulting in failure to undergo maturation. They could also arise as a consequence of blockade by antigen, or by surface bound immune complex, and in the case of Hogg this clearly occurred/...

occurred. It is reasonable to predict that a proportion of B1 lymphomas will be due to transformation of B3.2 cells, with subsequent blockade. These lymphomas are not due to an intrinsic defect in the B1 lymphocyte. Blockade will fix immature B lymphocytes in the transformed state. Possible mechanisms are blockade by antigen antibody complexes on the cell surface. This hypothesis is eminently testable, as unblocking will result in reversion to a B4 or B5 cell (as in Hcgg) or to a B2.Fc or B3.1 cell (as in Velzian or Reid) depending on the derivation of the B1 cell in the first instance. Needless to say, the data presented indicate that conversion of a B1 lymphoma to a B2.Fc, B3.1, B4 or B5 tumour by therapeutic measures should result in a vast improvement in prognosis (e.g. patients Velzian and Reid).

The B2.Fc cell can arise from two separate segments of the maturation sequence: (1) it can derive by maturation from the B1 cell under the influence of antigen; (2) it may be a transformed B3.1 memory cell arrested in the transformed state; the subsequent cell may be B3.1 or B3.3.

The sequence with points at which blockade has occurred are shown below:

- 1) B1 - B2.Fc Block - B3.1
- 2) B3.1 - B2.Fc Block - B3.1
- 3) B1 - B2.Fc Block - B3.3

In this sequence, category 1 tumours should occur during a primary response - or first exposure of the neoplastic B cell clone to antigen. Category II tumours could occur in B3.1 memory cells transformed by antigen, particularly if antigen excess or immune complexes/...

complexes are present. Category II tumours could also occur during a normal primary immune response due to extrinsic blockade. The ability to test experimentally for these tumour categories is not currently available, although B2.Fc tumours due to maturation block should differentiate into B3.1, or B3.3 cells if the blocking agent could be removed. Agents causing B cell transformation (endotoxin Pokeweed mitogen or other polyclonal B cell activators) may be aetiologically related to the development of B2.Fc lymphomas.

The B3.1 lymphoma is quite common, and may be B predominant or mixed. B3.1 lymphocytes are unstimulated memory cells, derived from the transformed B2.Fc cell. They can arise directly from the antigen induced maturation sequence B1 - B2.Fc - B3.1 under genetic control (Gelfand et al., 1974). Failure of the B1-B2.Fc - B3.1 lymphocyte maturation sequence to progress beyond this point will result in accumulation of B3.1 lymphocytes. The B3.1 cell can also be derived from the transformed B2.Fc cell. This is a cyclical process where blast transformation of the B3.1 cell, with loss of C3 receptor produces the B2.Fc cell which matures again into the B3.1 cell. This would result in the expectation of cells showing B3.1 and B2.Fc profiles in B3.1 lymphomas where antigenic stimulation is still present (e.g. the nodular tumour of McKenzie), the role of antigen being to perpetuate the transformed state.

The B3.1 cell might also mature via the B2.Fc cell into the B3.3 cell, identified by Mason (1976) as the small IgM memory cell lacking C3 receptors. These various maturation sequences of the B3.1 cell indicate that the opportunities for transformation into more primitive forms (B2.Fc) are slight, whereas differentiation towards/...

towards the mature (B3.3 - B4 - B5) cells would be the natural tendency of B3.1 neoplasms. It is not surprising then that in this series the B3.1 neoplasms carried a good prognosis. The sequence B2.Fc - B3.1 - B3.3 is probably dependent upon co-operation between B cells and macrophages, in that differentiation of memory cells into antibody forming cell precursors depends upon appropriate macrophage presentation of antigen.

The B3.2 lymphomas, and the contentious blast transformed equivalent, the B2.C cell, have been referred to as the follicular sequence. Understanding of the sequence is limited by failure to comprehend the role of the germinal follicle in the immune response. Follicular lymphocytes can give rise to plasma cells, but the most reasonable explanation is that the follicle acts as a region for lymphoblast transformation of committed B3.1 cells (to B2.C cells) expressing a high affinity for antigen localised to the dendritic macrophage. T cell co-operation occurs possibly within the follicle - and T cell suppression, limiting generation of low affinity cells - could also occur in this site. Since one of the committed memory cells is of B3.1 receptor profile, the probable sequence is B3.1 - B3.2, followed by blast transformation of the B3.2 cell to either the B2.C cell, or to the B1 cell from which the whole sequence of B lymphocyte generation can be restarted. In this case transformation is via the Fc receptor rather than reception of a second signal via the C3 receptor.

B3.1 - B3.2 \rightarrow B2.C - B4 - B5
 \searrow B1 - B2.Fc - B3.1 - IgG memory cell
 \searrow B4 - B5 - IgG secreting plasma cell

The only two patients with tumours of B3.2 profile have died, with peculiar/...

peculiar receptor profiles and leukaemic transformation. One was diagnosed as nodular lymphoma and progressed rapidly to diffuse lymphoma with leukaemia. This aggressive behaviour is comprehensible if transformation of B3.2 to B1 cells could be demonstrated experimentally. It is doubtful, if B2.C lymphomas occur, that any better prognosis would result from the sequence B3.1 - B3.2 - B2.C. The only pathways for the differentiation of the B2.C cell lie in the sequence B2.C - B3.2 - B4 - B5 with the risk of transformation into a B1 neoplasm. It is of interest to note that most nodular lymphomas have a B3.1 or B4 profile, and do not share the characteristics of the true follicular neoplasm - B3.2 lymphoma.

The B4 - B5 sequence can occur in theory from the primary response resulting in IgM secreting B5 cells, or the secondary response resulting in IgG secreting B5 cells. The first sequence is probably most common in non-Hodgkin lymphoma, and does not involve derivation of the B4 cell from the suspect B3.2 precursor. The sequence is B3.1 - B3.3 - B4 - B5, with loss of complement receptor possibly by an intermediate B2.Fc transformation induced by antigen (B3.1 - B2.Fc - B3.3 - B4 - B5). In a progressive primary response rapid evolution of B4 and B5 cells could occur by the sequence B1 - B2.Fc - B3.3 - B4 - B5 without involving the B3.1 cell. Diminished availability of antigen in the later stages of this sequence would allow B3.1 cells to accumulate.

There is a further pathway by which B4 and B5 cells of the secondary response type (IgG secreting) must be generated, and this involves the generation of the B1 cell via blast transformation of the/...

the B3.2 cell with loss of C3 receptor. This sequence may be implicated in Burkitt's lymphoma since the Burkitt's derived cell lines all showed either B1, or B4 receptor profiles.

B3.2 - Loss of C3 receptor, transformation - B1 - B4 - B5
This sequence clinically should arise in chronic, long standing immune responses and should be preceded by follicular hyperplasia.

In none of the lymphomas examined was there clear evidence of the sequence B3.2 - B1 - B4 - B5, and none of the non-Hodgkin lymphomas showed intracellular IgG as might be expected in mature secondary response cells. There was, however, clear evidence of an aberrant follicular response in Hodgkin's disease, with B2.C cells present in some samples and with many aberrant IgG containing plasma cells detected in Hodgkin tissue.

This exercise shows how it is possible to relate the known facts about B lymphocyte development in the immune response, to human lymphoma. By assuming a simple system of maturation blocks, it is possible to predict the origins and likely outcome of any lymphoma profile which is of B cell predominant, or of mixed cell type. It is also possible that in the future manipulation of the immune response may give rise to rational lymphoma therapy based on the identification, staging and sequencing of B lymphocytic lymphoma. The following table summarises the relationship of B cell sub-types in non-Hodgkin lymphoma

Relationship/...

Relationship of B cell subtypes in lymphoma

<u>Precursor subclass</u>	<u>Subclass of tumour cell</u>	<u>Next cell in maturation sequence</u>
Stem cell	B1	B2.Fc
B3.2	B1	B4
B1	B2.Fc	B3.1
B1	B2.Fc	B3.3
(This represents an active primary immune response with antigen present)		
B3.1	B2.Fc	B3.1
(This represents blast transformation of B3.1 cells in B3.1 lymphoma)		
B2.Fc	B3.1	B3.3
B2.Fc	B3.1	B2.Fc
(This represents a quiescent phase between two transformation events)		
B2.Fc	B3.1	B3.2
(The possibility of developing into a B3.2 tumour is present)		
B3.1	B3.2	B2.C
B3.1	B3.2	B1
(In B3.2 tumours transformation into a form with worse prognosis is likely)		
B3.1	B3.3	B4
B1	B4	B5
(?) Burkitts' tumour)		
B3.3	B4	B5
B3.2/B2.C	B4	B5
(This may be the sequence in Hodgkin's disease)		
B4	B5	-
(multiple myeloma or Waldenstrom's disease)		

Differentiation of T lymphocytes and its relationship to tumours of this cell class

The T lymphocyte develops from a stem cell precursor in the bone marrow, and acquires the characteristic T cell surface antigen (thy 1) in the mouse before entering the thymus to proliferate. Such cells have a low density of thy 1 antigen, and do not recirculate in thoracic duct lymph. Their life span is 1-2 days, and they rapidly accumulate following surgical removal of the thymus. Implantation of a thymus graft produces a fall in bone marrow release of these cells and control over bone marrow production is probably mediated via a humoral factor. They also express the TL antigen normally present only on thymocytes in TL + mice (Roelants, et al., 1976).

The thymocyte itself shows characteristics in which it differs from the "peripheralised" T lymphocyte. Thymocytes can bind IgM antibody (Webb and Cooper, 1973). They express the TL antigen strongly whereas peripheral B lymphocytes do not, and this antigen is also found on leukaemic cells in TL + mouse strains. However, peripheral T lymphocytes can also carry the TL antigen, although it is masked and not detectable by conventional techniques (Komuro, Boyse and Old, 1973). Thymocytes express little H2 antigen in mice, and do not recirculate, most dying in situ shortly after formation (Stobo, Rosenthal and Paul, 1973). The thymic epithelium acts as an "inducer" of thy 1 and TL antigens on thymocytes in the mouse (Mosier and Pierce, 1972).

The peripheral lymphoid tissues in mice contain T cells which are distinguished by their expression of the Ly antigens. Immature T/...

T cells are Ly 123 positive, as found in neonatal mice. In adults Ly 2 and Ly 3 positive, and Ly 1 positive T cells occur, and these show different functional characteristics. The cell with Ly2/Ly 3 surface phenotype is the cytotoxic T lymphocyte, the cell with Ly 1 surface phenotype shows helper cell activity (Cantor and Boyse, 1975a,b). These phenotypes are stable, even in leukaemia the neoplastic cells preserve their Ly surface phenotype (Takahashi, 1972). There have been several reports that "activated" (that is mature reactive T lymphocytes) express receptors for the Fc portion of IgG (Yoshida and Andersson, 1972). In the mouse 20% of thymus cells expressed Fc receptor (Andersson and Grey, 1974), and Fc receptors have been found on human T cells (Ferrarini et al., 1975). IgM receptors were described on human T cells (thymocytes especially) (Moriotta et al., 1975) and in the mouse on cytotoxic T lymphocytes (Lamon et al., 1975). T lymphocytes are therefore of several different classes, pre-thymic, thymic, helper T cell, cytotoxic T cell, and transformed (activated) T cell.

The receptor techniques used can detect E rosetting cells, and show E rosetting cells expressing IgM and Fc receptors. The subclasses of T cell detectable by current technology in man, are therefore the E rosetting cell (including thymocyte and peripheral T cells of both helper cell and cytotoxic cell class) the E rosetting cell with Fc receptors (activated T cell, or T killer cell) and the E rosetting cell with IgM receptors (thymocyte and cytotoxic T cell). The only two neoplasms in which T cells were the predominant cell included a solid tumour in the neck (Wilson) in a young female with a raised toxoplasma titre, and the acute lymphoblastic leukaemia (Cooke)/...

(Cooke) in a boy with mediastinal enlargement on X-Ray (Sternberg sarcoma). These two tumours behaved very differently despite their common receptor features. The tumour of Wilson was removed, and has not recurred and she remains well. The leukaemia of Cooke proved rapidly fatal. Undoubtedly these were neoplasms of differing malignant potential. The lymphoma of Wilson arose in a local cervical node, with no blood or bone marrow disease detectable, and no other lymphadenopathy. The association with toxoplasmosis is problematical, but the response to intracellular parasites is T cell mediated, and toxoplasma may have either precipitated the presentation of this lymphoma, or may have occurred because of defective T cell mediated immunity. The case of Cooke is suggestive of a tumour of immature thymic T lymphocytes, or perhaps even of a pre-thymic lesion since bone marrow was also involved. The cell showed acid phosphatase activity, and this may correlate with primitive thymic or pre-thymic T cell tumours.

Although only two thymus derived lymphoid neoplasms occurred in this series, the estimation of T cells in each case turned up some interesting features.

1) High levels of T cells were found in the tumours of Bathgate, Swanson and McGrail. All were diffuse well differentiated lymphomas of B3 cells. In the nodular group Smith E., and Thompson showed high T cell levels with B3 profiles. In Thompson there was a slight predominance of T lymphocytes, but for practical purposes this tumour was regarded as being of mixed receptor profile.

2) In the case of Robertson J., T cells bearing Fc receptors were found in substantial numbers in the blood. The lymph node also/...

also showed the presence of Fc receptor bearing T cells, and allowing for their presence the receptor profile in the node was B2.C. This is clear evidence for the association of activated T lymphocytes with a B lymphoid population of follicular profile. In the other B3.2 follicular tumour (Robertson, A) T cell levels were high, but mixed E and Fc rosettes were not evaluated. There is a suggestion again that follicular B cell neoplasms (B3.2 or B2.C) show features unlike those of other tumours of similar histological type.

3) Receptor profiles in Hodgkin's disease also showed T cell abnormalities. There was a marked T lymphocyte predominance in involved tissues, and T cells of abnormal morphology, and expressing IgM receptors were detected in some cases. The B cell receptor profile was frequently follicular, with numerous B3.2 or B2.C cells present. The points of interest can be summarised in the table below.

Association of T cell subclasses and B lymphocyte subclasses in lymphoma.

<u>T subclasse</u>	<u>B subclass</u>	<u>Tumour Histology</u>
Peripheral T cell (E rosetting)	B3.1 or B3.3	NPDL/DWDL
Immature T cell (E + IgM rosetting)	B3.2, B2.C	Hodgkin's disease
Activated T cell (E + Fc rosetting)	B3.2/B2.C	NPDL/DPDL

The interactions of T cells with B lymphocytes is known to/...

to occur at certain stages of the immune response. T helper cells induce proliferation, with antibody secretion by committed secondary response IgG secreting B lymphocytes. T suppressor cells act by suppressing proliferation of the committed precursors of antibody forming cells. The B lymphocyte of the secondary response can exhibit the follicular profile (B3.2) and T cells are found in substantial numbers with B3.2 cells in the germinal follicles of tonsil. In subsequent differentiation (B4 - B5) T cells play no part. The tumours of B3.2 profile are in this series either associated with activated T cells, or with immature T cells as in Hodgkin's disease. These findings strongly imply that both Hodgkin's disease, and tumours of follicular profile occur at a level of B cell differentiation corresponding to the secondary immune response.

In tumours not of follicular profile, the association of T cells with B cells of B3.1 or B3.3 subclasses is very similar to that of normal lymph node. If it can be assumed that the normal node contains committed lymphocytes (B3.1) ready to respond by follicular transformation (B3.2 cells) to stimulus by antigen, T cells may be required to present antigen to B3.1 cells either directly or via the dendritic (? reticular) macrophage. The B3.3 cell profile may indicate cells arising from the alternative sequence B1 - B2.Fc - B3.3 and these may be precursors of IgM antibody producing cells in the primary response to antigen. Injection of an antigen experimentally is known to result in a non-specific sequestration of T cells into spleen and lymph nodes (Black, 1975) and this contrasts with specific sequestration of antigen primed B lymphocytes to the spleen in adoptive immune responses/....

responses (Sprent and Lefkowitz, 1976). High T cell levels might therefore be expected in any node undergoing primary stimulation, or in the resting phase after a primary response. T cell infiltration is only of pathogenetic significance where the B cell profile is follicular, as this indicates an active secondary immune response and implies the presence in the affected node of the initiating antigen. In the active phase of T cell/B cell interaction, T cells might be expected to express either Fc or IgM receptors.

The sequences of B lymphocyte development as might be encountered in the normal immune response are shown in the accompanying table. It is probable that receptor profiles in lymphoma identify cells arrested in one part of this maturation sequence:- e.g. the multiple myeloma might be represented by the top line of sequence (3). The Key summarises the events precipitating, and phenotypic changes associated with, the shift of each cell in the sequence and these are lettered above the arrows representing the sequence changes. The important conclusion is that probably only 5 sequences of B cell maturation exist, and therefore B cell tumours are tumours occurring during one of these 5 maturation sequences. In practical terms, this reduces the number of phenotypic subclasses of B cell lymphoma from 12 to 5 functional subclasses of B cell tumour.

Maturation sequences in normal immune responses by B lymphocytes
as characterised by surface phenotype changes

1) Normal Maturation sequence to primary response (IgM)

Stem cell \xrightarrow{A} B.1 \xrightarrow{B} B2.Fc \xrightarrow{C} B3.1 \xrightarrow{D} B2.Fc \xrightarrow{E} B3.3 \xrightarrow{F} B4 \xrightarrow{F} B5

2) Generation of primary IgM response from primed B lymphocyte challenged by antigen.

B3.1 \xrightarrow{D} B2.Fc \xrightarrow{C} B3.1
 \searrow
 E B3.3 \xrightarrow{F} B4 \xrightarrow{F} B5.

3) Maturation sequence of IgG memory cell to secondary response plasma cell, and regeneration of IgG memory cell in the follicular cycle.

(see 4) B3.2 \xrightarrow{D} B1 \xrightarrow{C} B3.2 \xrightarrow{D} B1 \xrightarrow{B} B1 \xrightarrow{C} B4 \xrightarrow{F} B5
 \searrow
 B B2.Fc \xrightarrow{C} B3.1 - Follicular cycle

4) Generation of IgG memory cells (B3.2) in the follicular cycle

B3.1 \xrightarrow{G} B3.1 \xrightarrow{H} B2.C \xrightarrow{C} B3.2

5) Production of plasma cells during follicular involution

B3.1 \xrightarrow{G} B3.1 \xrightarrow{H} B2.C \xrightarrow{C} B3.2 \xrightarrow{F} B4 \xrightarrow{F} B5.

KEY: Step A = Normal differentiation

Step B = Antigen drive with division and daughter cell transformation

Step C = Normal maturation of blast cell to small lymphocyte

Step D = Transformation by antigen and complement loss of C3 receptor.

Step E = Division and maturation of blast cell to produce memory cell

Step F/...

- Step F = Clonal expansion and maturation to antibody secreting cell (division without blast cell transformation)
- Step G = IgM to IgG switch
- Step H = Transformation by antigen alone in absence of complement (T cell/macrophage induced) loss of Fc receptor.

Receptor Overlap Tumours

Cells bearing both B and T cell markers, including Fc and C3 as well as E rosetting capacities were found in patients Baird and Laverick. The T cell bearing Fc receptor alone is not unusual, according to the literature already reviewed. T cells bearing complement receptors are rare, but were detected in Hodgkin's disease as cells exhibiting an affinity for bound IgM used in preparation of the complement sensitised cell. The cell expressing receptors for E, Fc and IgM could therefore account for tumours exhibiting overlap characteristics. The nature of this cell is unknown, but it could for instance be an activated T lymphocyte, of immature or recent thymic derivation. In the tumour of McKenzie a second type of overlap was observed, where E rosetting cells exhibited surface Ig. Two explanations of this phenomenon are known. Firstly antibody binding to the T cell may be formed by B cells in the lesion. In the mouse spleen a subpopulation of thy 1 positive and surface Ig positive cells are detected, and T cell cytophilic antibody may play a role in T-B cell co-operation (Playfair, 1974). The second possibility is that B lymphocytes were manufacturing antibody with anti-sheep red cell specificity, giving rise to immune E rosettes (Steel, et al., 1974).

Lin and Hsu (1976) demonstrated dual E and complement receptors on peripheral blood leukocytes from a patient with lymphosarcoma and leukaemia. The same cells expressed IgM and IgD immunoglobulins on the cell membrane. The receptor was not directed against IgM since the assay involved the use of zymosan coated with human complement directly. IgM receptor bearing T/...

T lymphocytes form a subpopulation of T cells which fail to exhibit C3 receptor when tested with zymosan coated with complement. Mitogen activated T lymphocytes, which express Fc receptors failed to express receptors for IgM (McConnell and Hurd, 1976).

In summary receptor overlap tumours can be shown to be of the following types

- a) T cell expressing IgM receptors (E, IgM, spurious C3 rosette).
- b) T cell expressing Fc and C3 receptors (E, Fc, C3, No IgM receptor).
- c) T cells expressing surface Ig and C3 receptors (E, surface Ig, C3)

The presence of an Fc receptor on T cell tumours indicates "activation" and is a normal T cell characteristic. The presence of IgM receptor indicates a T cell subclass, which does not correspond to the activated T cell, and is probably immature.

On the basis of the two cases reported here, it is clear that receptor overlap tumours show histiocytic, or mixed lymphocytic and histiocytic histological pattern. They may be leukaemic (Lin and Hsu, 1976). The prognosis in the two cases in this series has been notably good, despite the primitive appearance of the cells in diagnostic biopsy. Most other cases of receptor overlap tumours reported have been in poorly differentiated lymphocytic or histiocytic tumours.

Summary of proposed classification scheme based on surface phenotype in non-Hodgkin lymphoma. The present histological equivalents are shown in brackets.

1) Receptor silent tumours

- a) Lymphoid stem cell (ALL)
- b) Lymphocyte - poorly differentiation ("null cell tumours) (DPDL)
- c) Mononuclear phagocyte stem cell (ALL)
- d) Reticular cell tumours (HL)
- e) Reticulin fibre forming cell tumours (Fibrogenic) (HL)
- f) Undifferentiated epithelial tumours (undifferentiated carcinoma, included only in view of the difficulty in diagnosis from true lymphoma) (U)

2) Tumours of Macrophages

- a) Promonocytic and monocytic leukaemia (SAME)
- b) True histiocytic lymphoma (macrophage tumour) (HL)

3) Tumours of B lymphocytes

- a) B1 tumours (HL, DLL (PD), CL, ALL)
- b) B2 tumours (NPDL, HL, DWDL, DPDL, CLL)
- c) B3.1 tumours (CLL, NPDL, DWDL, DPDL)
- d) B3.2 tumours (true follicular tumours) (NPDL, HL, DPDL)
- e) B3.3 tumours (NPDL, DWDL)
- f) B4 tumours (NPDL, DWDL)
- g) B5 tumours Multiple myeloma, plasmacytoma
Waldenstrom's disease

4)/...

4) Tumours of T lymphocytes

- | | |
|---------------------|--------|
| a) Prethymic/thymic | (ALI) |
| b) T predominant | (DPDL) |

5) Receptor Overlap Tumours

- | | |
|--|-----------------------------------|
| a) T cell with IgM receptor | (? Hodgkin's disease) |
| b) T cell with Fc and C3 receptors | (DM (L + H), HL) |
| c) T cell with surface Ig and C3 receptors | (Lymphosarcoma cell
leukaemia) |

6) Leukaemic Reticulo-endotheliosis (LRE)

- | |
|--|
| a) Polyclonal surface Ig with IgM receptor |
|--|

Note: that B2 and B3 tumours may be either predominant or mixed.

Hodgkin's disease is omitted, but shows both B and T cell abnormalities and must be classified separately.

The practical implications of this work

Receptor studies are sophisticated and expensive of time, money and materials. Whether such work will continue in the future obviously depends upon the value of such studies today. The exercise should be shown to be relevant to medical practice, and not merely of academic interest.

The surgeon or physician treating a lymphoma has a right to the best advice the pathologist can offer. Because of the unpredictable clinical behaviour of this disease, most classification schemes emphasise prediction of prognosis, and response to therapy as their goal. The study of surface phenotype is of better predictive value than current histopathology. It clearly demonstrates the differing prognosis of non-capping and capping B lymphocyte tumours, and even in a small series studied over a short time, highly significant ($p < 0.02$) differences in survival characteristics in these two subclasses were shown. The ominous prognosis in receptor silent, and B1 lymphoid neoplasms has been justified in nearly every case. The solid lymphomas showing T cell predominance and receptor overlap have responded better than current histopathological experience could predict. Finally the local lymphoma group in practice find value in the surface phenotype studies; for the first time physician, surgeon and radiotherapist know the class of cell they are dealing with, and can plan treatment accordingly. If macrophage tumours prove resistant to radiotherapy and susceptible to chemotherapy, valuable clinical time is not lost in irradiating an unresponsive tumour - as would probably happen if the lesion was diagnosed simply as Histiocytic/...

Histiocytic lymphoma. On the other hand, if the clinician knows that the tumour is of mononuclear phagocytes, and treats the patient as for monocytic leukaemia considerable benefit to the patient might result. The two patients with receptor overlap tumours responded well to the treatment they received, in other cases of receptor overlap tumour the same treatment can be recommended as and when they occur. In B1 lymphomas, and receptor silent tumours none of the present regimes offers a satisfactory chance of cure. New thinking about the therapeutic challenge of these lesions is long overdue. In lymphomas showing a follicular profile (B3.2) it would now be considered essential to monitor the patient closely, with repeat biopsy if necessary to anticipate conversion to a B2.C or B1 lymphoma even if the histology is that of a nodular tumour. As these examples show the subclassification of lymphoma by surface phenotype must now be regarded as an acceptable and desirable clinical investigation.

Medicine is about the diagnosis and treatment of patients with disease. Foremost in the minds of many clinicians is the question of how to treat lymphoma in the best possible way; which treatment offers the greatest chance of survival? The problem is rendered more acute in lymphoma by the perplexing variety of response of such tumours to treatment, the large number of treatments available, and the occurrence of many cases of lymphoma in children and young adults. This natural concern has resulted in an extraordinary number of therapeutic trials of chemotherapy in lymphoma. Most trials compare treatment A with treatment B in lymphoma of the same histological type. A distressing feature of/...

of such trials is the regularity with which the pathological appearances are accepted as sacrosanct, while the response of individual patients is ignored as being "atypical".

As an example consider that in part of a recent, as yet unpublished, trial a comparison is currently being made of two cytotoxic drug regimes in histiocytic lymphoma. The patients' lesions have all been diagnosed by a panel of pathologists as this entity. Although marginal differences in response of all patients with H.L. between the two randomised treatment groups are observed some individual patients do appreciably better on one drug than the other.

In this thesis, as has been shown, disease diagnosed as histiocytic lymphoma contains receptor silent, receptor overlap, B1, B3, macrophage and B2.Fc lymphomas. In other words some of these tumours are not even tumours of comparable cells, yet all are subjected to the same drug treatments, with the predictable outcome that some patients improve and some do not. If accurate trials of chemotherapy are to be performed knowing at least the class of tumour cell in the lymph node is mandatory. No one would think of treating warts and carcinoma of the lung in the same way, yet it is possible that some B3.1 lymphomas are as "benign" as the former, and receptor silent tumours are as malignant as the latter. Both may be treated in the same way because their histological appearances indicate that they are the same lesion. Obviously future clinical trials of chemotherapy in lymphoma must include at the very least a determination of tumour cell class by phenotypic studies before any valid comparison can be made between two forms of chemotherapy. An emphasis must also be placed upon the response of the individual patient; those doing well on a particular drug must,..

must be assessed to discover why. Perhaps they have a responsive tumour (e.g. Laverick) or they may have a B3.1, B3.3 or B4 lesion, in which case the response is governed by the tumour cell subclass and may be unrelated to the type of treatment received.

The future identification subclassification and sequencing of lymphoma by phenotypic studies

There is a limited area in which phenotypic studies, in the future, can contribute substantially. The techniques developed for the identification and subclassification of lymphoid cells in the normal immune response could be the forerunner of "sequencing" techniques applicable to B lymphocytic lymphomas. The material so far presented in this thesis is concerned with (a) identification of cell class and (b) subclassification of B lymphocyte tumours. Sequencing is a natural extension of subclassification techniques to identify the precursor cell of the lymphoma, and its successor in the differentiation sequence. Once the sequence of surface phenotype expression is established it can be compared with the differentiation sequence of normal B lymphocytes and the pathological lesion identified in terms of function as well as surface phenotype.

Sequencing methods

One technique has already been described; that of unblocking "blocked" B lymphocyte tumours by proteolytic digestion of the cell surface. This produced changes from receptor silent - B1 - B3 cells (Velzian), B1 - B5 cells (Hogg) and B1 - B2.Fc cells (Reid). These small fragments of differentiation sequence could correspond to normal maturation sequence of cells (a) from stem cell to blood B lymphocyte (Velzian: Stem cell - B1 - B2.Fc - B3.1), (b) in Hogg the generation of secondary response plasma cells from IgG memory cells (B3.2 - B1 - B1 - B4 - B5) part of the follicular cycle, and /...

and (c) in Reid (B1 - B2.Fc) a restricted part of the normal maturation sequence.

Identification of precursor cells cannot yet be achieved, although it is to be hoped that antisera might establish in which part of the differentiation pattern the sequence lay. The presence of secretory lymphocytes containing IgM or IgG antibody clearly indicates late primary (B3.3 - B4 - B5) or secondary response (B3.2 - B4 - B5) sequences. The association of non-capping cells of B2.Fc or B2.C pattern theoretically corresponds to a blast transformation event in either primary or secondary response. Identification of the next cell in the sequence allows definitive localisation of the event in the pathway of B lymphocyte differentiation. Much work has yet to be done. Firstly the establishment of the normal sequence of B lymphocyte development must be achieved. Secondly methods must be developed for inducing the tumour cell to differentiate into its end cell, or into the subsequent phenotype. Thirdly having achieved this the site of maturation arrest must be identified. Finally the event leading to maturation arrest must be investigated.

In this thesis I have shown that lymphomas can be classified according to their phenotypic features by the means of simple functional tests. The identification of subclasses of B lymphocytes has been demonstrated. I have attempted to illustrate the value of regarding the lymphoma as an abnormal immune response with reference to actual cases, and have shown the relationship between subclasses of B lymphocytes and prognosis. I have developed a theoretical scheme based on current knowledge which shows the likely sequence of/...

of surface phenotype changes in the various maturation pathways of the normal B lymphocyte. The potential for sequencing of lymphomas based on this scheme is shown. It is to be hoped that further development of phenotypic analysis will lead to a better understanding of the nature of malignant lymphoma and to significant improvements in diagnosis and treatment.

END

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It is valuable for any research worker to be associated with a group of individuals sharing a common interest. In this regard the physicians, surgeons, haematologists and radiotherapists of the Edinburgh Lymphoma Group provided both the material on which this work was carried out, and the necessary stimulus needed to ensure progress. The founding of the Lymphoma Group and its organisation was largely the achievement of one man, Dr. A.E. Stuart, who was also appointed supervisor of this thesis. It would not be possible to itemise all the points at which I have received help and support from Angus Stuart. He was the first to suggest that I let Nature do the experiments and use her results. He provided material support for this work, and was always ready to suggest sensible and worthwhile areas of activity. We collaborated closely over much of the work, specifically that on Hodgkin's disease, and wrote several papers together. It is difficult to imagine what might have been but the existence of this thesis is in major part due to the valued support of Dr. Angus Stuart and I am pleased to record this tribute to him.

It is sad to relate that my thanks to one individual should be recorded after his tragic death at the age of 47. John Mackay, Senior lecturer in the department of Bacteriology and I used to meet nearly every week to discuss common interests in immunology and virology relating to lymphoma, and to his main interest, rheumatoid arthritis./...

arthritis. Over a number of years his criticism and advice proved valuable to me on many occasions and I valued him as a close friend. His loss marks the end of an era, which I should like to close by dedicating this thesis to his memory.

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Cell receptor studies on seven cases of diffuse histiocytic malignant lymphoma (reticulum cell sarcoma)

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Cell receptor studies on seven cases of diffuse histiocytic malignant lymphoma (reticulum cell sarcoma)

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SYNOPSIS Expression of B and T lymphocyte receptors has been studied in seven cases of reticulum cell sarcoma. In one case, surface receptors and tests of phagocytic function demonstrated the histiocytic origin of the neoplastic cells. In four cases, tumour cells expressed both B and T lymphocyte markers (two cases) or showed a normal pattern of expression of B and T lymphocyte markers. In the other two cases, lymphocyte receptors were not detected, and there was no evidence of phagocytic function: this class of receptor-silent tumours is of uncertain pathogenesis. The significance of these observations is discussed.

In man the different classes of lymphocyte are morphologically indistinguishable by light microscopy, but can be detected by tests which show differences in the reactivity of the cell surface. B lymphocytes carry stainable surface immunoglobulin (Fröland, Natvig, and Berdal, 1971) and express receptors for complement and the Fc portion of the immunoglobulin molecule (Shevach, Jaffe, and Green, 1973). T lymphocytes lack stainable surface immunoglobulin but show the ability to bind washed sheep red cells (Fröland, 1972) under experimental conditions. There is also evidence to show that T lymphocytes carry surface antigens which are not present on B lymphocytes but which they share with brain (Brown and Greaves, 1974). By exploiting these differences in surface reactivity, or antigenicity, B and T lymphocytes can readily be identified and quantitated in human peripheral blood (Brown and Greaves, 1974; Habeshaw and Young, 1975).

There have recently been attempts to identify neoplastic cells of B and T derivation in neoplasms of the lymphoid and haematopoietic systems (Smith, Barker, Clein, and Collins, 1973; Shevach *et al* 1973; Edelson, Kirkpatrick, Shevach, Schein, Smith, Green, and Lutzner, 1974; Jaffe, Shevach, Frank, Berard, and Green, 1974). In chronic lymphocytic leukaemias most of the examples so far studied have shown the neoplastic cells to be B cell

in type (Pincus, Bianco, and Nussenzweig, 1972; Piessens, Schur, Moloney, and Churchill, 1973; Mellstedt and Petterson, 1974). These cells express generally a single heavy chain component indicating a clonal origin (Silberman and Schrek, 1974). In some cases the neoplastic cells lack receptors for complement (EAC) (Shevach *et al*, 1973) or carry HTLA (human thymic lymphocyte antigen), a T cell marker (Edelson *et al*, 1974). In other leukaemias, a prolymphocytic leukaemia (Dewar, Habeshaw, Young, Stuart, Parker, and Wilson, 1974) and a variant of chronic lymphocytic leukaemia (Shevach *et al*, 1973) cells bearing both B and T lymphocyte markers have been described.

In solid tumours of lymph node, lymphocyte receptors of both B and T type have been described. In a lymph node secondary associated with thymic lymphoid tumour the cells were of T lymphocyte derivation (Smith *et al*, 1973). In nodular lymphocytic lymphoma, the tumour cells were of B derivation, corresponding to the follicular cells of the normal germinal centre (Jaffe *et al*, 1974).

Cellular infiltrates in skin associated with mycosis fungoides, Sezary cell leukaemia, and lymphosarcoma have also been studied (Edelson *et al*, 1974). In all cases the infiltrating cells show the characteristics of T lymphocytes.

Macrophages, which may be confused with lymphoid cells, can also be identified in cellular infiltrates, for example, in grafts during rejection and in malignant leukaemic reticuloendotheliosis. This

technique makes use of the IgG-sensitized red cells which adhere to macrophages in frozen sections of the appropriate tissue (Shevach *et al.*, 1973). This technique can be used to identify complement (EAC) or immunoglobulin (EA) receptors in tissue, but it is not suitable for the localization of the sheep receptor (E) expressed by T cells (Shevach *et al.*, 1973; Jaffe *et al.*, 1974).

There is no firm conviction as to the derivation of the less well differentiated lymphoreticular neoplasms classified as reticulum cell sarcoma. Gall (1958) proposed that within this group of tumours was a group characterized by the resemblance of tumour cells to histiocytes (macrophages), an entity which he termed 'histiocytic malignant lymphoma' (see review by Stuart, 1974). Lukes (1968) suggested that the neoplastic cells were of lymphoid origin from their morphological resemblance to follicular centre cells. If either of these opinions is correct, a study of the surface receptors of tumour cells from reticulum cell sarcoma might show conclusively their origin either as T or B lymphoid, or as histiocytic, thus allowing conclusions to be drawn as to the pathogenesis of individual tumours.

In this study an attempt has been made to identify the neoplastic cell population of reticulum cell sarcoma as being either of T or B lymphoid origin as proposed by Lukes, or as being truly histiocytic, i.e. macrophage-like, as proposed by Gall (1958).

Materials and Methods

CONTROL LYMPH NODES

Control lymph nodes comprise two groups, the first consisting of nine nodes from groin, axillary, cervical, or abdominal groups removed from patients with non-neoplastic conditions. These were classified on the basis of site of origin and light microscopic appearance. The second group consisted of five nodes removed from patients with systemic neoplasia (carcinoma or Hodgkin's disease) which were not microscopically involved by the tumour. These were classified according to site and diagnosis of the primary condition. All nodes were obtained as fresh surgical specimens within minutes of their removal.

NEOPLASTIC LYMPH NODES

These were received as fresh surgical specimens, and were immediately received into Hanks BSS or medium 199 and chilled in ice. The diagnosis of histiocytic lymphoma (reticulum cell sarcoma) was made by frozen section and subsequently confirmed by paraffin section and electron microscopy. The source was inguinal (case 4) cervical (cases 1, 3, 7), and axillary (cases 2, 5). An enlarged spleen, almost

totally replaced by tumour, was the source of cells in case 6.

PREPARATION OF LYMPH NODE CELL SUSPENSIONS

The lymph node was sliced into small fragments and immediately transferred to Hepes buffered medium 199, containing 10 units heparin/ml. The fragments were further teased under the medium and cells washed out by gentle pipetting. Cells were filtered through stainless steel gauze, washed twice in medium 199, and the concentration was adjusted to 3×10^6 /ml. Samples were smeared and stained with Giemsa, and viability was assessed by trypan blue dye exclusion.

PREPARATION OF SPLEEN CELL SUSPENSION (CASE 6)

Albumin gradients were used to concentrate neoplastic cells from the crude spleen suspension. Discontinuous gradients of bovine serum albumin were set up at 2, 4, 8, 12, 17, 22, 28, and 35% from stock bovine serum albumin (Armour) diluted with medium 199. Each layer was 4 ml in volume, and the gradients were loaded with 5 ml of cell suspension containing 100×10^6 cells. Sedimentation was allowed to continue for two hours at room temperature under gravity. All cell suspensions were tested for viability and smears were made at each fractionation step to ensure that suspensions were representative. Fraction 6 (22% albumin) contained 60% large abnormal cells, and was used in the receptor studies.

PREPARATION OF RED CELLS AND ROSETTES

Washed sheep red cells (E), sheep red cells sensitized with IgG antibody (EA1gG), and sheep red cells sensitized with IgM antibody and complement (EAC) were used in the rosette tests.

E rosettes

E rosettes were prepared by centrifuging washed sheep erythrocytes, together with lymph node cells in a ratio of 40:1, at $80 \times g$ for five minutes. Incubation was continued for two hours at 4°C, and lymph node cells binding three or more erythrocytes were counted.

IgG rosettes

IgG rosettes for detecting Fc receptors were prepared by sensitizing sheep cells with purified anti-sheep RBC IgG for one hour at 37°C. Sensitized cells were washed once in phosphate-buffered saline and screened for microscopic agglutination. Rosette formation was assessed as for E rosettes.

EAC rosettes

Sheep red cells were sensitized with rabbit anti-sheep RBC IgM for one hour at 37°C, and then reacted with either guinea-pig or human complement, using a two-stage sensitization procedure. The exclusion of sheep cell determinants was shown by rosetting with IgM-coated sheep cells alone. Sheep cells sensitized with complement were incubated with lymph node cells in a ratio of 40:1 at 37°C for 10 minutes, followed by two hours at 4°C. Quantitation of rosettes was as for E rosettes.

IMMUNOFLUORESCENCE

Detection of immunoglobulin-bearing cells was performed using a sensitive 'sandwich' technique. The morphology of immunofluorescent cells was assessed by dark ground and phase-contrast microscopy.

PHAGOCYtic CELLS

Phagocytic cells were assessed functionally by their ability to ingest neutral red.

The techniques employed here have been described in detail elsewhere (see Habeshaw and Young, 1975).

Results

MICROSCOPY

Microscopy of our seven cases shows that all of them conform to the usual view that reticulum cell sarcomata are composed of large cells with moderate amounts of cytoplasm and large vesicular nuclei which frequently contain nucleoli (fig 1). Electron microscopy of five tumours confirmed that certain features were common to all although differences in the degree of cytoplasmic differentiation were noted. As a rule the cytoplasm contained large numbers of ribosomes with quite variable amounts of rough-

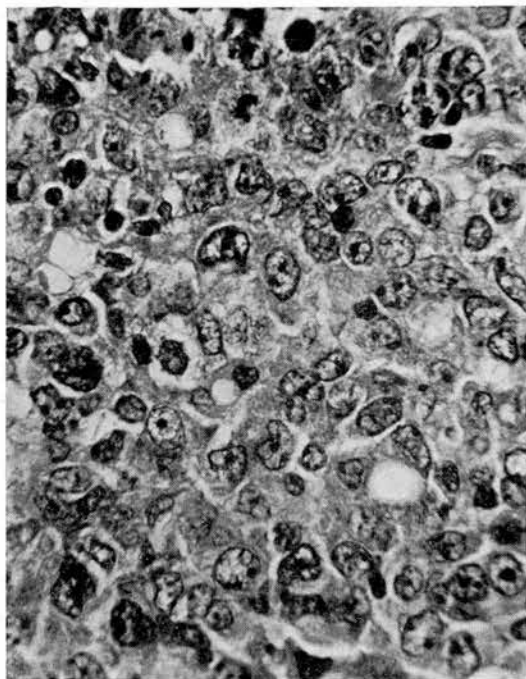


Fig 1 Representative light microscopic appearance of reticulum cell sarcoma. H & E \times 600.

surfaced endoplasmic reticulum. Also mitochondria were abundant. All cases showed prominent nucleoli and the nuclei often contained deep clefts or had a lobulated appearance (fig 2). The neoplastic cells did not form fibres to any conspicuous degree and when dendritic cells were found they were interpreted as part of a population of normal residual cells. Ingestion of dead cells by macrophages was noted but there was no indication of overt phagocytosis by

Patients	Diagnosis	Site	E (%)	EAIgG (%)	EAC (%)	NR (%)	Fluorescence (%)	Viability (%)
1	Reactive sinus hyperplasia	Coeliac	55	18	40	27	32	65
2	Normal	Groin	42	70	46	1.0	—	80
3	Reactive	Axilla	41	8	—	—	22	82
4	Follicular reactivity	Mesentery	38	13	52	27	35	80
5	Sarcoid reaction	Cervical	41	24	31	—	40	76
6	Sinus hyperplasia	Mesentery	49	45	—	—	22	93
7	Reactive	Groin	51	7	9	11	13	84
8	Reactive	Groin	32	11	24	10	53	86
9	Reactive	Groin	30	16	20	12	27	50
10 ¹	Carcinoma	Neck	42	18	15	18	32	68
11	HDLP ²	Mesentery	56	—	—	—	29	66
12	HDLP	Groin	31	29	49	1	27	55
13	HDLP	Groin	24	12	—	—	37	87
14	HDNS ³	Mesentery	37	27	16	11	33	78

Table I Control lymph nodes (non-neoplastic, reactive, or normal on histological examination)

¹Additional nodes from patients nos 10-14 with carcinoma or Hodgkin's disease were normal or reactive on histological examination.

²HDLP = Hodgkin's disease lymphocyte predominance; ³HDNS = Hodgkin's disease nodular sclerosis.

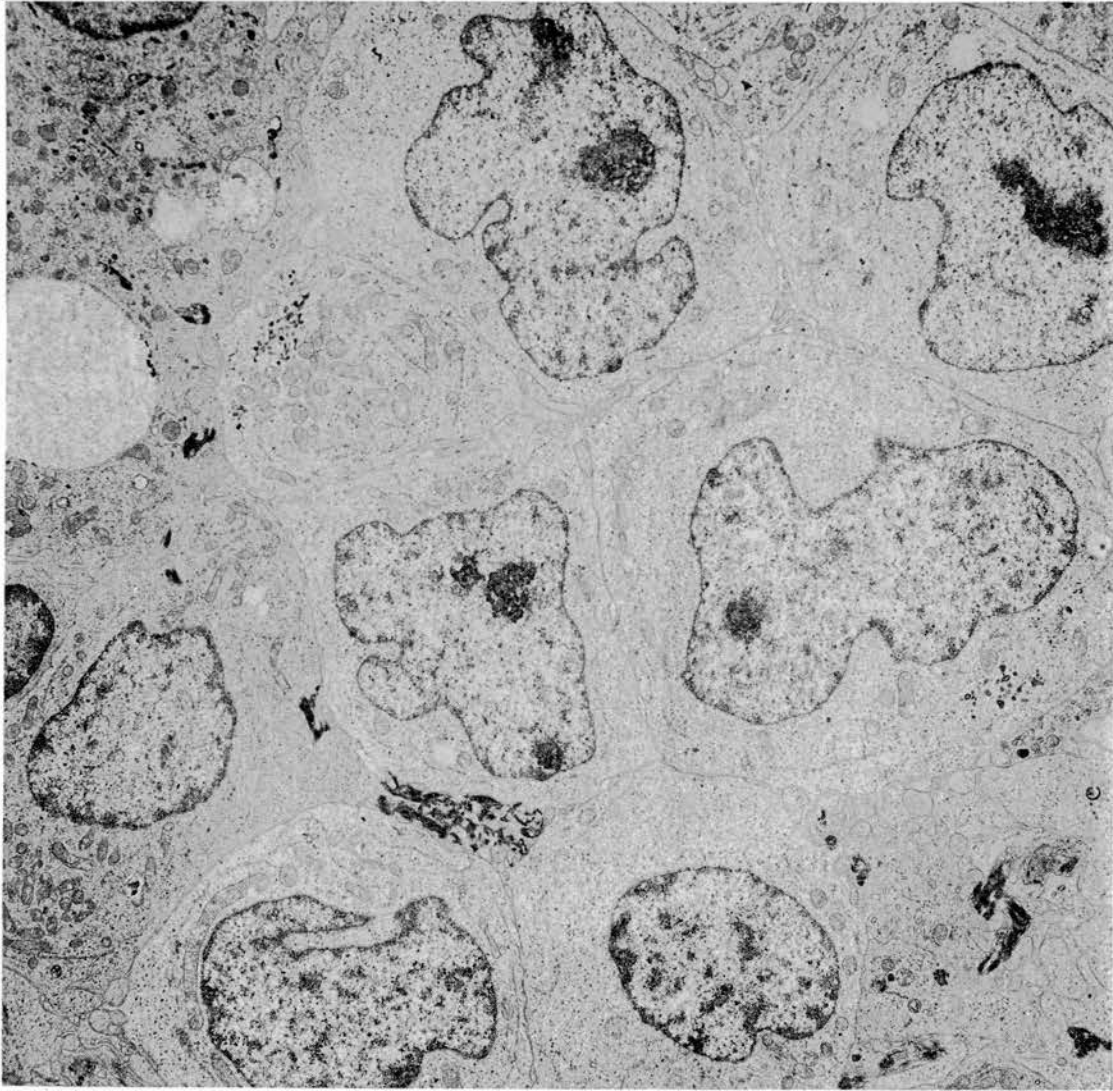


Fig 2 Representative electron microscopic appearance of reticulum cell sarcoma. $\times 3750$.

tumour cells. The degree of cytoplasmic differentiation varied between different tumours and between cells of the same tumour.

RECEPTOR STUDIES

The receptor pattern of lymphocytes from both groups of control lymph nodes indicates quite wide variability dependent upon the site of origin, viability, and reactive status of the node (table I). In general the proportion of T lymphocytes is lower and of B lymphocytes higher than in peripheral blood. The macrophages are more numerous than in

peripheral blood, although stromal macrophages are probably not represented in our preparations. The following ranges of cell receptors were obtained: E rosettes (T cells) 30-55%, EA IgG rosettes 7-70%, neutral red phagocytes 1.0%-27%, EAC receptors 9-52%, and immunofluorescent cells (B cells) 13-53%. In general the addition of totals of cells expressing receptors for sheep cells, cells showing stainable immunoglobulin, and the functional phagocytes ingesting neutral red gave a total equal to the percentage of viable lymph node cells.

The values for receptors in the neoplastic nodes

was at least as variable as in normal nodes (table II). In two cases (3 and 1) the receptor patterns were indistinguishable from those of normal nodes. In these cases, neoplastic cells expressed either B

Case No.	E Rosettes (%)	EAIgG (%)	Neutral Red Cells (%)	EAC (%)	Fluorescent Cells (%)	Viability
K1	67	28	—	—	20	—
R2	15	—	60	12	26	63
S3	37	9	1	—	39	76
L4	49	40	12	30	—	69
B5	72	43	22	68	32	85
O7	30	6	4	7	6	85

Table II Receptor pattern of reticulum cell sarcomas in lymph node

lymphocyte or T lymphocyte receptors. The phagocytic population was low in case 3, suggesting that these were normal lymph node macrophages and not of neoplastic derivation. In the first case (K1) the majority of cells carried T cell markers, but viability and phagocytosis were not assessed and it did not prove possible to distinguish morphologically the neoplastic cells from reactive lymph node lymphocytes.

In case R2 the proportion of T cells was low, as was the total of cells expressing EAC receptors. The percentage of neoplastic cells ingesting neutral red was significantly greater than in control lymph nodes, and these accounted for the bulk of the viable cells. Overnight culture of these cells resulted in a glass-adherent population of large cells with convoluted nuclei. Some of these ingested red cells coated with IgG when incubated with them for two hours at 37°C (fig 3). Many of the cultured cells were indistinguishable from normal lymph node macrophages, others had large basophilic nuclei, and a few were binucleate. Phagocytosis of cell debris was widespread in the culture preparation. The predominance of phagocytically active cells in the original cell suspension, and the presence of morphologically bizarre phagocytic cells expressing receptors for IgG, lead us to conclude that in this case the tumour cells were of histiocytic, not lymphoid, derivation. Of the population expressing immunoglobulin determinants by fluorescence a minor population only showed the confluent ring or cap staining associated with B lymphocytes, a point reflected by the low level of cells expressing complement receptors—also a marker of B lymphocytes. In case S3 the receptor pattern is within normal limits.

In case L4, the percentage figures for E, EAIgG,

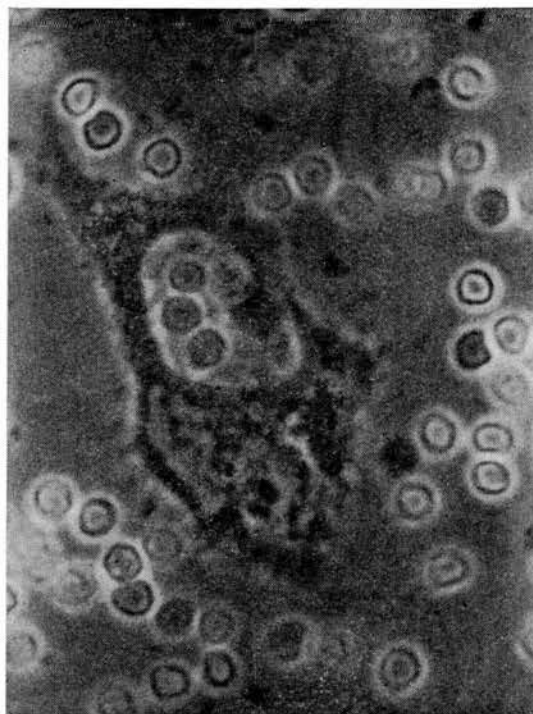


Fig 3 Very large cell in overnight culture of lymph node cells (case 2) which showed IgG receptors. Note phagocytic vacuole containing erythrocytes. Phase contrast $\times 700$.

EAC, and fluorescent cells also do not differ significantly from normal values. However, in this case the total of class-specific receptors expressed exceeds the total of viable cells and leads to the conclusion that at least one population of cells must express receptors not generally expressed by cells of that class. This is reflected in the totals for cells expressing T lymphocyte receptors (E) and receptors for EAIgG, which are normally expressed by a proportion of B lymphocytes and macrophages and not by T cells. Phase-contrast examination revealed rosette formation (by large neoplastic cells) with sheep red cells (E), immunoglobulin (EAIgG), and complement (EAC)-coated red cells, supporting the concept of 'receptor overlap' (figs 4, 5, 6).

In case B5, this overlap of receptors is confirmed, especially in relation to the T cell marker E, and the B cell receptor for EAC, whose combined total is 126% in a population where only 85% of cells were viable. Phase-contrast examination showed that in addition a minority of tumour cells failed to rosette with any of the particles (figs 7 and 8).

Cases R6 and O7 show the presence of a large

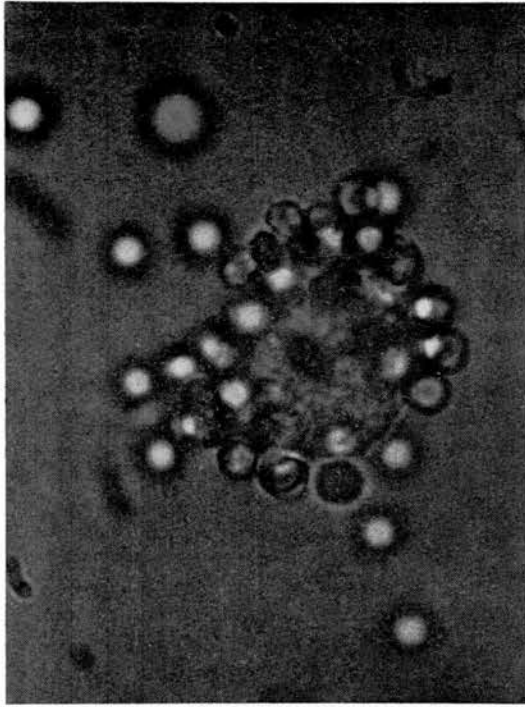


Fig 4

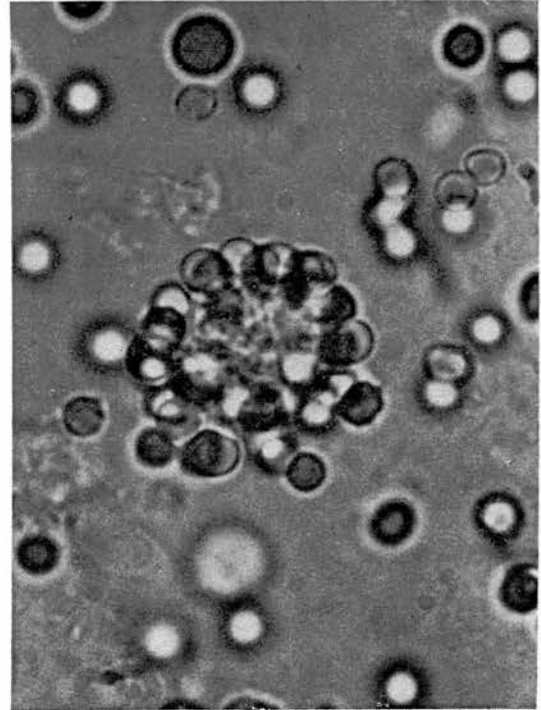


Fig 5

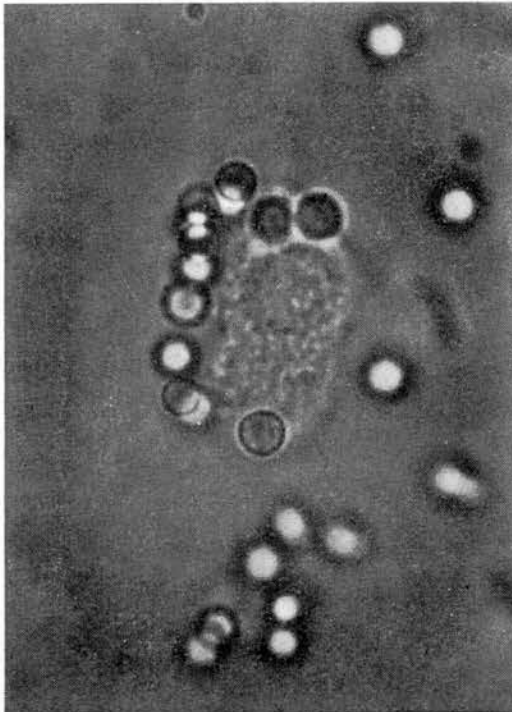


Fig 6

Fig 4 Tumour cell from case 4 showing rosetting with sheep erythrocytes. Phase contrast $\times 700$.

Fig 5 Large EA1gG rosette from case 4. Phase contrast $\times 700$.

Fig 6 Tumour cell from case 4, showing adherence of EAC-coated red cells. Phase contrast $\times 700$.

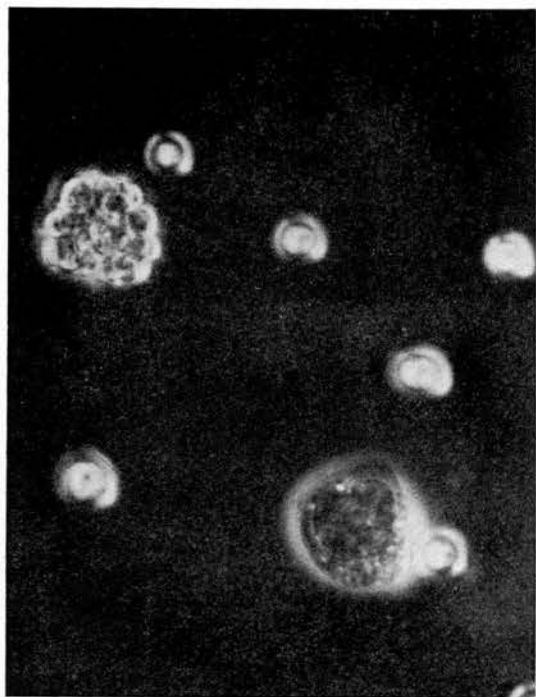


Fig 7 EAC rosette and large solitary cell from case 5. Dark ground illumination $\times 400$.

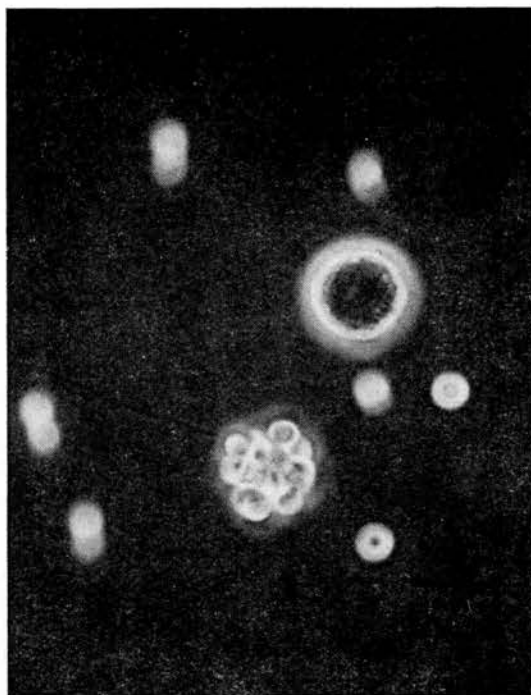


Fig 8 E rosette and large tumour cell from case 5. Dark ground illumination $\times 400$.

receptor silent population. Cells forming rosettes from the spleen preparation in case 6 can be accounted for by the contaminating lymphocytes and macrophages in the cell population (table III).

Receptors as Percentage		Differential Count as Percentage	
E	5	Tumour cells	60
EAIgG	18	Small mononuclear cells	23
EAC	18	Polymorphonuclear leucocytes	7

Table III Receptor studies on albumin gradient fraction of spleen cell suspension from case R6

In case 7, phase-contrast microscopy showed that readily identifiable viable tumour cells comprised over 60% of the cell population studied. The great majority of these cells failed to rosette with any of the test particles or to ingest neutral red, although some weak EAIgG and E rosettes were formed by tumour cells. A feature of interest in this case was the tendency of the tumour cells to show weak diffuse surface fluorescence with polyvalent antisera. This staining was not of the type associated with B lymphocytes, the staining being very weak and show-

ing no tendency to localize in caps or patches on the cell surface.

Discussion

This study of surface characteristics of malignant cells isolated from lymph nodes was facilitated by the choice of reticulum sarcoma where the cells are large and distinctive; furthermore in all our cases the entire node was replaced by tumour tissue obliterating the underlying architecture. Thus the receptor studies reflected the surface constitution of a predominantly tumour cell population. Nevertheless it may be difficult to distinguish between reactive lymphoid cells and neoplastic lymphocytes when the cells are in suspension and reference to histological sections is necessary to identify the nature of tissue taken for experiments.

In one case (2) we are confident in asserting a histiocytic origin for the neoplastic cells, since these were phagocytically active both in the original suspension and in tissue culture, and expressed receptors for EAIgG in culture. In addition, these cells lacked receptors for both E and EAC (T and B lymphocyte markers).

In two cases (4 and 5) overlap of receptors was noted. Since examining these nodes we have developed techniques which allow simultaneous detection of T and B cell receptors, and an additional case of prolymphocytic leukaemia has been described in which this phenomenon occurred (Dewar *et al.*, 1974). Other authors have also noted the expression of receptors for IgG (Yoshida and Andersson, 1972) by T lymphocytes undergoing transformation, by cells derived from chronic lymphocytic leukaemia (Shevach *et al.*, 1973), and by circulating cells in an uncharacterized lymphoproliferative disorder (Sandilands, Gray, Cooney, Browning, Grant, Andersson, Dagg, and Lucie, 1974). A small population of normal peripheral blood lymphocytes bearing both B and T cell markers has also been described (Dickler, Adkinson, and Terry, 1974). There are therefore two possible conclusions to be drawn from cases 4 and 5; either the neoplastic population is derived from a subclass of lymphoid cells bearing both B and T markers, or the population is derived from T lymphocytes which have acquired B cell markers during neoplastic transformation (or vice versa). In both cases the origin of the neoplastic cells is lymphoid rather than histiocytic.

In cases 6 and 7, the tumour cells were receptor silent, and therefore not classifiable as either lymphoid or histiocytic in origin. In case 6, and one other receptor silent lymphoma we have studied, progression of the disease was rapid and both patients died within six to nine months of diagnosis. This might suggest that receptor silence is an ominous prognostic sign in lymphoreticular neoplasia. It could be due to loss of receptor characteristics by dedifferentiation of the neoplastic cell, or suggest an origin from a receptor silent class of cell possibly of stromal origin (reticulum cell or reticular macrophage). The presence of small, but detectable amounts of immunoglobulin on the tumour cells in case 7 do not necessarily imply a B cell origin. Weak, diffuse fluorescence of this kind can be observed in carcinoma cells from secondarily involved lymph nodes, and may represent the presence of circulating anti-tumour-cell antibodies. It is worth mentioning that in our experience carcinoma, or malignant melanoma, cells from involved nodes are also receptor silent. In our two receptor-silent cases the mode of presentation, the histological appearances, and the subsequent clinical behaviour exclude the possibility of metastatic carcinoma or malignant melanoma.

Receptor studies are expensive in time and it is pertinent to ask what contribution they can make to the understanding of lymphoid neoplasms. The contribution of such studies to pathology and medicine has already been assessed (Wybran and Fudenberg,

1974). In the field of lymphoid neoplasia, the present study has confirmed the views of Lukes and of Gall in proposing lymphoid and histiocytic origins respectively for this group of neoplasms. The overall picture is far from clear; in particular no explanation for the neoplasms with normal ratios of B and T cell markers is satisfied by the current dogma of clonal origin from a single class of neoplastic lymphocyte. Two possibilities are perhaps worthy of further examination: first that the neoplastic state requires the participation of both B and T lymphocytes, as do the normal phenomena of antibody secretion and germinal centre formation. As an alternative, perhaps a fixed neoplastic population sequesters from the circulation a reactive lymphoid population which proliferates in concert in the environment of the node. It is hoped that further investigations of the solid tumours of lymph nodes will cast more light on a difficult subject.

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Extramedullary Plasmacytoma of Stomach

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A single case of gastric plasmacytoma showing unusual clinical and pathological features is described. The patient had gluten sensitive enteropathy, and showed increased circulating IgA levels prior to gastrectomy. Progression of the disease was associated with both K and L light chain proteinuria, hypoalbuminaemia, and vasopressin resistant polyuria. Pathological investigation revealed the coexistence of IgA secreting soft tissue plasmacytoma, with IgG secreting myeloma. The significance of these findings is discussed.

Key words: myelomatosis – plasmacytoma – immunoglobulin A (IgA) – malabsorption syndrome – vasopressin resistant polyuria

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Of all extramedullary plasmacytomas the majority occur in the submucosa of the upper air passages, less than 10 % occurring as primary neoplasms in the gastrointestinal tract (Wiltshaw 1971).

We report here a plasmacytoma of the stomach associated with gluten-sensitive enteropathy and diffuse, systematic myelomatosis of differing clonal derivation.

CASE HISTORY

(Mr. J.M. – Date of birth Oct. 19, 1926)

In 1967 the patient sustained a right hemiplegia related to a left carotid artery stenosis, followed by good recovery of function and he was subsequently given a two-year course of anticoagulants.

In 1969 he was diagnosed as having gluten-sensitive enteropathy with steatorrhea and total villous atrophy on jejunal biopsy; total remission of symptoms followed a gluten-free diet and vitamin supplements.

In Dec. 1971 an osteolytic lesion was demonstrated in the upper end of the left femur following pain in the left thigh. This lesion was presumed to be a metastasis from an undiagnosed primary tumour and was treated in April 1972 by local radiotherapy (to a total of 2,200 rads) followed by a rapid relief of symptoms and marked subsequent radiological improvement.

In July 1972 the patient was admitted for the investigation of persistent iron deficiency anaemia and because the stools were persistently positive for blood. A barium meal and endoscopy showed an infiltrative lesion in the region of the pyloric antrum. At this time there was a marked rise in

TABLE I
Levels of serum immunoglobulin during the course of the disease

	Date		
	May 31, 1972 (prior to removal of gastric primary)	Aug. 25, 1972 (after removal of gastric primary)	Febr. 14, 1973 (recurrence)
IgG	880 mg/100 ml	1,700 mg/100 ml	2,100 mg/100 ml
IgA	2,375 mg/100 ml	550 mg/100 ml	540 mg/100 ml
IgM	22 mg/100 ml	150 mg/100 ml	94 mg/100 ml

Normal values: IgG 800–1500 mg/100 ml, IgA 140–170 mg/100 ml, IgM 50–110 mg/100 ml.

the serum IgA fraction to a level of 2,375 mg/100 ml (see Table I). No protein was detected in the urine. At laparotomy the tumour was found to involve the pyloric region of the stomach, the base of the mesocolon and the origin of the middle colic vessels; there was no obvious glandular enlargement and the liver was macroscopically normal. A Polya gastrectomy was carried out and part of the transverse colon was resected. Histology revealed a plasmacytoma of the stomach (Figure 1). Postoperatively the serum IgA concentration fell to near normal levels (see Table I). At this time the liver function tests were normal except for a raised serum alkaline phosphatase of 26 KA units/100 ml.

He was first seen for assessment at the Royal Infirmary, Edinburgh, in Aug. 1972 when a skeletal survey showed no abnormality and histology of specimens from marrow obtained by aspiration and trephine was normal. No further treatment was given at that time and the patient remained in good health until late Dec. 1972 when he was referred because of marked lymphadenopathy in the right side of the neck and left axilla. Biopsy of a cervical node was consistent with a diagnosis of plasmacytoma. The serum alkaline phosphatase estimation was now 40 KA U/ml being of liver origin on electrophoresis. Repeated bone marrow smears were normal. The patient was started on prednisolone and melphalan in doses of 40 mg/day and 4 mg/day respectively. Therapy was discontinued after five days when signs of small intestinal obstruction developed. Because of copious vomiting, the patient was treated with i.v. fluids and nasogastric suction. Although there was radiographic evidence of hold-up at the site of the gastric anastomosis, the symptoms subsided gradually. On Febr. 15, 1973 a definite mass

was felt in the central epigastrium and the serum IgG was considerably elevated (see Table I). The serum albumin had fallen to 2.1 g% and sacral oedema was noted. For the first time, kappa and lambda chains were found in the urine and a cold haemagglutinin in the serum. The direct Coombs' test was positive to a titre of 1:80. The patient developed remittent fever and the peripheral white blood count rose to 27,000 μ l, 92% of the cells being neutrophils. Repeated blood cultures were sterile and culture of urine and swabs from the nose, throat and axilla yielded no significant growth. The patient was transfused with blood and given kanamycin i.m. 50 mg daily. He then developed hyponatraemia, the serum sodium falling to 114 meq/litre and serum chloride to 88 meq/litre. This was thought to be due partly to loss of sodium into the 'tissue compartment' because of hypoalbuminaemia. Treatment with salt-poor albumin and fluids was instituted and thereafter the serum sodium rose and serum albumin reached 3 g/100 ml. The patient then developed polyuria and within 10 days the diuresis had risen to 11 l. The urinary osmolality varied between 280 and 290 m.osm/l and administration of vasopressin tannate i.m. failed to induce reduction in urine volume or an increase in urine osmolality. Thereafter the patient's condition deteriorated rapidly due to a chest infection and he died on March 8, 1973.

PATHOLOGY

Postmortem findings

Specimens of duodenal and jejunal mucosa were obtained within 2 h of death, and

immediately fixed for microscopic examination. Postmortem was performed 12 h after death showing an emaciated man, with enlarged cervical (1–2 cm), carinal (3–5 cm), mesenteric (3–5 cm) and para-aortic lymph nodes. A tumour measuring $3 \times 5 \times 2$ cm was present in the upper anterior mediastinum, secondary nodules were present in the pancreas, both kidneys, and tumour infiltration was present in both lung bases. The tonsils were involved, but no macroscopic tumour deposits were found in sternal, vertebral, costal or pelvic bone marrow. No osteolytic lesions were found in the femora, or skull. The liver showed fine surface nodularity with dilation of in-

trahepatic bile ducts associated with a degree of obstruction at the porta hepatis by enlarged lymph nodes. The brain showed an old cystic infarct ($2.5 \times 1 \times 5$ cm). Ureters, bladder and prostate were macroscopically normal.

Microscopic examination

The primary tumour was an infiltrating poorly differentiated plasmacytoma replacing gastric mucosa and infiltrating muscle coats (Figure 1).

In all the effected lymph nodes, tonsil, pancreas, and kidney the histological features were those of a poorly differentiated

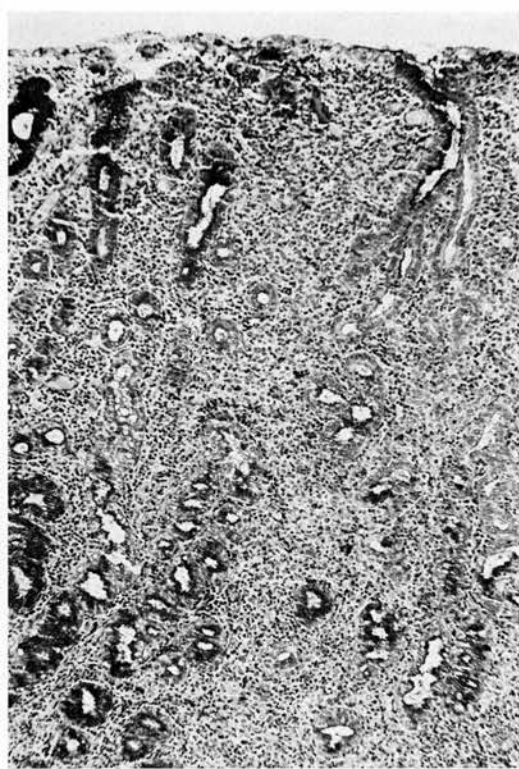


Figure 1. The primary tumour in stomach. The gastric epithelium is darkly staining, and the glands separated by a dense infiltrate of mononuclear cells. — P.A.S. $\times 95$

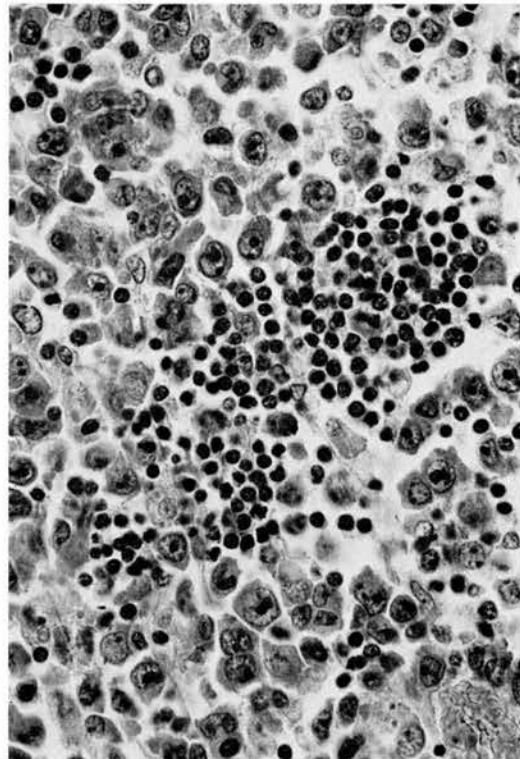


Figure 2. The appearance of tumour in lymph node. A focus of normal lymphocytes contrasts with the large neoplastic cells, some of which show the features of plasma cells. — H. & E. $\times 425$

plasmacytoma composed of large pleomorphic cells with abundant basophilic cytoplasm (Figure 2). Methyl-green pyronine stains showed a number of small islands of tumour cells staining intensely with pyronine scattered in a uniform population of poorly pyroninophilic cells. No stromal component could be identified in reticulin stained preparations. The tumour in the mediastinal mass, tonsil and cervical nodes was less well differentiated than that in other sites. Necrosis was obvious in mesenteric and para-aortic nodes.

The bone marrow from ribs, sternum and vertebral column was excessively cellular, and showed diffuse infiltration with immaturity, though well differentiated plasma cells,

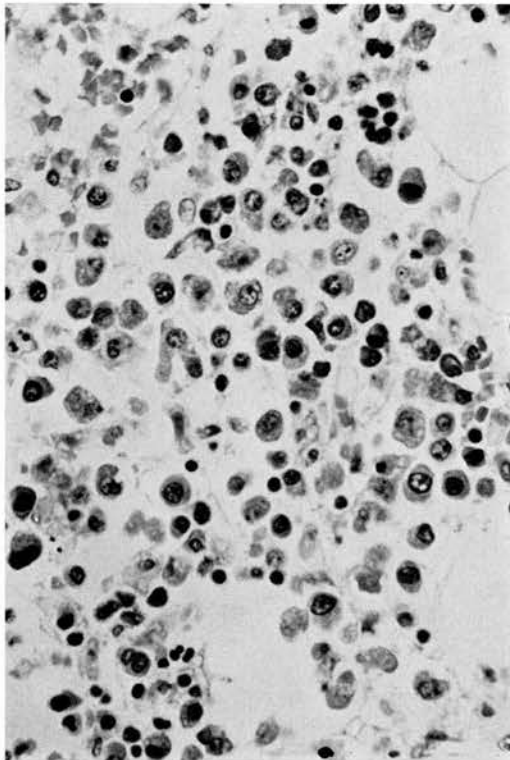


Figure 3. Bone marrow. Numerous immature plasma cells are present as a diffuse infiltrate. — Giemsa stain $\times 425$

quite unlike those found in the lymph nodes (Figure 3).

The portal tracts in the liver were expanded by loose connective tissue containing plasma cells, lymphocytes, and neutrophil polymorphs. The pancreas showed, apart from tumour infiltration, prominent islet tissue and some interlobular fibrosis.

The jejunal mucosa showed the typical light and dissecting microscope appearance of sub-total villous atrophy.

In the kidney the principal features were widespread interstitial oedema of the medulla, with 'myeloma kidney' changes in the proximal cortical tubules, and the presence of cortical tumour nodules. Amyloid was not present.

Immunological studies

Clinical quantitation of immunoglobulin was by the method of radial gel diffusion. In addition, urinary and serum components were identified by immunoelectrophoresis with monospecific antisera.

Immunofluorescent studies

Material was obtained post-mortem from lung, kidney, liver, lymph-nodes and bone marrow. It was immediately snap frozen onto dies. Frozen sections and freeze dried paraffin-impregnated sections were cut and stained for IgM, IgA, IgG and kappa and lambda light chains by both direct (rabbit anti-immunoglobulin — FITC conjugate) and indirect (goat anti-rabbit — FITC conjugate) methods. Appropriate controls were included at each step.

Immunofluorescent studies showed the tumour in lymph nodes to stain exclusively for IgA and lambda chains. Staining was not uniform, occurring in clumps of cells

corresponding to the patchy pyroninophilia already described (Figure 4).

In the portal tracts, IgG and IgA staining cells were both present, IgG cells predominating, and both classes of light chain were found.

In the kidney there was strong tubular staining for both kappa and lambda chains, and tubular IgA was present in the medulla.

The bone marrow showed a very pronounced predominance of well differentiated plasma cells staining for IgG; although both classes of light chain were present there was a marked excess of kappa over lambda chain.

Control sections showed weak non-specific staining in kidney, liver and lung, but lymph nodes showed very little non-specific staining.



Figure 4. Typical appearance of tumour in lymph nodes by immunofluorescence. Indirect staining for IgA. - $\times 800$

Electron microscopy

The plasmacytoid nature of the tumour cells in the lymph nodes was confirmed. Furthermore, several of the tumour cells contained crystalline linear deposits showing a light band periodicity of 11 nm, and a dark band periodicity of 11.8 nm. This material occurred exclusively in dilated endoplasmic sacs, related to the Golgi areas of the tumour cells (Figure 5). In addition nearly all tumour cells contained prominent intranuclear inclusions.

DISCUSSION

This case adds one more report of gastric plasmacytoma to the 21 reported in the



Figure 5. Endoplasmic crystalline inclusion in a tumour cell. - $\times 68,750$

world literature up to 1971. (For reviews see Hampton & Gandy 1957, Annamunthodo & Robertson 1958, Remigo & Klaum 1971). However, the case presented here contributes some further details to the assembling clinico-pathological picture of the entity of gastrointestinal plasmacytoma.

In this case gluten-sensitive enteropathy existed before diagnosis of plasmacytoma. This was proven by biopsy, clinical investigation, and response to therapy. The pathological features of sub-total villous atrophy were also present at autopsy. In the literature, most authors stress gastrointestinal symptoms – often associated with gastric ulceration – and loss of weight as presenting symptoms of gastric plasmacytoma, although documentation of malabsorption syndrome has not been reported to date. Rappaport et al (1972) described the pathological features and demonstrated severe plasma cell infiltration of the intestinal mucosa and submucosa in Mediterranean abdominal lymphoma with malabsorption. In this series 16 out of 20 cases subsequently developed malignant lymphoma. Although no plasma cell tumours were reported, the authors state that in Mediterranean lymphoma with malabsorption – ‘the possibility is suggested that this abnormal, though probably not neoplastic proliferation of plasma cells is a morphological manifestation of an immune deficiency state which predisposes the patient to the development of malignant lymphoreticular neoplasms’.

In contrast to the case of gastric plasmacytoma reported here, the features of Mediterranean abdominal lymphoma are particularly well documented in regard to the abnormal proteins secreted by the tumour cells. In the majority of cases the paraprotein consists of alpha chains only (α -chain disease)

(Seligmann et al 1971). In our case the abnormal protein appeared as a fairly diffuse, myeloma band on electrophoresis, and reacted both with anti- α -chain antisera and with anti-lambda-chain antisera. By immunofluorescence the tumour cells expressed both α -chain and lambda-chain determinants.

In two cases, reported by Hefferman (1947) and Ruland (1954) primary gastric plasmacytoma was followed by the development of skeletal plasma cell tumours. In this respect Line & Lewis's case (1969) is also of interest as a mediastinal tumour was present, with a clavicular deposit of tumour, and light chain of immunoglobulin in the serum. In our case, the patient in the later stages of his illness developed a raised serum IgG, and at this time both kappa and lambda light chains were present in the urine. The direct Coombs' test became positive. The demonstration, post-mortem, of the IgA, lambda chain secreting nature of the plasmacytoma does not account for the presence of the urinary kappa chain, nor the elevated serum IgG. Serum M protein components (light chains) were present in the cases described by Line & Lewis (1969) and Remigio & Klaum (1971). Urinary M proteins, or Bence Jones proteinuria are generally not present in soft tissue plasmacytoma (Snapper 1953, Dolin & Dewar 1956), but may be so when skeletal ‘metastases’ or co-existent multiple myeloma is present. In our case serum IgA was elevated initially but fell when the primary tumour was resected. Light chains were present in urine only when elevated IgG levels were found, and then both classes of light chain were present. This finding supports the view that soft tissue plasmacytomas are not generally associated with Bence Jones proteinuria. This is probably

due to the relatively small mass of plasma cells, but in cases of α -chain disease renal excretion of α -chain is notably poor (Seligmann et al 1971). Lymphomas of the gut, for example Waldenström's disease of the stomach (Froget et al 1972) or Mediterranean abdominal lymphoma (Rappaport et al 1972), are frequently associated with abnormal serum IgM or IgA paraproteins, which are poorly excreted in the urine.

In view of the post-mortem appearance of diffuse myelomatosis of bone marrow, the great preponderance of cells secreting IgG and kappa chain in the bone marrow, the elevated serum IgG and the kappa chain proteinuria, we believe that these features indicate myelomatosis of different clonal origin from the primary plasmacytoma. It is also possible that the previous reports of bone involvement in soft tissue plasmacytoma might, in some instances, have been due to co-existent myelomatosis of different clonal origin. The late onset of light chain proteinuria was related to a period of marked renal dysfunction, ADH resistant polyuria, and low serum albumin. The pathological features found in the kidney were those of myeloma kidney and similar to those described by Remigio & Klaum (1971). Amyloid was not present in our case although para-amyloid deposits were present in cases described by Ruland (1954) and Annamunthodo & Robertson (1958) in lymph nodes. Low serum albumin can exist without the nephrotic syndrome in patients with primary amyloidosis with renal involvement, the serum albumin level being below the threshold value for renal filtration. Although amyloid was not present in this case, both kappa and lambda chains and IgA were present in the renal tubules and interstitium, and may have produced

an equivalent effect to the deposition of amyloid.

Finally, attention is drawn to the crystalline deposits observed in the cells of this tumour which are identical with those described in the cells of acute promyelocytic leukaemia (Cawley & Hayhoe 1973). It is relevant to note that myelomatosis terminating in acute leukaemia has been described in four cases (Videbaek 1971) and monocytic leukaemia in three (Osserman 1971).

The case described conforms to the general features of previously described cases of plasmacytoma of the gastrointestinal tract, notably in respect of widespread lymph node metastasis with haematogenous spread to kidney, lung and pancreas (Wiltshaw 1971). The clinical features illustrate the well documented initial favourable response of a solitary myeloma of bone to radiotherapy, and the relentless progression of a soft tissue plasmacytoma. In retrospect it seems possible that the patient would have benefitted from a course of prednisolone and melphalan following the resection of the gastric tumour, despite the initial good subjective response to surgery and the fall in serum IgA.

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Quantitation of Subclasses of Mononuclear Cells in Normal Human Blood by Membrane Receptor Studies

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SUMMARY. A technique for quantitating mononuclear cells expressing E, EA_(IgG), EAC receptors, immunoglobulin carrying cells and phagocytic cells in normal human venous blood is described; normal values for each of these classes of mononuclear cell are established. The effects of different methods of cell preparation have been considered; these were found not to have a significant effect on the final values. In addition, we describe a second category of 'fluorescent non-capping' cells and show that these are numerically equivalent to cells which ingest neutral red.

Much recent progress in cellular immunology has been based upon the concept of the thymic dependent 'T cell' and thymic independent 'B cell' lymphoid systems. Currently, attempts are being made to explore the heterogeneity of lymphocyte populations in man, by use of surface receptor detecting techniques believed to identify specifically either class of lymphocyte. It has also been shown that monocytes, macrophages and their precursors share some of their surface receptor properties with lymphocytes. When polymorphonuclear leucocytes and red cells are removed from the venous blood, the residual mononuclear cell population (lymphocytes and monocytes) is rich in the class specific receptors for sheep erythrocytes (E), the receptor for IgG immunoglobulin (EA_(IgG)), and the receptor for complement (EAC).

The Complement Receptor (EAC)

The EAC receptor is found on B lymphocytes (Lay & Nussenzweig, 1968; Pincus *et al.*, 1972). In chronic lymphocytic leukaemia lymphocytes expressing EAC receptors are increased, as are cells carrying surface immunoglobulin (Wilson & Nossal, 1971; Pincus *et al.*, 1972). The macrophage also expresses a receptor for EAC (Mantovani *et al.*, 1972).

The IgG Receptor (EA_(IgG))

Receptors for the Fc fragment of IgG are present upon blood monocytes, hepatic, and splenic macrophages (Huber *et al.*, 1969). Both IgG and EAC receptors on macrophages are important in phagocytic reactions. Monocyte receptors for IgG are labile, and are regenerated after phagocytosis (Schmidt & Douglas, 1972). Most antibody-forming cell precursors also have receptors for IgG (Basten *et al.*, 1972).

The Sheep Erythrocyte Receptor (E)

A large proportion of human blood lymphocytes express a receptor for sheep erythrocytes

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(E) (Brain *et al*, 1970; Coombs *et al*, 1970). In primary immunodeficiency states, with absent or depressed T cell function, E rosetting lymphocytes were reduced or absent, while in T cell immunodeficiencies E rosetting cells were normal or increased (Auiti *et al*, 1973).

Surface Immunoglobulin

Immunoglobulin is readily detectable on the surface of B lymphocytes by direct or indirect immunofluorescent staining (Papamichael *et al*, 1971; Frøland & Natvig, 1972). Aggregates of immunoglobulin and fluorescent anti-immunoglobulin occur as 'dots', 'rings' or 'caps' on the lymphocyte surface. The reaction seems to be due to the expression of light chains rather than heavy chain determinants (Biberfeld *et al*, 1971).

Phagocytic Cells

In the study of mononuclear cell receptors most authors remove phagocytic cells by adherence to plastic or glass surfaces, or to nylon wool columns, or by carbonyl iron ingestion followed by magnetic removal (Cutts, 1970). Effective markers for phagocytic populations are colloidal carbon (de Halleux *et al*, 1973), amaranthus starch and neutral red dye (A. E. Stuart and E. Dewar, personal communication).

EDTA Sensitive Receptors

EDTA has been used in receptor studies to prevent binding of immune complexes to macrophages (Bianco & Nussenzweig, 1971) and to inhibit spontaneous sheep cell rosetting (Jondal *et al*, 1972). It does not inhibit the binding of C3 by human monocytes (Shevach *et al*, 1972).

Class specific receptors on mononuclear cells differ from the receptors for antigen upon antigen binding lymphocytes, both in the number of cells reacting, and in the function of the receptors. Receptors for antigen are present upon few lymphocytes and these antigen reactive cells initiate the immune response. In human mononuclear cell populations the function of receptors for sheep cells (E), Fc fragment of IgG (EA_(IgG)), and complement (EAC) are not clear.

Knowledge of the number of each class of mononuclear cell expressing these receptors provides a powerful diagnostic tool for the pathologist in the fields of immune deficiency disease and lymphocytic or histiocytic neoplasms, and may give a more rational basis for the treatment of these disorders by the clinician.

PLAN OF EXPERIMENTS

Preliminary Experiments

Before proceeding to the experimental series reported here, the methods of preparation and standardizing the red cell suspensions, and the rosetting techniques used, were tested using cell suspension from fresh human thymus glands. In a series of seven, the mean values for E rosettes were 70%, EA_(IgG) rosettes 12.8%, and EAC rosettes 2.4%, the mean viability of the thymocytes being 84%. This showed that the methods employed for sensitization of red cells effectively excluded E rosetting determinants on cells sensitized with IgG or complement.

In peripheral blood, the numbers of cells forming rosettes with EAC cells sensitized with

sub-lytic concentrations of human or guinea-pig complement were shown to be unacceptably variable, due, it was thought, to variation in the numbers of monocytes expressing receptors for complement. The two-stage technique reported here detected numbers of rosetting cells numerically equivalent to the fluorescent B cell population detected by immunofluorescence, and in our hands proved less variable than the original single stage sensitization used before. The exact status of the complement receptor expressed is at present unknown, but the rosetting cells detected appeared to be B lymphocytes (which possess C₃ receptors) and the sensitized red cells were agglutinated by anti-C₃ (Meloy reagents) but not by anti-C₄.

Experimental Series

In the initial experiments venous blood samples from 15 healthy subjects (nine male and six female) aged 20–60 years were studied in respect of total white cell recovery, mononuclear cell recovery, E rosetting, EA_(IgG) rosetting and surface immunofluorescent cell populations. In addition, the reproducibility of the technique was assessed by repeat determinations of these characteristics at weekly intervals in three individuals.

Following this, a second series of samples from 12 different healthy individuals was studied. A comparison of the number of cells was made for sheep cell (E) rosetting, surface immunofluorescent, and phagocytic mononuclears following dextran sedimentation and ficoll triosil separation. On ficoll triosil separated samples, in addition, EAC and EA_(IgG) receptor mononuclears were studied in the presence and absence of EDTA. From data accumulated in these two series quantitative values for the separate classes of peripheral blood mononuclears were calculated, together with standard deviations for both series.

MATERIALS

1) Ficoll/Triosil Mixture

10 parts of 33.9% 'Trisosil 350' (Nyegaard & Co., Oslo) were mixed with 24 parts of 9% Ficoll' (Pharmacia), sterilized by membrane filtration (pore diameter 0.22 μ) and stored at room temperature in 10 ml quantities in siliconized universal containers. The specific gravity was 1.076.

2) Sheep Red Blood Cells (E)

20 ml blood from a single sheep was obtained by venepuncture and mixed with 5 ml Alsever's solution containing penicillin 100 iu/ml, and streptomycin sulphate 200 μ g/ml in the final mixture. Sheep cells were obtained weekly, and stored at 4°C. Cells were used within 1 week.

3) Other Reagents

Stock solutions of veronal buffered saline (VBS), 0.10 M trisodium ethylenediaminetetraacetate (EDTA) and 1.0 M MgCl₂ + 0.15 M CaCl₂ were prepared (Rapp & Borsos, 1970).

VBS-G-EDTA. 100 ml stock VBS was mixed with 395 ml glass distilled water, and 5 ml of warmed stock 10% gelatin solution, to give a final gelatin concentration of 0.1%. The gelatin used was acid ossein (Credo Food Products Ltd), pI 7.1, and was devoid of anti-

complementary activity. 450 ml of VBS-gelatin was mixed with 50 ml of stock EDTA solution (VBS-G-EDTA). This solution was freshly prepared each week, sterilized by filtration, and stored at 4°C.

VBS-G-Ca²⁺Mg²⁺. 20 ml stock VBS was added to 0.1 ml of stock 1.0 M MgCl₂ + 0.1 M CaCl₂, and 1 ml of 10% gelatin solution (warmed) added. The total volume was made up to 100 ml with glass-distilled water. This solution was prepared fresh each day.

(4) *Rabbit Anti-Sheep-RBC-IgM Haemolysin*

This was prepared by the immunization of rabbits intravenously with sheep red cell stroma according to the method of Kabat & Mayer (1961). The serum obtained was titrated and IgM antibody separated by gel filtration on Sephadex G-200, in Tris-HCl buffer, pH 7.3. The first peak effluent contained most of the antibody activity as judged by haemolytic titration. Titres were 1 in 8500 for 100% lysis with 1 in 10 guinea-pig serum (complement Wellcome) diluted with VBS-G-Ca²⁺Mg²⁺. Sheep cells were sensitized with a 1 in 4000 dilution of antibody.

(5) *Rabbit-Anti-Sheep-RBC-IgG*

This was prepared by the intraperitoneal injection of 10% suspension of washed sheep red cells (5 ml) weekly for 6 weeks. Agglutination titres of purified IgG antibody were in 2000. In some of the later experiments rabbit anti-sheep RBC serum (Wellcome Laboratories) was used, at a concentration of 80% of the minimal agglutinating titre.

(6) *Fluorescence Antisera*

Indirect immunofluorescence used polyvalent rabbit anti-human-IgG+IgM+IgA prepared by immunization of rabbits with purified pooled immunoglobulins separated from myeloma sera. FITC coupled goat anti-rabbit antiserum (Meloy reagents) was used in the second stage of the procedure. Commercially available polyvalent rabbit anti-human IgG+IgM+IgA was also used (Sera Services Ltd). Aggregated material was removed by centrifugation for 2 h at 40 000 g before use.

(7) Neutral red was made up as a 0.01% solution in 9 g/l NaCl (Saline), and sterilized by filtration.

METHODS

PREPARATION OF MONONUCLEAR CELLS

(A) *Ficoll/Triosil Separation*

20 ml of venous blood from each subject was taken into 200 units preservative free heparin (Evans Medical) or mixed with lithium heparin (Searle) in a plastic tube. A white cell count was done and smears made for later differential counting. 10 ml of anticoagulated blood was then carefully layered onto 10 ml of ficoll/triosil mixture, and centrifuged at 4°C for 25 min in standard siliconized universals at 800 g at the blood/ficoll interface.

The whole leucocyte band was carefully removed and pipetted into plastic tubes containing ml medium 199 (Wellcome) with heparin 10 units/ml. Cells were then washed three times in a large excess of medium 199 (280 g for 5 min), and resuspended in a known volume of medium 199. Smears were made, and the total white cell count and recovery calculated. The total recovered cells were divided by the original blood volume to give the values per ml of recovered leucocyte population, from which data the mononuclear cell recovery could be calculated. Viability was assessed by trypan blue dye exclusion, in all cell preparations.

B) Dextran Sedimentation

Blood was obtained as before; white cell counts and smears were made. 20 ml of blood was mixed with 6.7 ml of 6% Dextran 250 (Pharmacia) in saline (filter sterilized before use) and incubated for 1 h at 37°C. The total supernatant plasma was removed and the leucocytes were washed as before. Smears of the cell suspension were made, and total white cell count and recovery were calculated.

C) Ammonium Chloride Lysis

20 ml of heparinized venous blood was obtained as before. A white cell count was performed and smears were made. The blood was then centrifuged, the supernatant discarded and the cells washed twice in a large excess of medium 199 + 10 units heparin/ml to remove all traces of serum. The third wash was in phosphate-buffered saline (PBS). Cells were resuspended in a small volume of PBS and added dropwise with stirring to a mixture of 9 parts 0.83% NH₄Cl solution + 1 part 0.85 M Tris buffer, at 4°C (see Dioguardi *et al*, 1963). The optimal blood/NH₄ volume ratio for complete lysis should be determined by experiment (about 10 ml for 1 ml blood). After incubation at 4°C for 10 min, the white cells were recovered and washed three times in medium 199. Smears of the recovered suspension were made, and total white cell counts and recovery calculated.

All smears were stained with Giesma's stain, and differentials (500 cells counted) were performed on whole blood and on the separated leucocyte populations.

PREPARATION OF RED CELLS

(1) Sheep cells (E) were washed four times in PBS, and made up to a standard concentration of 400×10^6 /ml.

(2) EA_(IgG) (IgG sensitized cells). Washed sheep cells were incubated in 7S rabbit-anti-sheep-RBC-IgG or rabbit-anti-sheep-RBC serum, in a concentration equivalent to 80% of the minimum agglutinating titre for 1 h at 37°C. Sensitized cells were then washed twice in PBS and resuspended at standard concentration in PBS (for EA_(IgG) rosettes) or VBS-G-EDTA (for EA_(IgG)-EDTA rosettes).

(3) EAC sensitized cells. Washed sheep red cells were sensitized in 1 in 4000 dilution of rabbit-anti-sheep-RBC-IgM for 1 h at 37°C. Sensitized cells were then washed once in ice cold VBS-G-Ca²⁺Mg²⁺, and incubated on ice for 30 min with either fresh human serum, or guinea-pig serum (Wellcome) diluted 1 in 10 with VBS-G-Ca²⁺Mg²⁺ as a source of complement. They were then washed three times with ice cold VBS-G-EDTA,

allowed to reach room temperature, and then mixed with human or guinea-pig serum diluted 1 in 10 with VBS-G-EDTA. Incubation was then continued for a further 30 min at 37°C, followed by washing twice in VBS-G-EDTA. The red cells were resuspended at standard concentration in either PBS (EAC cells) or in VBS-G-EDTA (EAC-EDTA cells). Minimal lysis of sensitized cells occurred. Sensitized cells were prepared fresh and were not stored in excess of 24 h.

Preparation of Rosettes

In each case 1×10^6 leucocytes were mixed with 40×10^6 red cells of the appropriate suspension, in siliconized Wasserman tubes. The total volume was made up to 1 ml, with medium 199, for E, EA_(IgG), EAC rosettes, or with PBS for EA_(IgG)-EDTA, EAC-EDTA rosettes. EAC, EAC-EDTA and E rosettes were prepared by incubation for 5 min at 37°C, while EA_(IgG), EA_(IgG)-EDTA preparations were incubated for 10 min at room temperature. All rosette preparations were then cooled to 4°C, centrifuged at 180 g for 5 min, and incubated for 2 h at 4°C.

Resuspension and Counting

E rosettes: great care is necessary to ensure complete resuspension without disruption of rosettes. Rosettes were resuspended by rotation of the tube on its long axis for a standard number of turns ($\times 10$). Other rosettes were resuspended by end over end inversion of the stoppered tube five times.

Suspended cells were drawn into a Pasteur pipette by capillary action. A single drop just sufficient to fill the chamber was placed on either side of a counting chamber (Improved Neubauer) and the coverslip lowered into place and pressed down. Rosettes were enumerated as lymphocytes binding three or more red cells, in at least three large squares on each side of the counting chamber. E rosettes were quantitated as a direct percentage of viable mononuclears. All counts were performed in duplicate (two observers) and the values expressed as the mean of the two counts; those varying by more than $\pm 10\%$ were repeated. Finally, white cell counts were performed on each of the rosette preparations. Rosettes were quantitated as percentages of the recovered cell population.

Neutral Red Ingestion

1×10^6 white cells were mixed with 0.5 ml of neutral red solution and incubated at 37°C for 10–15 min. The cells were pelleted gently and resuspended in a small volume of medium 199. A drop was placed on a slide, covered with a coverslip and sealed with nail varnish. The percentage of cells ingesting neutral red was quantitated excluding polymorphs (if present) and dead cells.

Immunofluorescence

The indirect (sandwich) technique was used in the series reported here. Optimal dilution (as previously determined) of polyvalent rabbit anti-human IgM + IgG + IgA antibody was added to 2×10^6 washed leucocytes, the cells evenly suspended, and incubated for 10 min at 37°C, in siliconized Wasserman tubes. The cells were then washed twice (280 g for 5 min) in PBS, and cooled to 0°C in melting ice. To the cell suspension was added a 1 in 20 dilution of

FITC coupled goat anti-rabbit-serum, and incubation at 0°C was continued for a further 30 min. The cells were then washed twice in PBS, resuspended in a small volume, a drop placed on a slide and sealed under a coverslip. Slides were examined immediately, using a Reichart 'Zetopan' research microscope, with dark ground condenser, HBO 200 mercury vapour lamp, FITC-3 and BG-3 filters and amber eyepiece filter. Conventional illumination was employed alternately for cell counting and morphological assessment. Viable mononuclears only were counted in 20 high power fields (glycerol immersion $\times 800$), and the fluorescent cells expressed as a percentage of mononuclears, omitting polymorphonuclears.

Two categories of cells were counted. Fluorescent capping cells had to show clear caps, or confluent ring fluorescence to qualify (see Fig 1a, b, c) Fluorescent non-capping cells were classified as mononuclears showing small dots of fluorescence diffusely localized over the cell surface (Fig 1d). This category of cell does not cap during prolonged incubation, but fluorescent material gathers in a ring of small vacuoles just beneath the cell membrane.

Quantitation of Receptors in Whole Blood

From the white cell count, and the blood volume, the total WBC in the original blood sample were calculated. The total of recovered white cells following separation was noted from the cell count and volume of the washed cell suspension. Differential counts in whole blood and in the separated fraction enabled the mononuclear cell recovery to be calculated. The numbers of mononuclears expressing each receptor in the recovered fraction were calculated. This numerical value, divided by the mononuclear recovery per cent, multiplied by 100, gives the value for each receptor per ml of whole blood.

Formula

Receptor per ml whole blood

$$= \frac{\text{No. of mononuclears with receptor in recovered population} \times 100}{\text{Percentage mononuclear recovery}}$$

In practice the numbers of polymorphs in ficoll/triosil separated blood were very small, and for this method of separation correction for polymorphs in determining total mononuclear recovery was not usually necessary, total recovered white cells being virtually equivalent to total recovered mononuclears.

RESULTS

1) Viability

By all methods of preparation more than 95% of cells excluded trypan blue in all cell samples.

2) Differential Counts

On whole blood all total white cell counts, and absolute counts of each cell type, fell within the range of normal values.

(a) *Ficoll/triosil suspensions.* The mean value for polymorphonuclear contamination was 0.9%, representing in absolute numbers a range from 0.0017×10^6 to 0.2844×10^6 cells/ml

of the recovered suspension. Mean values for mononuclear cells were $0.96 \pm 0.53 \times 10^6/\text{ml}$ in the same suspensions.

(b) *Dextran suspensions*. Mean values for recovered mononuclears in white cell suspension prepared by dextran were $1.12 \pm 0.48 \times 10^6$ cells/ml. Polymorphonuclear contamination was high, occasionally exceeding the differential counts in whole blood. (Mean values for polymorphonuclears 32%, $1.16 \times 10^6/\text{ml}$.)

(c) *Ammonium chloride*. Polymorphonuclear contamination was intermediate between dextran and ficoll/triosil preparations, but still substantial. Leucocytes prepared by this method tended to clump.

(3) Mononuclear Cell Recoveries

Percentage values for mononuclear cell recoveries in both series are shown in Table I

TABLE I

Series I. Percentage white cell recovery, and percentage of mononuclear cell recovery from ficoll/triosil gradients

Subject No.	% White cell recovery	% Mononuclear cell recovery
1	6.3	34.4
2	15.0	34.1
3	23.1	20.2
4	17.7	40.3
5	12.6	31.8
6	10.4	20.0
7	14.0	30.3
8	11.0	35.7
9	10.0	33.2
10	12.5	30.1
11	19.7	37.0
12	12.7	33.3
13	17.2	43.7
14	13.7	20.3
15	20.9	23.1
Mean	14.52	33.34
SD	± 4.38	± 6.65
SE	4.96	11.42

Series II. Percentage of white cell recovery and mononuclear cell recovery following separation on ficoll/triosil or dextran; series of 12 subjects, mean values with range of values given in parentheses.

	% Recovery white cells dextran	% Recovery mononuclears dextran	% Recovery white cells ficoll/triosil	% Recovery mononuclears ficoll/triosil
Mean	52.0	46.8	16.4	34.0
Range	(32-68)	(37-68)	(6-41)	(14-61)
SD	± 11.6	± 12.0	± 9.65	± 17

Classification of Mononuclear Cells

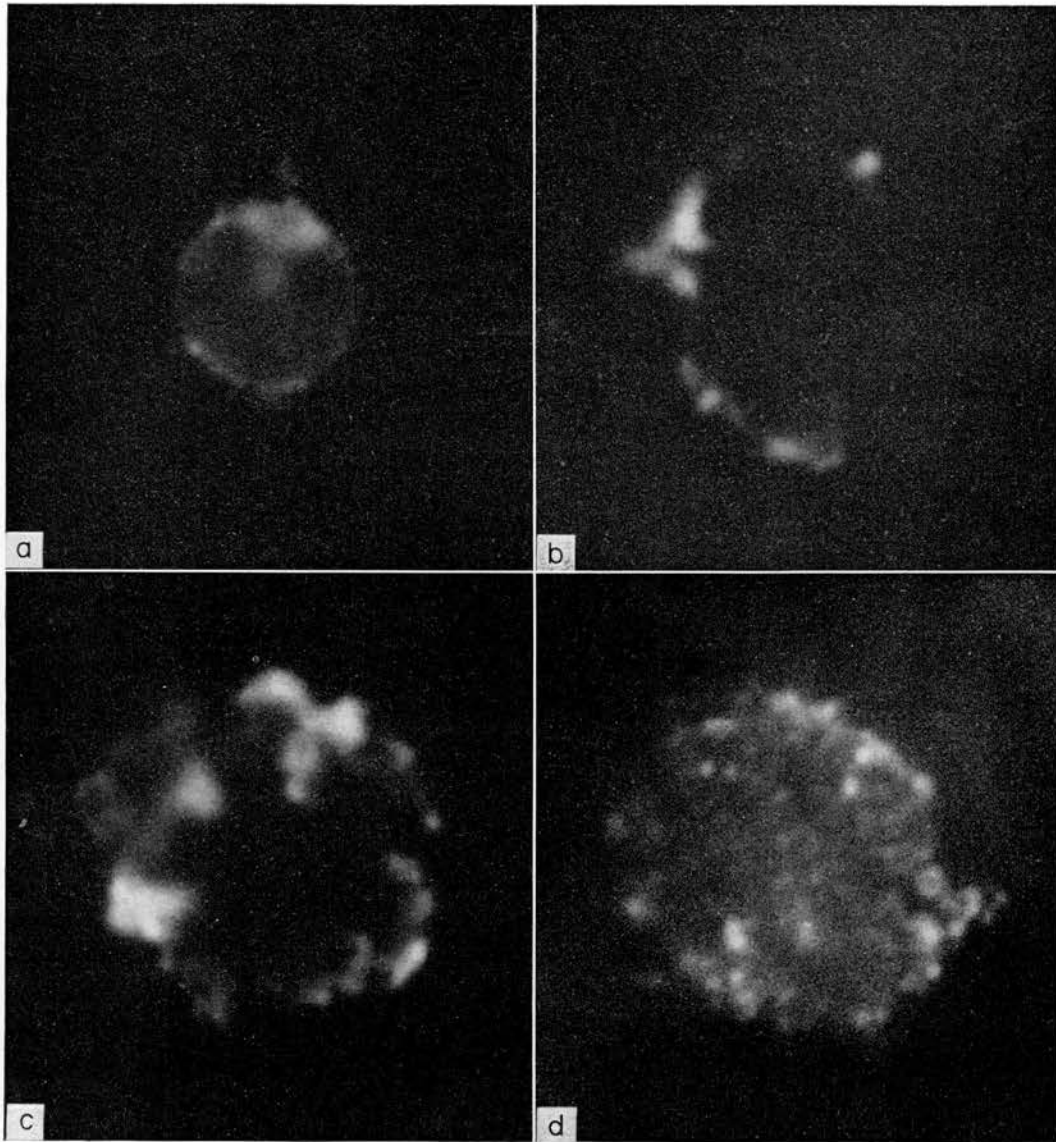


FIG 1. Patterns of membrane fluorescence. (a) Confluent ring fluorescence, with early capping (small B lymphocyte). (b) Fluorescent capping cell (large B lymphocyte). (c) Patchy membrane fluorescence (large B lymphocyte). (d) Fluorescent non-capping cell. All photographs $\times 2000$; living cells, indirect membrane immunofluorescence.

TABLE II. Mean percentage values of mononuclear cells reacting with sheep (E), antibody coated erythrocytes (EA_(1gG)) and of fluorescent capping cells and fluorescent non-capping cells obtained by repeat determinations on three individuals: Subject I studied six times, Subject II studied on five occasions, and Subject III on four occasions, with ficoll/triosil recovery, and WBC counts. Range of values for each subject given in parentheses.

Subject	E receptor cells	EA _(1gG) receptor cells	Fluorescent capping cells	Fluorescent non-capping cells	Recovery of white cells	WBC count/ml blood
I	67% (58-70)	24.8% (16-40)	14.4% (11-19)	9.8% (6-19)	10.62% (6.3-15)	5.99×10^6 (5.2-6.1)
II	55.1% (48-64)	39.2% (31-47)	10.6% (7.2-14)	9.56% (7-21)	14.2% (11.6-17.7)	5.85×10^6 (5.6-6.2)
III	60.0% (55-63)	32.25% (21-40)	14.25% (13-15)	12.8% (9-16)	14.25% (12-17)	4.28×10^6 (3.25-5.83)

By either method of separation only a minority recovery of mononuclear cells was achieved, and mononuclear recoveries with dextran and ficoll/triosil separations were similar. The range of particles which could be studied was greater for the ficoll/triosil method than for dextran, owing to the presence of receptors for EA_(1gG) and EAC on the contaminating polymorphonuclear populations.

(4) Individual Variability

Repeat determinations were made on three subjects in this study at weekly intervals. The data are given in Table II. Mean values, recovery, and white cell count are shown together with the total range. Subject II is female, Subjects I and III male.

(5) Effect of Different Methods of Separation

In view of the potential criticism that the recovered cell population did not reflect accurately the receptor status in whole blood, a comparison was made between dextran and ficoll/triosil separated mononuclears in respect of fluorescent cells, neutral red phagocytic cells, and spontaneous sheep cell rosettes. The assumption was that if one method of separation favoured one class of cell, this should be reflected in the compared figures. We believe the data presented (Table III) suggest that E rosettes tend to be higher, and B fluorescent capping cells lower in ficoll/triosil separated than in dextran sedimented blood, but this trend is not statisti-

TABLE III. A comparison of E rosetting cells, fluorescent cells and phagocytic cells in samples separated by dextran (12 individuals), ficoll/triosil (12 individuals) and ammonium chloride lysis (three individuals); results given as cells/ml whole blood

	Dextran sedimentation	Ammonium chloride	Ficoll/triosil
E rosettes	$0.83 \times 10^6 \pm 0.099$	0.89×10^6	$1.0 \times 10^6 \pm 0.23$
Neutral red	$0.52 \times 10^6 \pm 0.15$	0.38×10^6	$0.37 \times 10^6 \pm 0.12$
Fluorescent capping	$0.76 \times 10^6 \pm 0.15$	0.50×10^6	$0.55 \times 10^6 \pm 0.26$
Recovery of mononuclears	$46.8 \pm 12\%$	53.0%	$34.0 \pm 17\%$

TABLE IV. Values of subclasses of receptor expressing mononuclears $\times 10^6$ per ml normal venous blood. Cells separated on ficoll/trisil gradient. Series 2, 12 subjects: \pm SD and SE

	No. $\times 10^6$ /ml	SD	SE
E	1.01	± 0.23	± 0.0532
EA _(1gG)	0.73	± 0.23	± 0.0529
EA _(1gG) -EDTA	0.94	± 0.34	± 0.1186
EAC	0.59	± 0.30	± 0.0922
EAC-EDTA	0.94	± 0.53	± 0.2809
Neutral red	0.38	± 0.12	± 0.0137
Fluorescent capping	0.55	± 0.26	± 0.0658
Fluorescent non-capping	0.31	± 0.11	± 0.0122
Recovery of white cells	16.4% \pm 9.65%		
Mononuclear recovery	34.0% \pm 17%		
WBC count	5.98 \pm 1.28		

cally significant. The choice of receptors for comparison is limited to those in which polymononuclears can be excluded, by direct microscopy (as with neutral red or fluorescence) or by absence of receptor (as with the E rosette).

(6) Normal Values of Receptor Mononuclears in Whole Blood

These are given in Table IV, and a comparison between the first series (15 subjects) and the second series (12 subjects) is given in Table V. Two unexpected features are presented. Firstly, the fluorescent non-capping population is quantitatively similar to the neutral red phagocytic population. Secondly, the mononuclears expressing receptors for EAC and EA_(1gG) are numerically equivalent, but only in the presence of EDTA. A comparison between the two series (Table V) shows that with normal subjects, using the standard techniques described, reproducible results can be obtained on separate occasions.

TABLE V. Comparison of first and second series of E rosettes and EA_(1gG) rosettes and fluorescence

	Receptors Series 1 (No. $\times 10^6$ /ml)	Receptors Series 2 (No. $\times 10^6$ /ml)
E	1.09 \pm 0.27	1.01 \pm 0.23
EA _(1gG)	0.73 \pm 0.36	0.73 \pm 0.23
FC	0.42 \pm 0.14	0.55 \pm 0.26
FNC	0.18 \pm 0.09	0.31 \pm 0.11
Recovery (whole WBC)	14.52 \pm 4.4%	16.14 \pm 9.69%
Recovery (mononuclears)	33.3 \pm 6.6%	34 \pm 17%
Mean WBC count	4.89 \pm 1.18	5.98 \pm 1.28

This table shows the reproducibility of the technique in assessing numbers of receptor expressing mononuclear cells in two independent series.

DISCUSSION

Techniques for the quantitation of subclasses of human mononuclear cells have been only recently established, and their value in clinical medicine has yet to be assessed. Before useful conclusions can be drawn from the 'experiments of nature' encountered clinically, the normal data must be established. Early in this study it was appreciated that percentage quantitation alone, without reference to white cell count, or recovery of mononuclears was of very limited value. It was also clear that the simultaneous study of a wide range of receptors was more likely to provide the basis necessary for the future interpretation of changes in disease.

One of the least satisfying findings was that none of the separation techniques employed gave more than a minority recovery of mononuclear cells. The quantitative study therefore had to be based upon white cell counts on whole blood, and raised the question of selective depletion of one cell class over another as a consequence of the cell separation procedure. We were unable to show that significant alterations in classes of receptor bearing cells were related to the separation procedure, although in our hands ficoll/triosil separation resulted in a relative enrichment of E rosettes and corresponding depletion of fluorescent capping cells.

Variability between individuals (Table II) can be accounted for by variation in white cell count, mononuclear recoveries, and possibly other changes related to exercise or stress at the time of sampling (Steel *et al.*, 1974). This does not entirely explain the source of 'internal variability' noted in respect of certain classes of receptor in these experimental series. Within the limits of experimental error it is usually possible to account for all peripheral blood mononuclears by adding the totals of E rosetting, EAC rosetting (or fluorescent capping cells) and neutral red ingesting cells. Both B cells and monocytes have receptors for C₃ and for IgG. It appears from our data that for the monocyte to express these receptors by an adherence reaction, EDTA must be present. Divalent ions stimulate phagocytic uptake by these receptors, but not adherence (Rabinovitch, 1967; Mantovani *et al.*, 1972). In the absence of EDTA, EAC-receptor bearing cells are quantitatively similar to the fluorescent capping cell (B lymphocyte). There is, however, a lack of correspondence between receptor expressing mononuclears of EA_(IgG), EAC-EDTA, and EA_(IgG)-EDTA subclasses and fluorescent capping cells, implying that a population other than the B lymphocyte may (a) carry surface immunoglobulin but not 'cap' and (b) express IgG receptors. Recently a population of lymphocytes in normal human peripheral blood expressing E and IgG receptors, and showing surface fluorescence, has been described (Dickler *et al.*, 1974). Our observations support the concept of a fourth class of mononuclear cell, but owing to the technique used for quantitation statistical verification of this concept is not possible. An additional cause of variation between individuals observed in this series, lies in the reciprocal expression of E and EA_(IgG) receptors. In both series, some individuals had high EA_(IgG) rosetting, and low E rosetting cells, and others had low EA_(IgG) and high E rosetting cells. These trends were not statistically significant in a small series, but they indicate that the patterns of E and EA_(IgG) rosetting may distinguish between individual subjects (e.g. Subjects I and II, Table II). The fluorescent non-capping cell has not previously been described as a separate subclass of immunoglobulin carrying mononuclear cells. It is quantitatively similar to the population of cells ingesting neutral red, and might be regarded as monocytic. In our experience, cells from monocytic

leukaemia which retain their capacity to ingest neutral red generally fail to stain for surface immunoglobulin, even though they express IgG receptors. Other authors describe fluorescent cells similar to those described here, and regard them as B cell in type (Papamichael *et al.* 1971; Frøland & Natvig, 1972).

The spontaneous rosetting of sheep cells with human peripheral blood mononuclears can undoubtedly define a subpopulation of lymphocytes. The evidence for the E receptor lymphocyte being a 'T' cell is based largely upon studies of human thymus (Jondal *et al.* 1972; Silveira *et al.*, 1972; Whittingham & Mackay, 1973) and of rosetting in parallel with T cell quantitation by anti-thymocyte antiserum (Yata *et al.*, 1973; Aiuti & Wigzell, 1973). In our view it is premature to regard the sheep cell rosette test as an indicator of cellular immunity in man as there is only circumstantial evidence linking deficiency of the E rosetting population with absent or deficient T cell function. The usefulness of the E receptor lies in the consistent values obtained for E rosetting cells in normal venous blood, and it remains to be seen whether depletion of this class of cell is associated with clinical evidence of disordered T cell function.

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ROSETTING AND OTHER REACTIONS OF THE REED-STERNBERG CELL

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PLATES XXXVII-XL

FRESH unfixed biopsy material of spleen and lymph nodes are routinely made into single cell suspensions in this laboratory for diagnostic tests. This material comes from a variety of conditions. It has been noted that cell suspensions from tissues subsequently found to be marked with Hodgkin's disease show a characteristic clustering of lymphocytes around larger cells which can be identified as Reed-Sternberg cells. This clustering is visible within moments of preparation of the cell suspension, as soon as it is placed under the microscope. Similar clusters of lymphocytes can often be seen on imprints of Hodgkin tissue stained with Giemsa, and it is likely that the phenomenon is representative of some specific process occurring *in vivo*. Control suspensions of normal spleen removed incidental to abdominal surgery, or after traumatic rupture, reactive tonsillar tissue, and spleens, lymph nodes and peripheral blood leucocytes from a variety of other conditions including malignancies have not been observed to show clustering of this type. In this paper we explore the hypothesis that the lymphocyte rosetting reaction may be useful in identifying the precursor of the Reed-Sternberg cell. Because much of the work on the Reed-Sternberg cell has been contradictory, the findings of others in relation to cytochemistry and immunology have been correlated with our own. A brief account is given of staining with anti-human monocycle serum.

MATERIALS AND METHODS

Source of material

Four lymph nodes and one involved spleen were obtained from patients with Hodgkin's disease of mixed cellularity or lymphocyte depletion type. All contained numerous uninucleated and multinucleated Reed-Sternberg cells on histological examination. The surface was cut with a sharp knife and gently but firmly scraped. Cells removed in this way were harvested in Medium 199 and washed once. Cytocentrifuged preparations contained 0.5×10^6 cells/ml which were centrifuged at $3g$ for 10 min. in a volume of 0.5 ml.

Reed-Sternberg cells are defined as large cells, either uninucleate or multinucleate and measuring 20–50 μ diameter. The nucleoli are characteristically prominent and eosinophilic, the cytoplasm is extensive and appears pale pink after staining with haematoxylin and eosin and light blue after Giemsa's method. The nuclear membrane is distinct and the nuclei are often lobulated.

Chamber culture technique

The chambers consisted of a piece of perspex measuring 7.6 cm \times 2.5 cm \times 0.15 cm with a circular hole of 1.25 cm diameter in the middle. The hole was covered on one side with a

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coverslip and sealed with silicone grease. The chambers and coverslips were exposed to UV light overnight. They were filled with cell suspension using a sterile Pasteur pipette and covered gently with another coverslip, taking care not to leave air bubbles in the chamber. Time lapse cinephotography was done using a phase contrast inverted microscope fitted with a warm stage. Time lapses of 5 s or 10 s were selected.

Cell receptors and membrane fluorescence

E rosettes were prepared by incubating washed sheep erythrocytes and lymph node cells in a ratio of 40 : 1 for 10 min. at 37°C. The mixture was centrifuged at 30 g for 5 min., cooled at 4°C and incubated at 4°C for 2 hr. The cells were gently resuspended in 5 ml of Medium 199 before cyto centrifugation.

IgG rosettes for detection of the Fc receptor were made by sensitising ox red cells for 1 hr at 37°C with purified IgG obtained by injecting rabbits with ox erythrocytes.

Complement coated erythrocytes were prepared by mixing washed ox red cells with purified IgM antibody and fresh human serum adsorbed with acetylated yeast cell walls. The mixture was incubated for 30 min. at 37°C and washed in appropriate veronal buffers containing calcium and magnesium ions.

Cells bearing surface immunoglobulin were identified in suspension preparations by indirect immune fluorescence using both polyvalent and monovalent rabbit anti-human immunoglobulin (for detail see Habeshaw and Young, 1975).

Anti-monocyte serum. Cyto centrifuge specimens fixed in ethanol were stained with an anti-human serum (Stuart, Young and Grant, 1976) using indirect fluorescence combined with phase contrast.

Cyto centrifugation of rosette preparations. Rosettes were formed by the incubation of the appropriate red cell suspensions with lymph node cell suspensions, or pronase-treated cell suspensions in the ratio of [ten RBC] to one [leukocyte (10×10^6 red cells + 1×10^6 lymph node cells)] in a volume of 1 ml of Medium 199. The cell mixtures were incubated for 10 min. at 37°C, centrifuged at 80 g for 5 min., cooled to 4°C and incubated at 4°C for 2 hr. The rosettes were gently resuspended, and diluted to 5 ml by the addition of Medium 199. 0.5 ml was added to each cyto centrifuge chamber and spun at 150 r.p.m. for 5 min. in the cyto centrifuge (Shandon-Elliott Cytospin). The specimens were then fixed in methanol and stained with Giemsa's stain.

Cytochemistry

Preparation of substrates

Non-specific esterase. 20 mg naphthyl acetate were added to 15 ml of 0.1M phosphate buffer (pH 7.3) and 15 ml distilled water. 20 mg "Fast Red TR" were added and stirred thoroughly. The resulting solution was filtered and used almost immediately.

Acid phosphatase. 20 mg sodium naphthyl phosphate were added to 20 ml HCl-veronal buffer mixture pH 5. Polyvinyl pyrrolidone and 20 mg "Fast Garnet GBC" were added, stirred, filtered and used fresh.

Alkaline phosphatase. 20 mg sodium naphthyl phosphate were added to 20 ml TRIS buffer pH 10. 20 mg "Fast Red TR" were added, and after stirring the solution was filtered and used.

Peroxidase. 0.2 g benzidine was moistened with a few drops of distilled water and ground in a mortar and pestle. 200 ml of distilled water were added and the solution filtered. 0.25 ml fresh 3 per cent. H_2O_2 was added to the filtrate.

Demonstration of enzyme activity. All smears, dabs and cyto centrifuge preparations were fixed in 90 per cent. ethanol for 2 min. except those investigated for peroxidase activity, which were left unfixed. Non-specific esterase was demonstrated by incubation with the substrate for 20 min. at 37°C. The slides were washed, counterstained with haematoxylin and mounted in glycerine jelly. Acid and alkaline phosphatases were incubated for 30 min. at 37°C and their subsequent treatment was identical to that of the esterase preparations. Peroxidases were treated with 0.5 per cent. copper sulphate for 1 min. Benzidine solution was then

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applied for 5–8 min. Thereafter the slides were washed in water and counterstained with neutral red. With these methods non-specific esterase acid and alkaline phosphatases appeared as brownish-red areas. Peroxidase activity appeared blue.

Immunoperoxidase technique. (Taylor and Burns, 1974). Formalin fixed paraffin sections were brought to alcohol. Endogenous peroxidase was inactivated with a methanol solution of H_2O_2 and the sections treated with normal swine serum (1 in 20 in TRIS buffered saline). Excess serum was poured off after 10–15 min. at room temperature. Specific rabbit anti-human serum diluted in 1 in 20 normal swine serum in TRIS saline was applied at an optimal concentration for 30 min. Purified antisera with activity against IgG, IgA, kappa and lambda chains were used. Swine anti-rabbit serum, diluted 1 in 20 in TRIS saline, was allowed to react with the sections for a further 30 min. at room temperature. After washing, peroxidase rabbit anti-peroxidase immune complex solution (1 in 100) was applied for 3 min. At the end of all stages the sections were thoroughly washed in TRIS buffered saline. The sections were then exposed to DAB solution (3,3'-diamino benzidine) prepared by adding 6 mg of DAB to 10 ml of TRIS buffered saline. Two drops of fresh 10 vol. H_2O_2 were added to this solution which was used within 20 min. Colour reaction was developed for 3 min.

Pronase treatment of cell suspensions. Separation of lymphocytes from Reed-Sternberg cells was performed by incubating a part of the fresh suspension with pronase solution at a final concentration of 100 μ g/ml at 37°C for 1 hr with intermittent agitations. This was followed by washing three times in buffered saline pH 7.4. The cells were counted and their viability assessed by exclusion of trypan blue. IgG-coated ox erythrocytes were used to test for the Fc receptor followed by cytocentrifugation, ethanol fixation and staining by Giemsa's method.

RESULTS

Cinicrophotography. Time lapse films taken of the living cell suspensions revealed a dynamic interaction between lymphocytes and Reed-Sternberg cells. This intercourse was observed to be of two different types. Some lymphocytes slide steadily over the surface of the large cells in a vermiform manner, never ceasing their gliding movements around the central cells (figs. 1 and 2). Other lymphocytes appear to form immobile attachments by their uropods to the Reed-Sternberg cell. These cells are actively motile, but are attached by a narrow thread of cytoplasm; this attachment seems firm—lymphocytes can be seen tethered by an elongated process to the cell surface, with their nucleus and most of their cytoplasm some distance away. Even when accidental currents of fluid flow through the culture chamber these motile cells are not swept away, and maintain contact with the Reed-Sternberg cell. The surface of the central cell often becomes completely covered with lymphocytes, forming a structure like a bunch of grapes (fig. 3). Lymphocytes arrive and can burrow their way into this mass of cells. Once a lymphocyte becomes a participant in such a mêlée around the Reed-Sternberg cell, it can still detach itself, but very often it appears to have difficulty in doing so.

Although the Reed-Sternberg cell does not seem to suffer from these inquisitive interactions, one typical cluster was observed and filmed in which disintegration of the morula occurred, followed by desertion by the lymphocytes of several dead Reed-Sternberg cells.

On several occasions cytoplasmic processes have been observed extending from the Reed-Sternberg cell. These are of two quite different types. The first type are long and thin, emerging from the surface like quills from a porcupine; they do not branch and most adhere to glass (fig. 4). The other type are not

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adherent to glass, and resemble fronds or veils of membrane floating freely from the surface of the Reed-Sternberg cell. They often show branching (fig. 5).

Macrophages do not participate in these reactions; they are more active than usual, adhere rapidly to glass, and commonly show multinucleation.

Morphology of cytocentrifuge preparations and short term cultures

Cytopentrifugation of the cell suspensions followed by Giemsa staining allowed morphological assessment of all the cells present in the suspension, in particular the cells at the centre of the clusters which cannot be seen in normal smear preparations. Binding of lymphocytes to those central cells appeared to be strong, as the procedure of cytopentrifugation did not disrupt the majority of rosettes and only infrequently were Reed-Sternberg cells seen without an almost complete halo of surrounding lymphocytes. Cells in the centres of these lymphocyte clusters showed a wide spectrum of shape and size. Large typical Reed-Sternberg cells, ten times the diameter of adherent lymphocytes, were always rosetted by lymphocytes (figs. 6 and 7). In addition to classical Reed-Sternberg cells, smaller cells with large nuclei and with an abundance of cytoplasm were part of the same central rosetting cell class. Smaller cells with basophilic cytoplasm and nuclei only slightly larger than lymphocytes also showed this distinctive rosetting reaction (figs. 8 and 9).

A subpopulation of lymphocytes was observed which showed a distinctive lobulation of the nucleus. Some showed segmentation of the nucleus into three or four lobes in a characteristic "clover leaf" form whereas others showed irregular buds which projected from the surface (fig. 10). The cytoplasm did not contain granules. Often these hyperlobulated forms were seen as participants in the lymphocyte rosette around Reed-Sternberg cells.

Cultures of cell suspension maintained over 4 days showed greater numbers of lymphocyte rosettes than did the cytopentrifuge preparations. Macrophages were readily identified as single cells in these cultures and did not form rosettes with lymphocytes as did the Reed-Sternberg cells. Dense clustering of lymphocytes around small mononuclear cells was especially evident in cultures; the nature of this central cell is not known but their appearance was quite different from the mature Reed-Sternberg cell.

Tests with anti-human immunoglobulins. Reed-Sternberg cells, identified under phase contrast illumination by their size, nuclear morphology and their rosette of lymphocytes, were not seen to show any specific fluorescence with polyvalent anti-human Ig serum. Occasionally a faint speckled fluorescence was observed and dead Reed-Sternberg cells showed diffuse cytoplasmic fluorescence. Antisera prepared against individual subclasses of immunoglobulin did not react with Reed-Sternberg cells. Although many lymphocytes showed bright specific fluorescence it was exceptional to find fluorescence on those lymphocytes surrounding Reed-Sternberg cells.

Tests with anti-human monocyte serum. The serum gave brilliant fluorescence with glass adherent cells, macrophages and granulocytes. No fluorescence was observed with Reed-Sternberg cells or attached lymphocytes.

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Membrane receptor tests. The ever-present lymphocyte rosette around the Reed-Sternberg cell often prevented attempts to characterise the cell's receptor profile. In fresh suspensions treated with sheep erythrocytes, E rosettes were shown by nearly all lymphocytes forming clusters. E rosettes are fragile and consequently the number seen in cytocentrifuge preparations is less than that obtained by counting fresh cell suspensions. It was not possible to say whether the Reed-Sternberg cell itself possessed receptors for sheep erythrocytes, but where the lymphocyte rosette was incomplete no sheep cells were adherent. The cytocentrifuge preparations revealed that a significant proportion of the hyperlobulated lymphocytes described above were T cells. E rosettes were seen around them in many cases and they often participated in the apparently exclusive T cell rosette around Reed-Sternberg cells. It was not possible to determine whether they were all T cells because, although no atypical lymphocytes were seen with IgG or complement receptors, it is possible that disruption of these rosettes occurred during centrifugation.

Pronase treatment of cell suspensions was successful in stripping the lymphocytes from the surface of the Reed-Sternberg cell. After this treatment, IgG sensitised red cells adhered to the surface in an apparently specific manner (fig. 11). Attempts to demonstrate an Fc receptor on adherent T lymphocytes were unsatisfactory.

Cytochemistry. Strong esterase activity, manifested by dense granular brownish-red deposits of reaction product was seen in a population of large round cells without adherent rosettes of lymphocytes, which were judged to be macrophages. Macrophages from one patient who had been subjected to lymphangiography before excision of his lymph node, showed very strong esterase activity. In this case the macrophages formed esterase-positive multinucleated giant cells.

The majority of mature Reed-Sternberg cells were negative for esterase activity, but in a significant number definite activity could be seen, consisting of a diffuse faint reddish-brown stain. In a number of cases a small central rosetting cell was seen to be esterase positive. Lymphocytes never showed esterase activity and neutrophil polymorphonuclear leucocytes showed granular positive activity.

No acid phosphatase activity was seen in any of the large number of Reed-Sternberg cells examined. Specific activity was noted within large cells which did not form rosettes and these were thought to be macrophages. No alkaline phosphatase activity was observed in any cells although positive controls showed strong activity. Reed-Sternberg cells were uniformly negative for peroxidase. Macrophages showed faint activity and neutrophil polymorphonuclear leucocytes showed strong activity.

Immunoperoxidase. The method consistently demonstrated the presence of immunoglobulin in plasma cells whose cytoplasm was stained deep brown. Staining of Reed-Sternberg cells was very variable and frequently negative; some uninucleate Reed-Sternberg cells gave a diffuse brown colour but this was never as deep as that of plasma cells. Occasionally, control slides in which saline was used instead of rabbit anti-human immunoglobulins antiserum

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showed a mild diffuse staining. This represents a non-specific adherence of antibody at some stage in the procedure rather than a failure to block endogenous peroxidase activity. Controls using DAB solution only on untreated sections revealed no endogenous peroxidase activity within Reed-Sternberg cells. The conclusion reached was that some, but not all, Reed-Sternberg cells contained IgG but the reaction was weak when compared with plasma cells.

DISCUSSION

Histological examination of Hodgkin's lymph node tissue sections has failed to identify a convincing precursor of the Reed-Sternberg cell. We believe, however, that the precursor can be recognised in viable single-cell suspensions. A consistent interaction of lymphocytes around both classical Reed-Sternberg cells and also around smaller mononucleated cells makes it possible to demarcate a family of cells illustrating the maturation of the Reed-Sternberg cell from its precursor. Cyto-centrifugation of such suspensions allows the morphology of the cells forming these rosettes with lymphocytes to be examined. Preliminary observations have revealed a cell measuring 12–14 microns diameter with a rounded nucleus and clear cytoplasm without granules which is moderately basophilic. It appears lymphoid, but further characterisation in terms of cytochemistry and immunology is obviously necessary.

Interaction of lymphocytes and Reed-Sternberg cells was described by Pulvertaft (1959) who filmed the migration of lymphocytes over the surface of the cell in cultures of tissue from Hodgkin's disease. He noted that no other cell type formed such prolonged attachments but made no comment on the significance of the phenomenon as the function of the lymphocyte was still obscure at that time. Clustering of T lymphocytes around Reed-Sternberg cells is now a well-documented finding but interpretation of the significance of the phenomenon has received scant attention. Perhaps the most acceptable interpretation is that an immune response is mounted by cytotoxic T cells against the Reed-Sternberg cell because of "foreign antigens" on its surface. Order *et al.* (1971) have produced evidence which does suggest the presence of a tumour-associated antigen in Hodgkin's disease to which lymphocytes may be reacting. Furthermore, it is well known that lymphocyte predominance in this disease correlates well with the patient's prognosis. Archibald and Frenster (1973) believe they can show a correlation between the fraction of the Reed-Sternberg cell surface occupied by lymphocytes and the amount of ultrastructural cytotoxic damage in the cell. The mechanism of T cell cytotoxicity is still largely unknown but recently some evidence has accumulated. Intimate contact between the T cell and the target cell is required and the cytotoxic event is probably secretory in nature; active protein synthesis by the T cell is necessary. The result is a drastic increase in the permeability of the target cell membrane followed by lysis (reviewed by Henney, 1973). Kinetic evidence strongly suggests that cytolysis results from the collision of a single lymphocyte with a single target cell (Wilson, 1965; Henney, 1971). If the process we have observed does represent T cell cytotoxicity it is probably not the same process which Henney has described.

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It is certainly not a "one hit" phenomenon and the fact that clusters have been observed in culture after 48 hr tends to refute the idea that an efficient cytotoxic reaction is being mounted. It is possible that far from representing aggression by T cells the clustering is a manifestation of a deranged process of intercellular co-operation in which the Reed-Sternberg cell is a pathological variant of a cell type which normally co-operates with T lymphocytes. Cell clusters are well recognised in experimental immunology, with the central cell being either glass adherent (and thus presumably monocytic in type) (Neilson *et al.*, 1974) or B cell lymphoblastoid (Jondal *et al.*, 1975). The absence of activity of the mature Reed-Sternberg cell with anti-monocyte serum makes it unlikely that it is derived from a monocyte precursor, and it may be that the Reed-Sternberg cell is of B cell origin. The segmented lymphocyte of clover leaf or budding appearance was a regular feature in cytocentrifuged specimens (Stuart, 1976). Egan and Garrett (1974) noted similar cells in the peripheral blood of patients with advanced Hodgkin's disease, and Lucas (personal communication, 1976) suggests these are reactive cells since he has observed them in children with gastro-enteritis. We believe they may represent a population of reactive T lymphocytes since they form rosettes with sheep erythrocytes.

Two different kinds of membranous processes were observed arising from the Reed-Sternberg cell; these may have significance with respect to lymphocyte interaction at the cell surface. It is perhaps premature to ascribe a function or significance to these at the moment since they have been observed only on a few occasions. However, it is tentatively suggested that one of the types may result from the interaction of T lymphocytes at the surface of the Reed-Sternberg cell. This is the branching veil-like structure seen waving about in the medium, obviously not adherent to glass. Film sequences show that these fronds are left behind when lymphocytes break free. The other kind of membranous processes are readily distinguished; they arise from the perimeter of the glass-adherent Reed-Sternberg cell, like quills of a porcupine. They also adhere to glass and do not show branching. Their significance is unknown.

In common with most other workers we found no esterase activity in the majority of Reed-Sternberg cells; however, appreciable activity was present in a significant minority. This activity was faint and diffuse, often located opposite a nuclear indentation. This demonstration of esterase activity agrees with the results of Braunstein *et al.* (1962) although these authors detected stronger activity in every Reed-Sternberg cell. However, it differs from the findings of Dorfman (1961) and Kadin (1973) who found complete absence of esterase. It may be possible to explain these contradictory findings in terms of a gradual loss of esterase activity as the Reed-Sternberg cell reaches maturity; less likely is the hypothesis that the Reed-Sternberg cell has more than one cellular origin.

Results with the PAP immunoperoxidase technique were similarly inconclusive, some Reed-Sternberg cells giving a positive reaction and many remaining negative. What is now required is evidence of the ability of some Reed-Sternberg cells to synthesise IgG since these tests fail to distinguish between synthesis and passive acquisition of immunoglobulin. Our experience with surface membrane fluorescence suggests that if immunoglobulin is present at all,

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it is there in only small amounts. Further investigations of the surface membrane properties of the Reed-Sternberg cell were difficult to carry out because of interacting lymphocytes. After treatment with pronase the Reed-Sternberg cell was able to bind IgG-coated red cells in a specific manner thus indicating the presence of the Fc receptor. The Fc receptor is possessed by monocytes and macrophages (Huber *et al.*, 1969) and most antibody forming cell precursors (Basten *et al.*, 1972). It has been described on T cells (Yoshida and Anderson, 1972; Dewar *et al.*, 1974). Of the reticulum cell class so far investigated, neither the human reticular cell of Stuart and Davidson (1971), the mouse dendritic cell of Steinman and Cohn (1974) nor the dendritic reticular cell of Nossal *et al.* (1968) express the Fc receptor.

The sheep erythrocyte rosetting test showed that most of the surrounding lymphocytes around the Reed-Sternberg cell were T lymphocytes. Fluorescence tests confirmed that B cells were only rarely present in the rosettes. The Reed-Sternberg cell itself did not possess a receptor for sheep red cells, and failed to react with anti-monocyte serum. Our results indicate that the Reed-Sternberg cell is neither a T cell nor a monocyte. Surface immunoglobulin and peroxidase reactions do not convincingly indicate a B cell origin but investigation of the precursor cell as revealed by lymphocyte rosetting may yield useful information.

SUMMARY

Clustering of lymphocytes around Reed-Sternberg cells was noticed in single cell suspensions made from viable Hodgkin's lymphoid tissue. Cyto centrifugation of the suspension showed that clustering also occurred around a smaller cell type, thought to be the precursor of the classical Reed-Sternberg cell. Time-lapse cine films taken of the clustering showed unceasing activity on the part of the lymphocytes migrating over the surface of the central cell.

Reed-Sternberg cells were reacted with anti-monocyte serum using indirect fluorescence techniques. In its mature form at least, the Reed-Sternberg cell showed no activity with the antiserum. No immunoglobulin was detected in the Reed-Sternberg cell using fluorescence techniques, but a few Reed-Sternberg cells showed diffuse cytoplasmic staining using the peroxidase-labelled antibody technique.

Membrane receptor tests showed the lymphocytes surrounding the Reed-Sternberg cell to be T-cells. After proteolytic enzyme treatment to free lymphocytes from the surface, the Reed-Sternberg cell bound IgG-coated red blood cells indicating a probable Fc receptor.

Cytochemistry demonstrated weak non-specific esterase activity in a small minority of Reed-Sternberg cells, and absence of acid phosphatase, alkaline phosphatase and peroxidase.

A subpopulation of lymphocytes with distinctive segmentation of the nucleus was noted. These were often to be seen participating in lymphocyte rosettes around the Reed-Sternberg cell.

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TWENTY-NINE CASES OF NON-HODGKIN LYMPHOMA

A correlation of surface receptors with
histological appearance using both the
Rappaport and Lukes and Collins classifications

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Summary

The receptor patterns of cell suspensions from 29 cases of Non-Hodgkin lymphoma were correlated with the histology of the nodes from which the cells were taken. Twentytwo were judged to be B predominant or B mixed, and because of this preponderance these ^{NODES} were ^{SUB} divided // by a method based on the distribution of surface immunoglobulin and the expression of Fc and C₃ receptors.

"Mature" B cell and B mixed tumours showing capping surface Ig with Fc and/or C₃ receptors correlated well with a nodular growth pattern and consisted of what Rappaport calls "poorly differentiated" lymphocytes equivalent to the "small cleaved" cell as defined by Lukes and Collins. Ten of the 14 patients in this receptor category are alive between 12 and 30 months after diagnosis.

Receptor silent and "immature" B cell tumours with non-capping surface Ig correlated predominantly with the Rappaport histiocytic lymphoma and Lukes and Collins' large cleaved and large non-cleaved lymphomas, though these // histological categories also included a wide variety of other receptor types such as T cell, Receptor Overlap and the single true Macrophage tumour. Five of the 11 patients with receptor silent or immature B cell tumours are alive between 7 and 15 months after the diagnosis.

Diffuse mixed and diffuse poorly differentiated lymphocytic lymphomas in Rappaport's classification correlated poorly with receptors, mature and immature B cell tumours being equally represented.

Introduction /

Introduction

The first purpose of this paper is to show how receptor studies on human lymphomata may be used to identify the cells present (Table I; Figure 1), the second is to assess the maturity of the B cell by a number of cell surface markers, and the third is to relate the surface markers to the histology of the tumour. Previous attempts to identify the cells present in human lymphomas by surface marker techniques (Brouet, Labaume and Seligmann, 1975; Cooper et al., 1975; Dorfman, 1975; Gajl-Peczalska, Bloomfield and Sosin, 1975; Habeshaw and Stuart, 1975; Jaffe et al., 1975, Huang et al., 1974; Peter, Mackenzie and Glassy, 1974; Stuart and Habeshaw, 1976; Smith et al., 1973) have all shown that most non-Hodgkin lymphomas consist predominantly of B cells with a minority of T cells; a few are derived from T cells and those arising from macrophages are distinctly uncommon. Tumours without B, T or macrophage markers, we call receptor silent. Some authors have encountered tumours in which the sum of B cells and T cells appears to exceed the total cell count. This suggests simultaneous expression by cells of both B and T characteristics, named by us as receptor overlap (Lin and Hsu, 1975; Habeshaw and Stuart, 1975; and Murphy, 1975).

The receptors expressed at different stages of B cell differentiation in the mouse have been well documented (Gelfand et al., 1975; Metcalf et al., 1975; Ramasamy, Munro and Milstein, 1974; Sidman and Unanue, 1975(a), (b)). These ^{authors} showed failure of surface Ig to 'cap' (aggregate at one pole of the cell) in the ~~most~~ ^{of B cell development} immature stages. As maturation proceeds /

proceeds, the cells 'cap' surface Ig and express first Fc and then both Fc and C₃ receptors. The plasma cells, or mature secretory B cells, have inconsistent surface markers, but contain intra-cytoplasmic Ig. (Corte et al., 1975). We and others have found identical variations in the pattern of surface Ig, Fc and C₃ receptors in human material such as normal peripheral blood (Habeshaw and Young, 1975), tonsil (Siegal, Grieco and Gupta, 1974), and in lymphomas (Brouet et al., 1975; Dorfman, 1975; Jaffe et al., 1975; Stuart and Habeshaw, 1976; Habeshaw and Stuart, 1975; Moscatelli, Bricarelli and Quartino, 1976). It therefore seems likely that the sequence of phenotype change seen in mature ^{clon of} mouse B lymphoid populations also occurs in man. If this were true, then the expression of surface Ig, Fc and C₃ receptors may be a means of assessing cellular differentiation in human B cell neoplasms. In this paper we propose a scheme of B cell differentiation in human Non-Hodgkin lymphoma based on the experimental and natural observations previously cited.

Tumours in which the B cells express an undifferentiated receptor profile should appear immature histologically and behave aggressively clinically. The opposite should apply to those tumours assessed as well differentiated by surface markers. In this paper we test this hypotheses in 25 tumours of B cell or receptor silence type.

MATERIALS /

MATERIALS AND METHODS

The techniques employed are described in outline since details have been reported previously (Habeshaw and Young, 1975; Stuart and Habeshaw, 1976; Dewar et al., 1975).

Lymph node cell suspensions

Cell suspensions in tissue culture medium were prepared from lymph node biopsies. Cells were not fractionated and when viability was less than 60 per cent, the suspension was discarded. The material is not selected in any way other than this and represents a continuous series of cases.

Antibodies:- Rabbit anti-sheep-red-cell and rabbit anti-ox-red-cell antibodies

IgM antibodies were prepared by the intravenous immunisation of rabbits with boiled ox or sheep erythrocyte stroma according to Kabat and Mayer (1961). The IgM was obtained by Sephadex G 200 gel chromatography in 0.1M tris/HCl or glycine buffer at pH 8.0 - 8.4. The first peak effluent contained most of the IgM and most antibody activity by tray titration.

Rabbit anti-ox-red-cell, anti-sheep-red-cell, and anti-human-red-cell IgG antibodies were made by conventional subcutaneous and intraperitoneal immunisation of rabbits with the appropriate stroma. Purified IgG was obtained by salting out, dialysis against distilled water, and batch chromatography on DEAE cellulose.

Rabbit anti-human-C₃ and C₄ antisera were used to demonstrate complement components on the surface of sensitised /

sensitised red cells.

Formation of Rosettes

In 23 cases in this series particles were prepared, and rosettes formed according to the standard methods described by Habeshaw and Young (1975). In 6 cases, Fc and C₃ receptors were prepared using rabbit anti-ox-rbc IgG (for Fc) and rabbit anti-ox-rbc IgM + human R₃ reagent (C₃d receptor) (Lachman et al., 1973). Controls showed that results obtained with sheep or ox red cell were comparable for Fc and C₃d receptors. A heavily sensitised red cell is needed to detect all cells expressing an Fc receptor. Mixed E and Fc receptors were detected using human red cells sensitised with rabbit anti-human-rbc IgG as previously described (Dewar et al., 1974). Rosette counting is facilitated by the addition of 0.1ml of 0.01% solution of acridine orange to the suspended rosettes immediately before examination by fluorescence and phase contrast microscopy. (Brostoff, 1974).

Immunofluorescent staining

Antibodies against human serum immunoglobulin were obtained commercially, these included polyvalent rabbit anti-human-immunoglobulin (Nordic), FITC coupled goat anti-rabbit serum (Meloy, Nordic), and monospecific antisera against heavy (IgM, IgA, IgG, IgD) and light (Kappa, Lambda) chains of immunoglobulins (Nordic, Meloy, Dakopats). Capping is a phenomenon dependent upon the degree of cross-linking in the surface Ig anti-Ig complex, and both temperature and time of incubation. The conditions cited are those which specifically favour the formation of 'caps' in normal B lymphoid tissue. Increasing times of incubation beyond 30 minutes can lead to cap shedding or interiorisation in normal lymphoid cells, and give /

give rise to an erroneous measurement of B lymphoid cell numbers.

All antisera were centrifuged at 110,000g for one hour before use to remove aggregates of Ig. They were then aliquoted in 0.1ml quantities and stored at -20°C for up to one month. It should be emphasised that removal of aggregated Ig from anti-human or anti-rabbit immunoglobulin antisera by high speed centrifugation is essential - otherwise B cells as well as all other cells expressing the Fc receptor will be stained, and any capping which occurs is then due to capping of Fc receptors rather than capping of surface Ig/anti Ig complex.

In two cases, the capacity of the tumour cells to synthesise surface Ig following trypsinisation was assessed. Cells were treated with 0.25% trypsin (Armour) in PBS for 30 minutes at 37°C , to abolish surface Ig staining entirely. After washing, the cells were cultured in medium 199 + 10% FCS for 24 hours and reassessed for presence of surface Ig, and for intracytoplasmic Ig.

Receptor Terminology

Using these methods the tumours are assigned to the classes indicated in Tables I and II according to the receptor pattern that predominates over the sum of all others.

The term "Mixed" implies that no single class exceeded the sum of all others. In each of these cases, the word "mixed" is preceded by the largest single cell class.

Receptor /

Receptor Overlap

The sensitisation procedure ~~controls~~ described by Dewar et al. (1975) ensure that single cells expressing both T cell and B cell characteristics are not overlooked.

Receptor Silence

Cells failing to express receptors by any of the methods outlined are clearly of unknown origin and may be derived from a metastatic carcinoma or sarcoma in a lymph node. Where this has seemed likely on clinical and histological grounds the case has been discarded, leaving, nonetheless, some cases of histologically undifferentiated neoplasms with an undoubted lymphomatous 'look' and clinical findings appropriate to that diagnosis.

Histological and Cytological Evaluation

Tissue for histology was taken from the slice of lymph node biopsy next to that from which the cell suspensions were made. Fixation was in 10% formaldehyde in saline. Sections were cut at 2.5 microns and stained with H & E, Gordon and Sweet's reticulin, Giesma and Methyl-green Pyronin stains. The tumours were classified using the Rappaport (1966) and Lukes and Collins (1975) classifications.

Dabs of freshly cut lymph node surface and smears of the cell suspensions were stained with May-Grunwald-Giesma. As well as giving increased cytological detail of the cells, these techniques helped us judge whether the cell populations in the tissue sections was equivalent to that in the suspensions. Intra cytoplasmic immunoglobulin content of the tumours was assessed by the P.A.P. ~~immunoglobulin~~ technique of Taylor and Burns (1974).

RESULTS

Receptor Analysis

The results of the receptor analysis are shown in Table I which shows that 22 of the 29 tumours were predominantly B cell. Nine of these 22 were classed as "B mixed" since they contained an appreciable number of T cells. Two showed receptor overlap, one was a macrophage tumour and the last a T cell tumour.

Table II shows how the B lymphoma^{ka} can be further subdivided according to their expression of surface Ig, Fc and C₃ receptors and cytoplasmic Ig. Although the origin of receptor silent cells is unknown they are included, for convenience, with the B cells. The horizontal line indicates an important division between tumours whose cells cap their surface Ig (B₃B₄B₅) called mature, and those that do not (B₁B₂) regarded as immature. The mature cells express receptors for Fc and C₃, immature cells may have no surface receptors or an Fc receptor only, but never both Fc and C₃. Cases expressing only C₃ receptors on non-capping B cells were not seen in this series but may well occur. Capping of surface immunoglobulin (Fig.1) occurs more ~~commonly~~^{frequently} in reactive than in neoplastic nodes. Nevertheless a higher proportion of non-capping cells were found in two highly reactive nodes with germinal centres giving a B₂ receptor profile. Eighty per cent of the control lymph nodes gave a mixed receptor pattern.

Comparison /

Comparison of Receptor Patterns with Rappaport's
Histological Classification (1966)

The correlation of histology with the B₁^{cell} surface phenotype is shown in Table III. Three of the four well differentiated lymphocytic lymphomas (Fig.2) had cells that were mature morphologically and by their expressed receptors. Two of these ^{patients} are alive and in remission, the third also in remission, died of a myocardial infarct. The fourth patient has clinical features typical of chronic lymphocytic leukaemia but, unlike the other three, his lymphocytes in both blood and lymph node biopsy expressed an immature receptor pattern.

In the nodular lymphomas, all were poorly differentiated lymphocytic histologically (Figs.3 and 4), all but one showed a mature receptor pattern and all but one are alive and in remission. There was a sequential change in the receptors of the patient who died. At biopsy, the lymph node cells were B₃ (with a high proportion ^{of} T cells), but the blood B lymphocytes were B₂ with a receptor silent population of 10%. In spite of histology that frequently indicates a good prognosis, the patient had rapidly progressive disease, and ten days later the receptor silent population in the blood had risen to 40%. The patient died soon after and autopsy showed a diffuse poorly differentiated lymphocytic lymphoma. We have since encountered a case of follicular (nodular) lymphoma where the follicle centre cells were unusually large and there were numerous mitotic figures. The receptor profile was B₂.

In /

In the diffuse poorly differentiated and diffuse mixed group there were tumours with both mature and immature B receptor patterns and poor correlation with histology. The Rappaport 'Histiocytic' group included five B cell tumours and two with receptor silence. Four of the B cell tumours had an immature receptor pattern. One patient with a B₁ tumour became leukaemic terminally with the majority of his peripheral blood cells showing receptor silence.

Comparison of receptor patterns with Lukes and Collins Classification (1975)

Table IV shows that 13 of the 15 small lymphocyte and small cleaved cell tumours consisted of mature B lymphocytes as judged by receptors. Five of the six large non-cleaved tumours (Fig.5) were either immature or receptor silent. If the large cell groups (both cleaved (Fig.4) and non-cleaved (Fig.5)) are taken together, eight of the ten show immature receptor profiles or receptor silence. The large cell groups showed the greatest heterogeneity of receptor expression. In contrast, the small cell tumours with two exceptions expressed mature B lymphocyte characteristics.

DISCUSSION

These studies amply confirm that B cell tumours are the commonest of the Non-Hodgkin lymphomas. However, a variable number of T cells was noted in more mature tumours where frequently neither T nor B cells predominated over the sum of all others present. This mixed pattern was noted especially in the follicular or 'nodular' lymphomas. It is not known if this represents contamination of a neoplastic B /

B population by reactive T cells. One may speculate that the physiological co-operation of T and B cells may be echoed in neoplasms and if so, the concepts of "neoplastic" and "reactive" populations may be functionally irrelevant.

Reservation must be expressed over the small number of T and macrophage derived tumours observed in this series. Reliance on E rosetting by itself as a method of identifying T cells is possibly insufficient and current techniques of identifying macrophages ignore the precursor cell.

Salmon and Seligmann (1974) proposed a scheme of B lymphocyte development in lymphoma based upon the class of secretory or surface expressed immunoglobulin. The classes of B lymphoma proposed were B_0 (B stem cells), B_1 (virgin B lymphocyte), B_2 (immunoblast), B_3 (memory B lymphocyte), B_4 (plasmacytoid lymphocyte) and B_5 (plasma cell). They suggest that the course of development of lymphoma involves the triggering of a clone of responsive cells by antigen, followed by a second oncogenic stimulus which leads to irreversible proliferation of 'committed' B cells. In their classification, the B_1 tumours are represented most commonly by CLL and the well differentiated lymphocytic lymphoma group. The B_2 tumours are derived by division from B_1 cells, and the B_2 component is the cell found in poorly differentiated lymphocytic lymphoma Burkitt's and some histiocytic lymphomas. The B_3 or 'memory' cell has no equivalent in the lymphomas but from it is derived the lymphocytoid plasma cell (B_4) which secretes IgM. The final stage, B_5 , is the plasma cell.

The classification employed by us has some similarities but all the steps in our proposed maturation sequence are defined by receptor patterns demonstrated by us and others in normal,

normal, experimental and neoplastic lymphoid populations. B₁ and B₂ tumours are those in which the majority of cells have non-capping surface Ig. B₂ tumour cells have, in addition, an Fc receptor. B₃ and B₄ tumour cells cap their surface Ig and have both Fc and C₃ receptors. B₄ tumours differ from B₃ by having few Fc and C₃ receptor bearing cells and sometimes showing intra-cytoplasmic Ig to a slight degree. Intracytoplasmic Ig is prominent in B₅ tumours.

In our scheme, the primary division is between B cell tumours that cap their surface Ig and those that do not. As experimental work previously cited suggests that this may be an indicator of B cell maturity, it seems justifiable to use capping as a criterion to divide human B lymphomas into 'mature and immature' groups. If this correlated with histology and survival, then receptor studies might prove clinically useful.

B₃ B₄ B₅ lymphomas correlate well with a nodular growth pattern (Rappaport, 1966) and with small cleaved follicular centre cells (Lukes and Collins, 1975). All have substantial numbers of T lymphocytes in the affected node, giving rise to a mixed rather than a B predominant receptor pattern in half the patients with nodular lymphomas. The similarity of this pattern to the reactive lymph nodes should be noted. These findings conflict with Rappaport's term 'poorly differentiated' lymphocytic as ten of the thirteen tumours of this cell type had mature receptor profiles.

Table III shows that B cell immaturity as assessed by surface phenotype correlates reasonably well with histology showing large cleaved and non-cleaved cells. This is in reverse order to the theoretical basis of the

cleaved cells are at the start of the transformation sequence and, therefore, should be less mature functionally than the large non-cleaved cells which are thought to be the penultimate stage before B lymphocyte differentiation into plasma cell. The large non-cleaved tumours are, broadly speaking, equivalent to what Rappaport calls histiocytic lymphomas. This group shows the greatest heterogeneity of receptor expression including two with receptor silence, five B cell tumours (4 immature, 1 mature), one with receptor overlap and only one true histiocytic or macrophage tumour. Brouet et al (1975) reported five histiocytic lymphomas, four with receptor silence and one with receptor overlap.

This heterogeneity is reflected in the varied clinical behaviour shown by these lymphomas in two large retrospective series (Durant et al., 1975; Schein et al., 1974). There is no way of separating the small number of histiocytic lymphomas that do well, even after local treatment, from the many that are rapidly fatal. Identification of distinct sub-groups by receptor studies could therefore be very valuable. It is noteworthy that the receptor overlap is the only receptor class in which there has been no deaths so far; one was a diffuse histiocytic lymphoma, the other diffuse mixed. Both were hard to classify histologically and a search of retrospective material is being undertaken for cases of similar histology.

Neoplastic cells which appear receptor silent may develop surface immunoglobulin following trypsinisation and overnight culture. This treatment is often not possible with large cell 'histiocytic' tumours whose cells in culture show /

show poor viability. Three of our cases were receptor silent at the time of initial biopsy and two became so as their disease progressed; all are dead within 2 years. This pattern of receptor silence must be distinguished from that seen in some patients with diffuse, well differentiated lymphocytic lymphoma and chronic lymphatic leukaemia whose small round morphologically mature lymphocytes in both blood and lymphnodes fail to express surface Ig, C₃ and Fc receptors. These are possibly 'null' cells. We have encountered one such case which after trypsinisation and overnight culture, expressed non-capping surface fluorescence (Table III). In two similar cases seen too recently to be included in this series the cells remained receptor silent after trypsinisation and overnight culture.

Correlation of receptors with clinical survival is not possible with any certainty in this small series due to the short period of follow-up in some cases. Nevertheless, ten of fourteen patients with 'mature' receptors are still alive (one died of a myocardial infarct) compared with five out of eleven whose receptors were 'immature'.

TABLE I

Scheme of receptor expression in non-Hodgkin lymphoma

<u>Category</u>	<u>Characteristics</u>			Number of cases in present series
	E Rosette	C ₃	Phagocytosis of Neutral Red	
B Lymphoma	-	+or-	-	22
T Lymphoma	++	-	-	1
Macrophage	-	+	++	1
Receptor Silent	-	-	-	3
Receptor Overlap	++	+or-	-	2

*Mark
G. H. H. H.*

- + Present on a substantial number of lymph node cells.
- ++ Present on the majority of the lymph node cells.
- Not present on the lymph node cells.

TABLE II

Sub-division of B cell lymphomata on the basis of surface phenotype and functional attributes of the neoplastic cells

<u>Category</u>	<u>Characteristics</u>			<u>Number Of Cases</u>
	<u>Surface Ig</u>	<u>Fc</u>	<u>C₃</u>	
Receptor Silence	-	-	-	3
B ₁)) Immature	Non Capping	-	-	5
B ₂)	Non Capping	+	-	3
<hr/>				
B ₃))	Capping	++	++	9
B ₄) Mature)	Capping	+	+	4
B ₅)	-	-	±	1
			+++	<u>25</u>

- Characteristic not present on B cells
 + Present on a substantial number of B cells
 ++ Present on the majority of B cells
 +++ Present on all B cells
 + Variable presence

TABLE III

Comparison of B Surface Phenotype and Receptor Silence
with Rappaport's Classification

	DWDL	NPDL	DM DPDL	DH
Receptor Silent			1	2
B ₁ B ₂	1	1	2	4
B ₃ B ₄ B ₅	3	8	2	1

D = Diffuse

N = Nodular

L = Lymphocytic

H = Histiocytic or Undifferentiated

WD = Well Differentiated

PD = Poorly Differentiated

M = Mixed

TABLE IV

Comparison of B surface Phenotype and Receptor Silence
with Lukes and Collins Classification

	<u>Small Lymphocyte</u>	<u>Small Cleaved</u>	<u>Large Cleaved</u>	<u>Large Non-cleaved</u>
Receptor Silent			1	2
B ₁ B ₂	1	1 (follicular)	2	3
B ₃ B ₄ B ₅	3*	10 (8 follicular) (2 diffuse)	1	1

* This group includes one Lymphoplasmacytoid tumour
Immunoblastic sarcomas have been classified with
the large non-cleaved tumours



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