Immunological Markers in Hairy-Cell Leukaemia

by

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Preface

This dissertation is the result of my own work and includes nothing which is the result of work done in collaboration. The work was carried out in the Department of Haematological Medicine, between October 1975 and September 1978.

Immunological Markers in Hairy-Cell Leukaemia

Gordon F. Burns

Summary

The dissertation consists of six chapters. Chapter 1 presents a brief introduction on immunological markers. Chapter 2 describes the discovery of a previously unrecognised marker in hairy-cell leukaemia (HCL), a receptor for IgM. The rosette method used to reveal this receptor, and the conditions affecting rosette formation are described. A kinetic study of this receptor is used to provide a measure of membrane turnover in HCL. The distribution of the IgM receptor among normal and malignant haemic cells is investigated. Chapter 3 considers a variety of other immunological markers in HCL. Previous controversy in the literature concerning the presence of a receptor for complement on hairy cells is resolved and new information is provided about the presence of other markers on hairy cells. Chapter 4 presents experiments designed to investigate conflicting evidence regarding the nature of the hairy cell. Monocytic properties are measured and weighed against features of hairy cells which align them with the B-lymphocyte series. The presence of intrinsic surface immunoglobulin (SIg) is used as evidence for the B-cell nature of HCL. The results obtained showing only IgG on the surface of some cases of HCL, and multiple heavy chain isotypes on others, provide a solution to the confused literature regarding the presence of SIg on hairy cells. Two unusual cases of immunoproliferative diseases are extensively investigated in Chapter 5 in order to demonstrate some of the problems associated with immunological marker studies. Chapter 6

reviews the thesis and draws conclusions about the nosology of HCL. A possible position for HCL in schemes of B-cell differentiation is given.

Several papers of unconnected work submitted during the period of research for the dissertation are also included for consideration by the Examiners.

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CHAPTER 1

INTRODUCTION

Immunological markers and lymphoproliferative disorders

INTRODUCTION

Immunological markers and lymphoproliferative disorders

Immunological markers have been used in the study of human lymphoproliferative diseases (LPD) for less than ten years, but the impact of these studies has led to a complete re-appraisal of traditional classifications of the LPD. The rapid pace of development and the importance of these marker studies is reflected in recent monographs and reviews on the subject (Greaves *et al*, 1974; Preud'homme *et al*, 1975; Dwyer, 1976; Siegal and Good, 1977; Cooper and Seligmann, 1977; Whiteside and Rowlands, 1977), and many of the simpler techniques are already in routine laboratory use (Rose and Friedman, 1976).

The terms B cells and T cells, originally coined by Roitt *et al* (1969) by extrapolation from animal studies, are now applied, albeit loosely, in descriptions of most human lymphoid malignancies, and by surface marker studies most LPD can be classified into B-cell proliferations or T-cell proliferations. The commoner B-cell proliferations such as chronic lymphocytic leukaemia, Waldenstrom's macroglobulinaemia and multiple myeloma are often considered as clonal expansions of cells blocked at various stages of normal B-cell development, and surface marker analysis of the cells from these diseases has given rise to schemes outlining possible pathways of B cell differentiation (Salmon and Seligmann, 1974; Davis, 1975; Sweet *et al*, 1977; Gathings *et al*, 1977).

Classification of T-cell disorders such as T-derived chronic lymphatic leukaemia and the Sezary syndrome is less sophisticated

although marker studies have revealed that some T-cell proliferations can be classified into subpopulations of T cells (Moretta et al, 1977a; Worman et al, 1978). Further cell surface analysis will probably enable more complete classification of T-cell proliferations to be made, and the expression and loss of various cell markers will allow more precise ontogenic staging of both B- and T-cell disorders.

The extensive study of a few patients with cells possessing unusual surface phenotypes has also revealed possible subsets of cells with markers which combine features previously thought to be restricted to either B or T cells (Shevach et al, 1974; Sandilands et al, 1974; Hsu et al, 1975; Siegal et al, 1976; Burns et al, 1977d; Toben and Smith, 1977). It is possible, however, that such unusual phenotypes may be due to neoplastic changes of the leukaemic cells (Brown et al, 1974; Preud'homme et al, 1975) and also that the population of leukaemic cells under study may change its phenotype with time (Hsu et al, 1975; Burns et al, 1977d). Before studies of this kind are undertaken, it is imperative therefore that a panel of markers is used (Brown et al, 1974; Preud'homme et al, 1975; Burns et al, 1977d); that follow up studies are pursued (Hsu et al, 1975; Burns et al, 1977d); and that careful consideration is given to various technical problems associated with surface marker work (Seligmann et al, 1977).

The term immunological marker remains to be defined. The synonyms - immunological markers, surface markers, membrane markers and differentiation markers - have all been used in an approximately similar sense, and none is entirely satisfactory. Perhaps the most widely used marker in clinical immunology is the receptor for sheep erythrocytes found on T lymphocytes, but the title given by Coombs et al, (1970) to their original description of the phenomenon clearly excluded

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involvement of an immunological process. One definition of immunological (Herbert and Wilkinson, 1971) is "referring to specific immunity ... or to the study of immunology". This broad definition of immunological is preferred here because it incorporates all the markers used by immunologists in the study of B and T cells, while the terms surface and membrane exclude such useful markers as cytochemical stains which differentiate B and T cells (Ranki et al, 1976; Kulenkampff et al, 1977; Higgy et al, 1977). Also, until much more information is gathered about the ontogeny of markers, it cannot be claimed that many markers are indicators of differentiation along a particular pathway of development.

The terms marker, receptor, structure, antigen, have all been used loosely to describe cell components which help to define the immunological status of the cell bearing them. This multiplicity of terms reflects the level of knowledge of markers at present, and Sela (1975) has stated that "the 'receptor' reflects a range of ignorance" rather than a definition. Some markers are well characterized: for example, membrane immunoglobulin is a protein which, like its serum analogue, has been extensively investigated; the receptor for cholera toxin is the ganglioside GMI (Cuatrecasas, 1973); many of the oligosaccharides comprising the blood group antigens are known; the receptor for sheep erythrocytes present on human T cells is probably a glycoprotein (Owen and Fanger, 1974). Other markers remain ill-defined and their chemical composition is unknown. Some apparent markers may be chance associations caused by the hydrophobic nature of a particular arrangement of molecules on the cell membrane surface.

From the above, it is evident that markers can function both as

receptors, in the sense that they receive, or bind, otherwise passive moieties, and as antigens. The name used depends upon whether a specific receptor-ligand interraction was first described and the membrane component subsequently isolated, or whether an antiserum was first raised to a membrane component later shown to possess some receptor function. Also, in cases where the receptor function is well defined and an antiserum to the putative antigen blocks receptor-ligand binding in an apparently specific manner, the antigen and receptor may not be identical membrane components. Recent work has demonstrated that several antisera thought to be identifying a specific receptor antigen were in fact blocking receptor-ligand binding by steric hindrance (Schirrmacher and Halloran, 1975; Woofsy et al, 1977; Galili et al, 1977; Gattringer and Wick, 1977).

Any definition of a marker must also consider the question of specificity. When a marker functions as a receptor, for example, is it specifically receiving the tool being used to detect it? Since the binding of components is the sum of non-covalent physical forces dependent upon fit and charge, absolute specificity cannot be claimed for any receptor. The situation is analogous to that proposed by Burnet (1967) for antibody affinity: " ... no combining site is in any evolutionary sense adapted to a particular antigenic determinant. The pattern of the combining site is there and if it happens to fit, in the sense that the affinity of adsorption to a given antigenic determinant is above a certain value, immunologically significant reaction will be initiated". In any event, it is difficult to imagine that all human T lymphocytes carry a receptor specifically designed to receive a determinant on sheep erythrocytes, or that a population of human B lymphocytes carries a receptor specific for a mouse

erythrocyte antigen. In these two instances the erythrocytes are merely the tools used to detect markers of unknown function.

It has been suggested that recognition is not sufficient for a structure to be described as a receptor (Sela, 1975) and that recognition must be followed by another event. This may be true for many membrane receptors, and Greaves (1976) has provided a model whereby the receptor signal may be translated and communicated to the cell interior. However, in most cases any event consequent upon receptor-ligand binding remains to be elucidated. For example, most nucleated animal cells have receptors for plant lectins, but the effects of lectin-binding by lymphocytes are unique (Ling and Kay, 1975). Furthermore, isolated glycoproteins which specifically bind lectins are described as purified receptors. In this work therefore, a receptor is defined as a structure on, or from, a cell which binds the ligand used to detect it.

Bearing in mind all the above considerations, an immunological marker can be defined as a particular, observable, reaction of a cell which is useful in defining the immunological status of that cell; this reaction need have no known specific function or be followed by another event, but it must be consistent and reproduceable under standard conditions.

Although the use of immunological markers has enabled most LPD to be classified into B-cell, or T-cell, proliferations, this classification is not entirely true since there are cells from diseases such as acute lymphoblastic leukaemia which bear none of the classical B or T markers, and others such as the Reed-Sternberg cells of Hodgkin's disease which remain perplexing. Hairy-cell leukaemia (HCL) has also proved difficult to classify. The cells from this disease

have a unique characteristic appearance as well as features in common with both B lymphocytes and monocytes (Catovsky, 1977), and a recent report from the World Health Organization and International Union of Immunological Societies (Belpomme et al, 1978) described HCL as unclassified in type.

The reasons leading to confusion about the classification of HCL will be discussed, in detail, in each of the succeeding chapters. In brief, however, there are four main reasons for the continued debate about the nature of the hairy cells (HCs) pathognomonic of the disease. First, HCs possess markers generally regarded as characteristic of B lymphocytes but also certain characteristics usually associated with monocytes (Fu et al, 1974; King et al, 1975; Catovsky, 1977), and this has led to HCL being placed in the B lymphocyte or the monocyte-histiocyte series (reviewed by Catovsky, 1977). The problem here is to determine the relative importance of each of the markers used in classification, and to decide which, if any, are definitive for either series.

Second, HCs possess several distinctive features which are almost unique to HCL (Yam et al, 1971; Katayama and Finkel, 1974; Catovsky, 1977; Higgy et al, 1978), and this has led to the suggestion that HCL is derived from cells unrelated to either B lymphocytes or monocytes (Daniel and Flandrin, 1974; Stein and Kaiserling, 1974). The question to be answered is whether these distinctive features are absolutely restricted to HCs, and whether they are important enough in themselves to reclassify HCL as deriving from a distinct and separate lineage.

The properties of the HC can vary from case to case (Burns et al, 1973; Burns et al, 1977a), and a third reason for the confused literature on the subject has been dogmatic statements based on a

single case of HCL. Fourth, technical difficulties particularly associated with HCs may account for several conflicting reports concerning some of the markers present on HCs (Burns et al, 1977c; Catovsky, 1977).

The work described in this dissertation was undertaken in order to classify the cells of HCL by means of immunological markers. Their position in an immunological scheme of ontogeny will be established, and technical problems associated with marker studies will be considered in detail. The results of a detailed characterization of HCL are of more than academic interest since HCL, although undoubtedly a distinct disease (Bouroncle et al, 1958; Catovsky et al, 1974b), presents problems of differential diagnosis (LoBuglio, 1976; Catovsky, 1977). Misdiagnosis of this chronic indolent form of leukaemia may have disastrous consequences, since the vigorous chemotherapy appropriate for certain other lymphoproliferative disorders may be lethal for the patient with HCL (Katayama and Finkel, 1974). Therefore any laboratory aid to the clinician is of value. The clinical criteria used to diagnose the patients with HCL studied are beyond the scope of this dissertation, but full clinical details of several patients are given in published papers containing some of the results of this thesis (Burns et al, 1977a; Cawley et al, 1978a).

CHAPTER 2

A RECEPTOR FOR IgM ON HAIRY CELLS

I Identification and characterization of the receptor

1. INTRODUCTION

2. MATERIALS AND METHODS

2.1 Patients

2.2 Cell preparation and mononuclear cell isolation

2.3 Indicator cells

2.4 Rosette tests

2.5 Fluorescein-labelled, monomeric human IgM

2.6 Scanning and transmission electron microscopy

2.7 Cell culture

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2.9 Blocking tests

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3.3 Conditions affecting EA_M rosette formation

3.4 Density of the receptor for IgM on hairy cells

3.5 Blocking of EA_M rosette formation

4. DISCUSSION

II Kinetic studies on the turnover of the receptor for IgM

1. INTRODUCTION

2. MATERIALS AND METHODS

2.1 Patients

2.2 Mononuclear cell preparation

2.3 Indicator cells

2.4 Kinetics of rosette formation

2.5 Concentrated μFcR material

3. RESULTS

3.1 Shedding of the μFcR

3.2 Receptor synthesis and turnover by HCs

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4. DISCUSSION

III Distribution of the μFcR on haemic cells and the relationship of the μFcR on HCs to that on other cell types

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2. MATERIALS AND METHODS

2.1 Patients

2.2 Cultured haemic cells

2.3 Detection of the μFcR

3. RESULTS

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3.2 Acute lymphoblastic leukaemia (ALL)

3.3 B-cell lymphomas and leukaemias

3.4 Terminal B cells

3.5 Monocytes and histiocytes

3.6 Granulocytes

3.7 Pathological T cells

3.8 Cultured cells

4. DISCUSSION

A RECEPTOR FOR IgM ON HAIRY CELLS

I Identification and characterization of the receptor

1. INTRODUCTION

In studies designed to investigate the presence of a receptor for fixed C3 on the hairy cells (HCs) of hairy-cell leukaemia (HCL) by the rosette method of Ross et al, (1973b), it was observed that a number of cells formed rosettes with the IgM-coated ox indicator erythrocytes used as a control.

This result raised the possibility that some HCs had a receptor for IgM. The investigation of this possibility forms the basis of this chapter.

2. MATERIALS AND METHODS

2.1 Patients

A series of 23 patients with HCL was studied. All the patients fulfilled the normal clinical and morphological criteria for the diagnosis of HCL (Bouroncle et al, 1958; Katayama and Finkel, 1974). In particular, the diagnosis was confirmed by the demonstration of the following: tartrate-resistant acid phosphatase (Li et al, 1970); a typical ultrastructure including the ribosome-lamellar complex (Katayama et al, 1972a; Daniel and Flandrin, 1974; Catovsky et al, 1974b); a high leucocyte alkaline phosphatase score (Katayama and Finkel, 1974; Hayhoe et al, 1977); and the characteristic pattern of esterase staining (Higgy et al, 1978).

Peripheral blood (PB) was obtained, on several occasions, from all 23 patients with HCL. In addition, the spleen of 10 patients, bone marrow cells from 3 patients and lymph node cells from 1 patient were studied.

2.2 *Cell preparation and mononuclear cell isolation*

Heparinized peripheral blood was obtained either on the same day as collection or after one day in the post. After more than one day the blood tended to clot and the recovery and viability of leucocytes was decreased.

In the initial experiments, the peripheral blood was allowed to settle and the buffy coat collected into Hanks' balanced salt solution buffered with 20 mM HEPES (HBSS). The cells were washed three times in HBSS and resuspended in HBSS with 0.2% bovine serum albumin (HBSSA) to a concentration of 2×10^6 cells/ml before testing. Examination of the cells obtained after Ficoll-Isopaque fractionation of whole blood, using the mixture made to a density of 1.077 g/ml (Boyum, 1968, 1976), demonstrated that HCs remained on the interface with virtually no loss of pathological cells. Thereafter, leukaemic cells were always isolated over a Ficoll-Isopaque mixture, or commercial lymphoprep (Nyegaard and Co., Oslo), before washing and resuspending in HBSSA as above.

Cytocentrifuge preparations made from cells isolated in this way demonstrated that, in general, over 95% of the cell preparations were mononuclear cells. The proportion of morphological HCs varied from case to case, but esterase staining of the preparations confirmed the virtual absence of monocytes characteristic of the disease (Seshadri et al, 1976; Burns et al, 1976; Higgy et al, 1977). Viability of the cell suspensions was assessed by trypan-blue dye

exclusion and by the ability of viable cells to convert fluorescein diacetate (0.5 µg/ml) to fluorescein (Celeda and Rotman, 1967). In all cases viability was greater than 95% unless stated otherwise.

After initial attempts to obtain viable cells from whole spleens processed in the laboratory had failed, the following procedure was found to be necessary. Immediately after its removal, the spleen was roughly sectioned and small pieces of tissue from different areas were forced through a 120 gauge stainless-steel mesh into a beaker containing ice-cold HBSS. The cell suspension was transported to the laboratory in an ice bucket (1 to 3 hours). The cells were then vigorously resuspended in HBSS with a pasteur pipette and washed in the same medium before centrifugation over Ficoll-Isopaque. The mononuclear cells thus isolated were washed and resuspended in HBSSA as for peripheral blood.

Using the above protocol, although the cell viability was generally high, it was found that the numbers of splenic HCs expressing the IgM receptor were low. This was later attributed to shedding of the receptor (see Section II of this chapter), and the final procedure was adapted to prevent this. Foetal-calf serum at a final concentration of 10% was added to the transport medium and this, to some extent, prevented shedding of the receptor.

Bone marrow specimens were collected into HBSS containing heparin (25 IU/ml) and either processed on the same day or after one day in the post. As for splenic cells, this transport medium was later modified by the addition of 10% foetal-calf serum. In the laboratory the bone marrow fragments were broken up with dissecting needles and forced through a 120 gauge stainless-steel mesh. Single cell suspensions were then processed in the same manner as peripheral blood and spleen cells.

2.3 *Indicator cells*

$\underline{EA}_M$ Ox erythrocytes coated with IgM antibody (EA_M) were prepared as follows. Primary antisera to ox erythrocytes were raised by immunizing rabbits intravenously with 1 ml, 2 ml and 7 ml of 10% washed ox erythrocytes in saline on days 1, 2 and 3 respectively before bleeding on day 8. Anti-ox erythrocyte IgM was isolated from the primary antisera by chromatography on Sephadex G200 in 0.5 M NaCl, 0.1 M Tris (pH 7.25). Fresh ox erythrocytes were washed five times in saline and any leucocytes present removed. The erythrocytes were suspended to 2% in saline (v/v) and sensitized with IgM by mixing with equal volumes of anti-ox erythrocyte IgM antibody and incubating at $0^{o}C$ for 15 min. The resultant EA_M cells were thoroughly washed and resuspended to 1% in HBSSA before use. Rosette titrations of ox erythrocytes sensitized with serial dilutions of IgM antibody indicated that a 1/10 dilution was within the plateau region, and this dilution was used for most studies. Higher concentrations of sensitizing IgM antibody tended to agglutinate the fresh, washed, ox erythrocytes.

$\underline{EA}_G$ Ox erythrocytes coated with rabbit IgG antibody (EA_G) were prepared in similar fashion. Hyperimmune rabbit antisera to ox erythrocytes were obtained by repeating five weeks later the course of injections given above for primary antisera. Rabbits were bled one week after the last injection and the sera were inactivated at $56^{o}C$ for 20 min for use as the source of anti-ox erythrocyte IgG antibody. EA_G indicator cells were prepared from this antiserum by the method of Hallberg *et al*, (1973) by sensitizing ox erythrocytes with serial dilutions for 40 min at room temperature. After washing three times with phosphate-buffered saline, indicator cells were finally resuspended to 1% in HBSSA before use. Titration experiments

with EA_G reagent indicated that a dilution of 1/50 was optimal for most sensitizing antisera. Unless otherwise stated, this dilution was used to prepare the indicator cells.

FEA_M Fluoresceinated (F) indicator erythrocytes for use in mixed rosetting assays were prepared by the method of Wolf et al (1971). Ox erythrocytes were washed in carbonate/bicarbonate-buffered saline, (pH 9.3) and directly fluoresceinated by adding 0.6 ml fluorescein isothiocyanate (1 mg/ml) to 0.2 ml of the packed cell pellet. The cells were gently resuspended and incubated at room temperature for 2 h. After washing, the fluoresceinated red cells were reacted with IgM antibody as described above to provide FEA_M.

E Spontaneous sheep erythrocyte (E)- rosette formation was used as a marker for T cells (Jondal et al, 1972). The method of Kaplan and Clark (1974) using aminoethylisothiouronium bromide (AET)-treated sheep erythrocytes was employed. Sheep erythrocytes were washed five times in physiological saline and 4 volumes of freshly prepared AET (0.143 M, pH 9.0) added to 1 volume of packed erythrocytes. The mixture was incubated at 37°C, with regular mixing, for 15 min before washing five times with ice-cold phosphate buffered saline (PBS). The washed cells were resuspended to 1% in PBS and filtered through a small cotton wool plug before use.

2.4 *Rosette tests*

EA_M rosettes After preliminary investigations, the following standard procedure for EA_M-rosette formation was adopted and used for all tests unless stated otherwise. Two drops of freshly separated leucocytes, at 2×10^6 cells/ml, were mixed with two drops of EA_M indicator erythrocytes (at 1%) in a plastic microtube and incubated

at 37°C for 10 min. The cells were then remixed by flicking the tube and centrifuged at 150 x g for 1 min and the tube containing the pellet was placed on ice for an hour or more to stabilize rosettes formed at 37°C. In all rosette tests, the pellet was gently resuspended with a pasteur pipette and two drops of fluorescein diacetate (diluted 10^{-4}) added to label live leucocytes (Ramasamy, 1974). Viable fluorescing cells were scored under combined phase contrast and incident U.V. illumination, and the percentage of rosette-forming cells was determined from counts of more than 200 cells. A control tube with unsensitized ox erythrocytes was always scored in parallel with each test. The morphological nature of EA_M rosette-forming cells was confirmed by cytocentrifuging the samples and staining with Giemsa.

EA_G rosettes Rosette formation with EA_G erythrocytes was performed in exactly the same way as in the EA_M test above, - except that the 10 min incubation was omitted.

Mixed rosettes Mixed rosettes were used to detect the presence of more than one receptor on the same cell (Wolf et al, 1971). Equal volumes of FEA_M and either EA_M, EA_G or E erythrocytes were mixed and rosetted with leucocytes as described above. In scoring these rosettes as mixed or single in type, fluorescein diacetate was not used and examination was carried out under alternate phase contrast and U.V. illumination. Cytocentrifuge preparations made from mixed rosettes examined under U.V. confirmed the suspension findings.

E rosettes The method described by Kaplan and Clark (1974) for E-rosette formation using AET-treated sheep erythrocytes, was employed. Initially, E (at 1%) and leucocytes (at 2 x 10^6/ml) were incubated together at 37°C for 15 min before centrifuging and incubating on ice as described for EA_M rosettes above. The incubation step at 37°C

was later found to be unnecessary and was omitted.

2.5 *Fluorescein-labelled, monomeric human IgM*

IgM was isolated from the serum of a patient with Waldenstrom's macroglobulinaemia by five cycles of euglobulin precipitation. No IgG was detectable by immunoelectrophoresis. The protein was fluoresceinated with fluorescein isothiocyanate (FITC) as follows. The FITC was made at a concentration of 2 mg/ml in 0.1 M sodium carbonate/bicarbonate buffer (pH 9.5) and added to the IgM as a single addition with stirring (16.8 moles FITC/mole IgM). Stirring was continued at room temperature for 4 h and then transferred to $4^{o}C$ overnight. After this time the preparation was Swinney-filtered and excess FITC removed by chromatography on a G25 Sephadex column. A high F/P ratio (8.58) was obtained for maximum sensitivity. This preparation of fluoresceinated IgM was then monomerized by reduction with 1.4 mM dithiothreitol in 100 mM Tris buffer, pH 8.0 for 1 h at room temperature. The reduced IgM was alkylated with an excess of 6 mM iodoacetamide and dialysed overnight against PBS. Final protein concentration was 2.57 mg/ml.

2.6 *Scanning and transmission electron microscopy*

The morphology of freshly isolated hairy cells, and of cells forming various types of rosette was examined by electron microscopy. Isolated mononuclear cells, or bulk preparations of rosettes formed as described above, were fixed in suspension for 24-28 h by adding at least 10 ml of 1.5% glutaraldehyde in 0.1 M cacodylate-HCl buffer (pH 7.4) containing 1% sucrose (Cawley and Hayhoe, 1973). The fixed cells were then washed in cacodylate-HCl buffer and the final cell concentration adjusted to approximately 1.5×10^{6} cells/ml.

Scanning electron microscopy (SEM) Fixed cells were collected on to polylysine-coated coverslips according to the modified method (Sanders et al, 1975) of Mazia et al (1974, 1975). This method involved placing a 20 µl drop of cell suspension on to coverslips previously coated with poly-L-lysine hydrobromide (Sigma) and the reverse side identified by a locating grid previously attached with epoxy resin.

The samples were then dehydrated in a graded series of ethanol, critical point dried using liquid CO_2 (Distillers water-free; Polaron E3000 critical point drier), and then sputter-coated with gold (Polaron sputter unit E5000). Finally, the coverslips were examined in an ISI Super 3A SEM with a 45° tilt and a usual operating voltage of 20 KV.

Some of the technical aspects of preparing haemic cells for SEM are discussed by Polliack (1977).

Transmission electron microscopy (TEM) Processing for TEM was carried out exactly as described by Cawley and Hayhoe (1973). The fixed cells were post-fixed for 1 h at room temperature in 2% osmium tetroxide in cacodylate-HCl (pH 7.4). The samples were then rapidly dehydrated in 70%, 90% and absolute alcohol and then in two changes of propylene oxide before infiltrating with Spurr resin and embedding in BEEM capsules in freshly prepared resin.

Thin sections were cut on an LKB ultratome III with a diamond knife, and then mounted on clean uncoated copper 200 mesh grids (LKB). Double staining with alcoholic uranyl acetate (15 min) followed by Reynolds' lead citrate (30 min) was employed, and the sections were examined in an AEI EM6B electron microscope at 80 KV.

2.7 Cell culture

A hairy-cell culture was established from the spleen of one patient by the method described by Karpas et al (1977). To provide a single cell suspension, small pieces of spleen removed aseptically in theatre were forced through a sterile stainless-steel gauze into RPMI-1640 medium supplemented with 10% foetal-calf serum and antibiotics (10 µg/ml streptomycin, 100 iu/ml penicillin, 1 µg/ml amphotericin). The cells were grown in stationary cultures in loosened screw-topped, 125 ml, Erlenmeyer flasks incubated at 37°C in 5% CO_2. The medium was changed every fifth day by aspiration of the upper half of the culture fluid from each flask and its replacement with an equal volume of fresh medium. The cell cultures were grown for about 3 months before they became contaminated and lost for further study. The characteristics of cell growth and the full surface-marker characteristics of these cells will be described in detail in Chapter 3. However, it should be stated here that almost 100% of the cells consistently maintained the characteristic hairy-cell morphology and continued to display tartrate-resistant acid phosphatase activity. Also, after 2 weeks in culture, virtually no T cells were identified in the cell population.

2.8 Blocking reagents

Various whole sera, heterologous antisera and immunoglobulin fractions were used to block EA_M-rosette formation in attempts to clarify the nature of the receptor. Before testing for blocking ability, all sera were heat inactivated at 56°C for 20 min and absorbed with washed, packed ox erythrocytes.

IgM IgM was isolated by repeated cycles of euglobulin precipitation from several sera obtained from two patients with Waldenstrom's

macroglobulinaemia. No IgG was detectable by immunoelectrophoresis. Monomerized IgM preparations were prepared by reduction of the IgM with 0.7 mM or 1.4 mM dithiothreitol in 100 mM Tris buffer (pH 8.0) for 1 h at room temperature. Free -SH groups were alkylated with a fourfold excess of iodoacetamide and dialyzed overnight against PBS. Aggregated IgM was prepared from the same sera by heat aggregation at 63°C from 30 min. $Fc_5\mu$ and $F(ab')_2\mu$ fragments were prepared from the same IgM source by the trypsinization procedure described by Shimizu et al (1974). Isolated IgM was made to a concentration of 10 mg/ml in 5M urea, 0.1M Tris (pH 8.0) containing 0.15M NaCl, 0.01M sodium azide and 0.05M $CaCl_2$. To 8 ml of the IgM was added 1/30 weight of trypsin (2.6 mg) and the reaction was allowed to proceed for 2 h at 25°C. After this time, ovomucoid trypsin inhibitor (Sigma) was added to stop the reaction and the trypsinized IgM was dialysed overnight against PBS. The preparation was concentrated by negative pressure filtration using the millipore 'immersible molecular separator' and fractionated on a column of BioRad A 1.5M. Six peaks were obtained as described by Shimizu et al (1974), and the peak corresponding to a molecular weight of 342,000 was selected as containing $Fc_5\mu$

IgG Monomeric IgG was isolated from pooled normal human serum by chromatography on QAE-Sephadex (Joustra and Lundgren, 1969). Aggregated IgG was obtained from this preparation by heating for 30 min at 63°C. Unfractionated human cord serum was also tested for blocking.

Antigen-antibody complex Crude toxoplasma antigen was obtained by repeated cycles of freezing and thawing of the whole organism

followed by centrifugation at 5000 x g for 10 min. The IgM fraction from a patient with acute toxoplasmosis was obtained by sucrose density gradient ultracentrifugation and titrated against the toxoplasma antigen to (visual) equivalence. This concentration of IgM, or the antigen-antibody complex diluted 1/2 (IgM-antigen), was used as blocking reagents. Dr. Harry Williams of Raigmore Hospital, Inverness, kindly supplied the toxoplasma antigen and antiserum.

Anti-hairy cell serum (AHS) A heteroantiserum to splenic hairy cells was prepared in rabbits by a two-pulse method (Levey and Medawar, 1966). Cells from the spleen of a patient with HCL were isolated and the mononuclear cells separated as described previously. As with most spleens from HCL patients, the mononuclear cell preparation contained over 90% hairy cells. After thorough washing, the cells were resuspended in sterile saline to a concentration of 1×10^7 cells/ml, and 1 ml of the suspension was injected intravenously into 2 rabbits. At the same time, a proportion of mononuclear cells was frozen down and maintained in liquid nitrogen. Two weeks later, the frozen cells were thawed rapidly at 37°C, washed in HBSS, and tested for viability by trypan-blue dye exclusion. The cells were 68% viable, therefore dead cells were removed by centrifugation over Ficoll-Isopaque (Hannestad et al, 1972) before resuspending in sterile saline at 1×10^7 cells/ml for injection. The 2 rabbits were then each given a 1 ml booster injection of this cell suspension. The rabbits were bled after 10 days and 20 days, and finally exsanguinated under anaesthetic a week later. The sera obtained from each rabbit were complement inactivated by heating at 56°C for 20 min and then rendered specific for hairy cells by a series of exhaustive absorptions. After

absorptions with red blood cells, normal spleen and tonsil cells, T-cell Sezary cells, cells from chronic myeloid leukaemias, acute monocytic leukaemias (both Schilling and Naegeli types) and cells from a myeloproliferative state with a large number of circulating normoblasts, the sera were tested for specificity and staining properties by an immunofluorescent technique. Details of the properties of these sera in staining reactions will be given in Chapter 3.

Anti-Ia-like antigen (anti-p29,34) Monospecific rabbit heteroantiserum against purified B-cell associated, Ia-like, membrane antigen (p29,34) was used in blocking studies. This antiserum (Springer et al, 1976) was the kind gift of Dr. Tim Springer of the Biological Laboratories, Harvard University, and its binding properties in HCL will be discussed in Chapter 3. For blocking studies, the serum was used at a dilution of 1/50, a concentration demonstrated to stain specifically HCL and chronic lymphocytic leukaemia (CLL) cells, but not T cells.

Anti-CIq serum In order to investigate the possibility of CIq on the hairy-cell surface binding EA_M and thus acting as an IgM receptor, blocking of rosette formation was attempted with various dilutions of an anti-CIq antiserum. The heterologous rabbit antiserum used was a kind gift from Dr. Ian McConnell, M.R.C. Group on Mechanisms in Tumour Immunity, The Medical School, Cambridge.

Chemical agents Various chemical reagents were tested for their effect on rosette formation. Rosette formation was carried out in the presence of ethylenediamine-tetraacetic acid (EDTA), 10 mM, at pH 7.0; puromycin at 2.5 µg/ml; sodium azide at 100 mM; and cytochalasin B.

The cytochalasin B was dissolved in dimethyl sulphoxide to a concentration of 10 mg/ml and further diluted in HBSSA to 2.5 µg/ml before use. The effect of pretreatment of leucocytes with papain was also examined. In this test leucocytes were treated with 1% papain for 6 min at room temperature and washed 2 times in HBSSA before rosette testing.

2.9 *Blocking tests*

In all of the blocking studies, one drop of leucocytes at 4×10^6 cells/ml was mixed with one drop of the reagent under test. The mixture was incubated for 15 min at room temperature and then rosette tested with 2 drops of indicator cells in the usual way. As there is shedding of receptors, even during this short incubation period (see Section II, Results), a control was always incubated in parallel and the % inhibition calculated by comparison of the 2 results.

The effects of various physical treatments such as the concentration of indicator erythrocytes and the temperature, time and pH conditions used for rosette formation were also considered as described in the Results below.

3. RESULTS

3.1 *Cells forming EA_M rosettes*

Peripheral blood Initial studies revealed that a proportion of mononuclear cells from the PB of all the HCL cases examined formed rosettes with EA_M. The percentage of cells forming EA_M rosettes varied widely from patient to patient and even within any one patient when the study was repeated on different occasions. With further

understanding of the nature of expression of the receptor for EA_M, and by carefully controlling the conditions of rosette formation accordingly, it was observed that the proportion of cells forming EA_M rosettes broadly reflected the proportion of HCs in that blood sample. Table 2.I presents some selected, but representative, results from each of the 23 patients.

As will be discussed throughout this thesis, peripheral blood involvement by HCs in HCL can vary from almost nil to florid leukaemia with almost 100% of HCs. However not all the leukaemic cells are particularly 'hairy' and in most patients peripheral blood involvement is greater than is apparent by examination of Romanowsky-stained smears. Determination of the leukaemic involvement depends on combined immunological, cytochemical and ultrastructural studies (see Chapters 3 and 6). In Table 2.I the % HC given is an estimate of the true leukaemic involvement based on the above criteria. Two points are clear from the Table. When the proportion of non-leukaemic T cells is high, the % EA_M rosette formation is low. This is particularly well illustrated by patients DC and LH whose peripheral involvement by HCs fell dramatically after splenectomy, and this was accompanied by a fall in the % EA_M rosette formation. Second, under the conditions employed for rosette formation, at any given time of testing not all hairy cells form EA_M rosettes. Patients KD, MB, LS, LP and AB, all with very high percentages of circulating HCs, illustrate this point.

Spleens The result obtained when splenic mononuclear cells from 10 patients were tested for EA_M rosette formation are shown in Table 2.II. The results from RH, MB and PS serve only to illustrate the precautions which must be taken in order to avoid receptor shedding,

Table 2.I EA_M rosette formation by peripheral blood leucocytes from HCL patients

Patient	Date	% of mononuclear cells		
		EA_M rosettes	T cells†	Hairy cells†
KD	20. 5.76	36	44	56
	2. 2.77	43	10	90
	18. 1.78	66	5	91
MB	4. 6.76	84	8	92
PS	27. 8.76	30	48	50
	19. 2.77	31	42	58
MH	16.10.76	23	47	53
HD	29.10.76	10	40	60
	13. 4.78	33	60	40
RH	5.11.76	20	70	30
	29. 4.77	5	87	13
LS	26.11.76	71	10	90
DC	13.12.76	64	30	70
	18. 5.77	24	85	15
FB	21. 1.77	43	50	50
	17. 6.77	55	50	50
AD	25. 1.77	65	18	78
	23. 1.78	27	30	40
LP	28. 1.77	78	11	83
LH	26. 2.77	66	33	77
	6. 2.78	10	70	30
FW	12. 3.77	52	45	55
	2. 2.78	13	56	37
BN	16. 6.77	37	44	56
NW	17. 6.77	12	87	13
RR	3. 9.77	36	60	40
HR	11.10.77	42	19	71

Table 2.I (continued)

Patient	Date	% of mononuclear cells		
		EA_M rosettes	T cells†	Hairy cells‡
AB	22.11.77	42	32	68
	23.11.77	56	0	98
GH	19.12.77	30	43	47
TN	15. 1.78	18	30	70
LK	18. 1.78	49	20	78
DA	2. 3.78	38	26	70
	6. 4.78	22	51	48
JC	27. 4.78*	15	9	90
	9. 5.78	74	<1	99

† The percentage of T cells in the mononuclear preparation was usually the % cells forming E rosettes (Chapter 3). In some cases this was confirmed by the % cells staining with an anti-T cell serum, or giving the T-cell esterase pattern (Chapter 3) and in patient LH this last marker was used as the only measure of T cells, since this patient's hairy cells also formed E rosettes (Chapter 5).

‡ The percentage of hairy cells given is that established by immunological techniques described in Chapter 4.

* This sample of peripheral blood was obtained, through the post, as a leucophoresis pack.

and this feature of EA_M rosette formation is considered in detail in Section II of the present chapter. The results from the other patients show that hairy cells can form EA_M rosettes and, as will be shown in Section II of this chapter, this lack of receptor expression by splenic HCs is probably not due to technical artifact.

Since HCs are probably produced in the bone marrow and accumulate in the spleen following sequestration from the PB, the possibility exists that HCs begin to lose their receptor for EA_M with ageing. A more detailed study of EA_M receptor expression by PB HCs following splenectomy would seem to substantiate this theory. Figure 2.1 illustrates that, following splenectomy, there is a sharp rise in the absolute numbers of HCs in the PB and that the absolute HC count quickly falls to pre-splenectomy levels. Examination of PB taken immediately before and after splenectomy demonstrated that this brief increase in cell numbers was not directly due to release of splenic cells during surgery, but took some hours to appear. It therefore seems that the sharp rise in HC numbers in the PB is caused by the appearance of HCs from some other extravascular site. During the time of study the proportion of HCs in the PB did not greatly alter, and consistently some 70-80% of PB mononuclear cells were HCs. The sharp fall in EA_M receptor expression corresponding to the supposed release of extravascular HCs (Figure 2.1), followed by receptor reexpression a week later when the absolute HC count had returned to normal, suggests therefore that these released HCs lack the EA_M receptor. Disappointingly, the single lymph node from one patient, and the bone marrow aspirates from 3 patients did not yield further information on this point, and further samples of biopsy material will be required. The results obtained from the lymph node and bone marrow specimens examined are

Table 2.II EA_M rosette formation by splenic leucocytes from HCL patients

Patient	Date	% of mononuclear cells			Comments
		EA_M rosettes	T cells	Hairy cells	
RH	12. 5.76	8	22	80	Poor cell viability obtained. This patient's spleen was transferred to the laboratory as a whole spleen before processing (see Methods). However, subsequent PB samples from this patient revealed that he may in fact fortuitously, have HCs of poor viability
MH	4. 6.76	0	3	97	Processed before appreciation of receptor shedding (Section II).
PS	19.10.76	5	13	86	Processed before appreciation of receptor shedding (Section II).
NW	19.12.76	37	16	82	
LH	12. 1.77	0	5	>90	Difficult operation requiring several hours. Also, splenic cell suspension transported to laboratory for 3 h in HBSSA without foetal calf serum (FCS) (See Methods).
	19. 1.77	77		>90	Result following 1 week of culture in RPMI 1640 + 10%FCS
DC	14. 1.77	58	22	80	
LP	3. 2.77	55	18	80	
AD	2. 3.77	47	9	90	
HR	10.10.77	35	6	91	
AB	22.11.77	46	10	90	

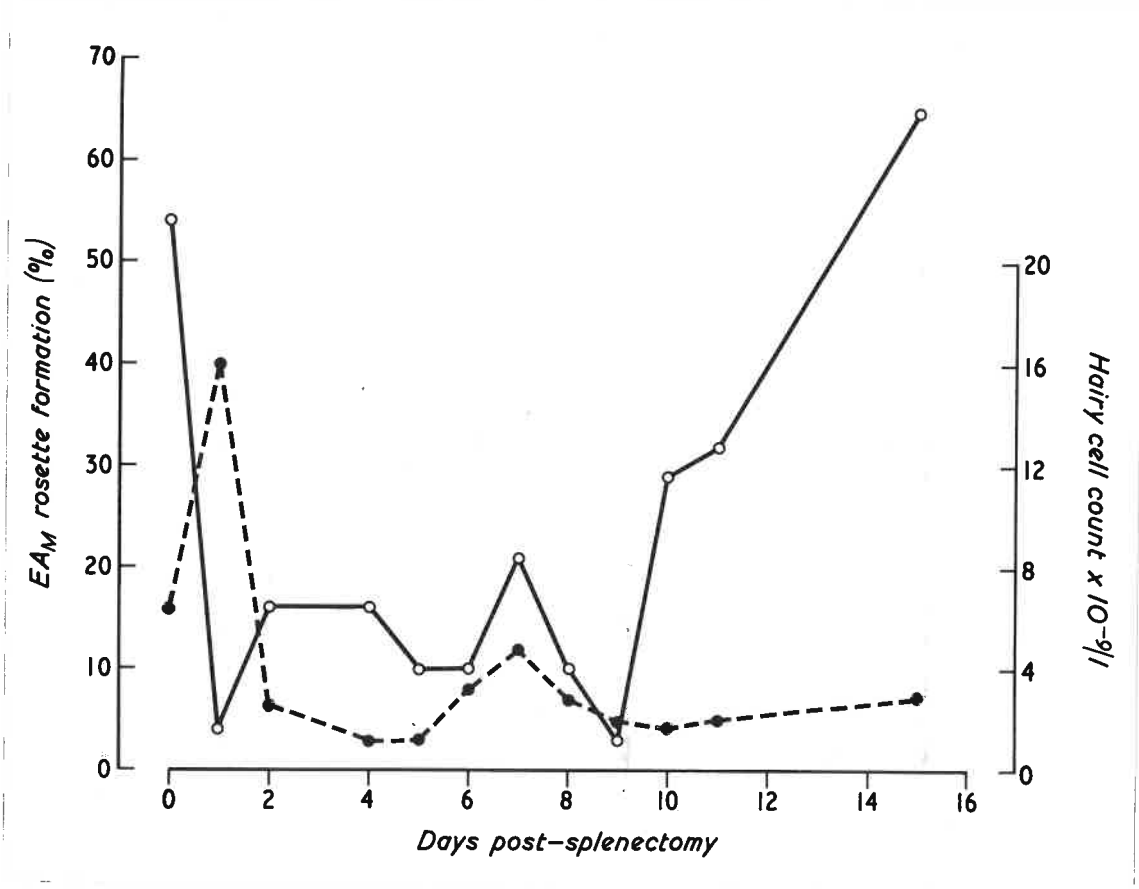

Figure 2.1 Absolute hairy cell numbers and EA_M receptor expression by the mononuclear cells from the PB of a patient (HR) with HCL in the immediately post-splenectomy period. Percentage of mononuclear cells forming EA_M rosettes (o—o); absolute count of HCs in the PB (•--•).

given in Table 2.III and demonstrate only that HCs expressing the EA_M receptor are present.

Cultured hairy cells The characteristics of the established hairy-cell culture will be considered in detail in Chapter 3. However, during the period of study (3 months) the cultured cells maintained a definite HC morphology and consistently displayed the cytochemical and immunological features of hairy cells. The cultured cells were examined for EA_M rosette formation after 18, 29, 46 and 66 days of culture, when 92, 95, 93 and 86% of cells respectively formed rosettes.

Morphological identification Cells forming EA_M rosettes were identified by morphology and cytochemistry of cytocentrifuge preparations, and by electron microscopy of bulk preparations. Figures 2.2 to 2.6 are typical of cells forming EA_M rosettes, and clearly demonstrate that hairy cells form EA_M rosettes.

Very few cells other than hairy cells were seen to form EA_M rosettes. The few monocytes and polymorphonuclear leucocytes present in rosetted mononuclear cell preparations were invariably negative. Occasional platelets were, however, seen to form EA_M rosettes.

Mixed rosettes By the use of mixtures of fluorescein-labelled EA_M (FEA_M) and unlabelled EA_G and sheep erythrocyte (E) indicator cells, it was found that the cells forming rosettes with FEA_M also invariably formed rosettes with EA_G. Control mixtures of FEA_M and unlabelled EA_M showed that the fluorescein labelling of the indicator erythrocytes did not affect the ability of the indicator erythrocytes to form rosettes. In the mixed tests with FEA_M and EA_G, adherent indicator erythrocytes on individual rosette-forming cells were scored as being all non-fluorescent (EA_G only), all fluorescent (FEA_M only), or mixed fluorescent and non-fluorescent (FEA_M and EA_G). In these tests the proportions of EA_G only

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Table 2.III EA_M rosette formation by bone marrow and lymph node leucocytes from HCL patients

Patient	Date	Material	% of mononuclear cells		
			EA_M rosettes	T cells	Hairy cells
HR	26.10.77	bone marrow	37	13	66
AB	22.11.77	bone marrow	37	38	50
	22.11.77	lymph node	25	31	70
LH	3.12.77	bone marrow	25	23	40

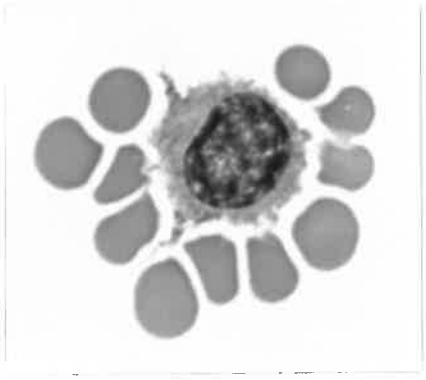

Figure 2.2

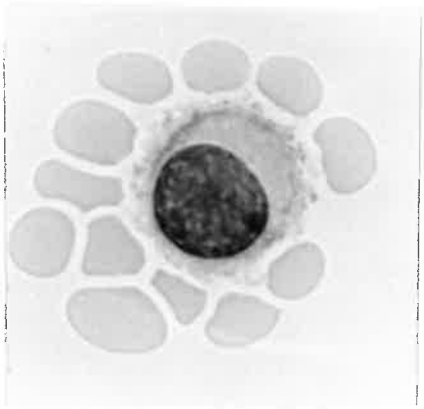

Figure 2.3

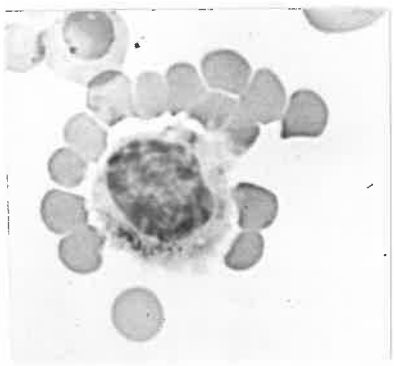

Figure 2.4

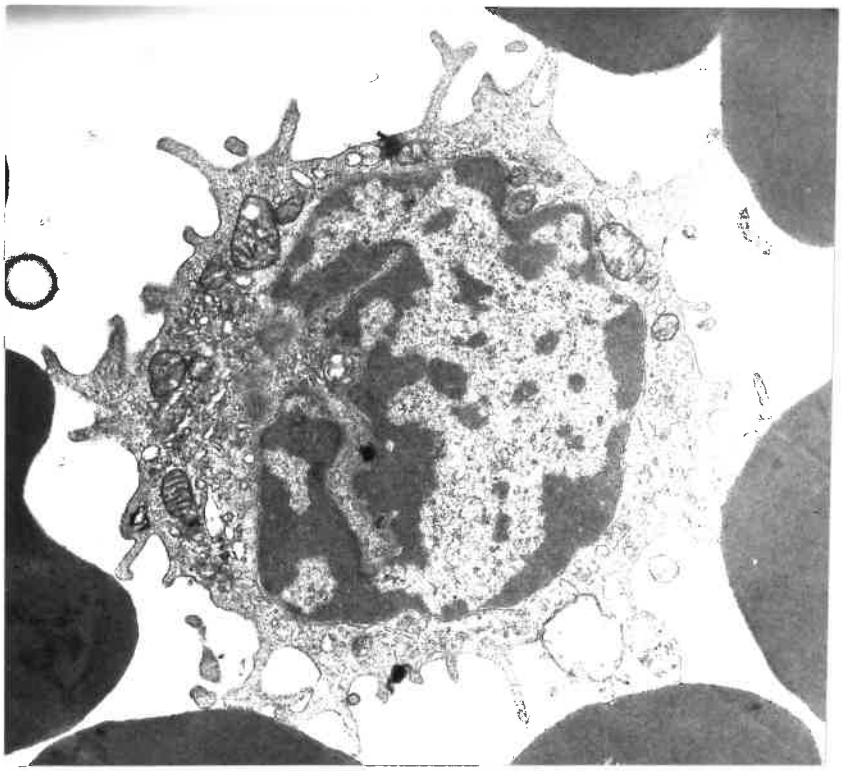

Figure 2.5

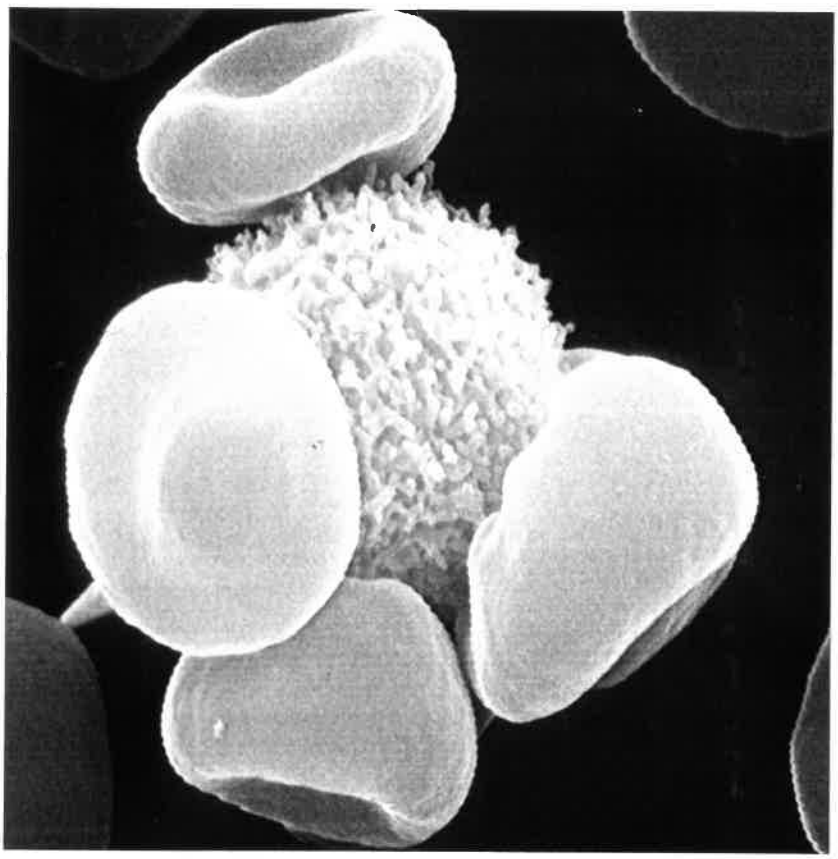

Figure 2.6

Figure 2.2 Cytocentrifuge preparation of an EA_M rosette-forming cell from the peripheral blood of a patient (LS) with HCL. The characteristic hairy-cell morphology of the rosetting cell is clearly illustrated. Stained with Giemsa.

Figure 2.3 Cytocentrifuge preparation of an EA_M rosette-forming cell from the peripheral blood of a patient (LK) with HCL. Stained with Giemsa.

Figure 2.4 Cytocentrifuge preparation of an EA_M rosette-forming cell stained for non-specific esterase by the method of Higgy et al, (1978). The rosetted hairy cell from the peripheral blood of patient LH shows the granular composition of the crescent of esterase positivity typical of hairy cells.

Figure 2.5 Transmission electron micrograph of a hairy cell from the peripheral blood of patient (LP) forming an EA_M rosette. The indicator erythrocytes make contact with the morphologically typical hairy cell mainly at the ends of the fine surface projections (x 7500).

Figure 2.6 Scanning electron micrograph (SEM) of a hairy cell from the peripheral blood of patient (PS) forming an EA_M rosette. The central cell demonstrates the typical hairy-cell morphology of both ridges or ruffles and microvilli seen under SEM (x 7000).

and mixed FEA_M and EA_G varied from case to case, but examples of FEA_M only were never seen. Mixtures of indicator cells in the proportions 1 FEA_M : 1 EA_G and 2 FEA_M : 1 EA_G were used and the 2 : 1 ratio was found optional for mixed rosette formation. Many individual rosettes from several patients were carefully examined for mixed and non-mixed rosettes. But because of the nature of examination percentage scores of mixed to non-mixed were not made.

When FEA_M and E indicator erythrocytes were mixed and read for mixed rosette formation as above, no mixed rosettes were observed although in all the HCL cases examined both fluorescent and non-fluorescent rosettes were present.

3.2 *Purity of the EA_M indicator system*

Since hairy cells have an avid receptor for the Fc of IgG as detected by EA_G rosette formation (above and Chapter 3), the possibility was considered that trace amounts of IgG in the IgM anti-ox erythrocyte antibody were causing apparent EA_M rosette formation. No IgG was detectable by gel diffusion of the IgM fraction of the primary bleed used to sensitize indicator erythrocytes. Also, the IgG fraction obtained by Sephadex G200 chromatography of this same primary bleed did not agglutinate ox erythrocytes, nor did it lyse ox erythrocytes in the presence of fresh complement. Neither normal monocytes, nor monocytes from 6 cases of myelomonocytic leukaemia, formed rosettes with EA_M indicator cells (Section III), but both normal and leukaemic monocytes had receptors for the Fc of IgG (γFcR) as demonstrated by their strong rosette formation with EA_G. The pathological T cells of Sezary's syndrome, on the other hand, did form rosettes with EA_M following culture (Section III), but did not form rosettes with EA_G.

EA_G rosette titration experiment If apparent EA_M rosette formation by

HCs was caused by trace amounts of IgG in the sensitizing IgM preparation, it would require that the γFcR on HCs be extra-sensitive. A titration experiment was set up to investigate this possibility. Ox erythrocytes were coated with serial dilutions of anti-ox-erythrocyte IgG antibody to form EA_G of progressively lower sensitivity. These cells were tested for rosette formation with the mononuclear cells from the peripheral blood of 5 cases of HCL and of 12 normal individuals. The results are illustrated in Figure 2.7. Since no attempt was made to remove monocytes from the normal leucocyte preparations, the normal titration reflects EA_G rosette formation by both monocytes and B lymphocytes. From the graph, it can be seen that the end point of the titration is similar for both HCs and normal leucocytes, indicating that HCs do not possess extra-avid γFc receptors. Also, since there is no tail of HCs forming rosettes at very high antibody dilutions, there is probably no subpopulation of HCs with extrasensitive receptors.

Morphology of EA_M and EA_G rosettes Further evidence for the specificity of EA_M rosette formation was obtained by the examination of EA_M and EA_G rosettes by electron microscopy. Figure 2.8 displays the morphology of typical EA_M and EA_G rosettes formed by HCs as viewed by transmission (TEM) and scanning (SEM) electron microscopy. It is clear from the figure that the two types of rosette are morphologically different. While receptor density cannot be excluded as the sole cause of this difference, the distortion of the EA_G indicator erythrocytes shown in Figure 2.8 is particularly characteristic of EA_G rosette formation by HCs.

Heterophil antibody The possibility that the IgM preparation used to make EA_M was detecting a heterophil antigen shared by HCs and ox erythrocytes was also excluded in the following way. Hairy cells from the peripheral blood of one patient (MB) were incubated with the IgM

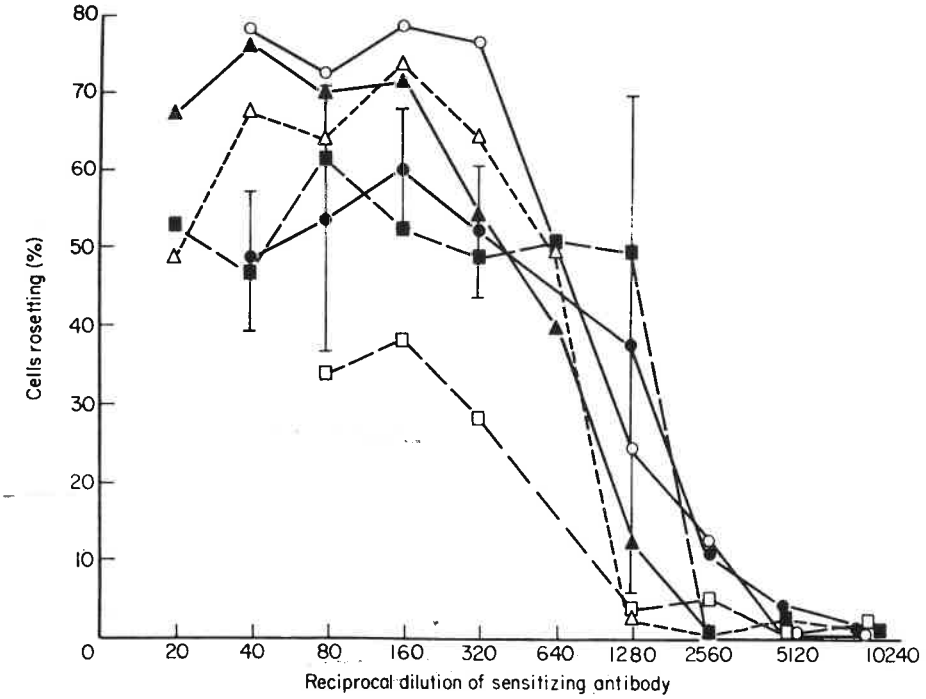

FIG 6. EA_{IgG} rosette titration. Percentage rosette formation with ox RBCs treated with serial dilution of IgG antibody. ○, △, □, ▲, ■, Leucocytes from individual LRE patients; ●, mean of 12 normal leucocyte preparations, error bars indicating ±1 SD.

Figure 2.7 EA_G rosette titration. Percentage rosette formation with ox erythrocytes treated with serial dilutions of IgG antibody mononuclear cells from 5 individual HCL patients; ●, mean of peripheral blood mononuclear cells from 12 normal individuals, error bars indicating ± 1 SD.

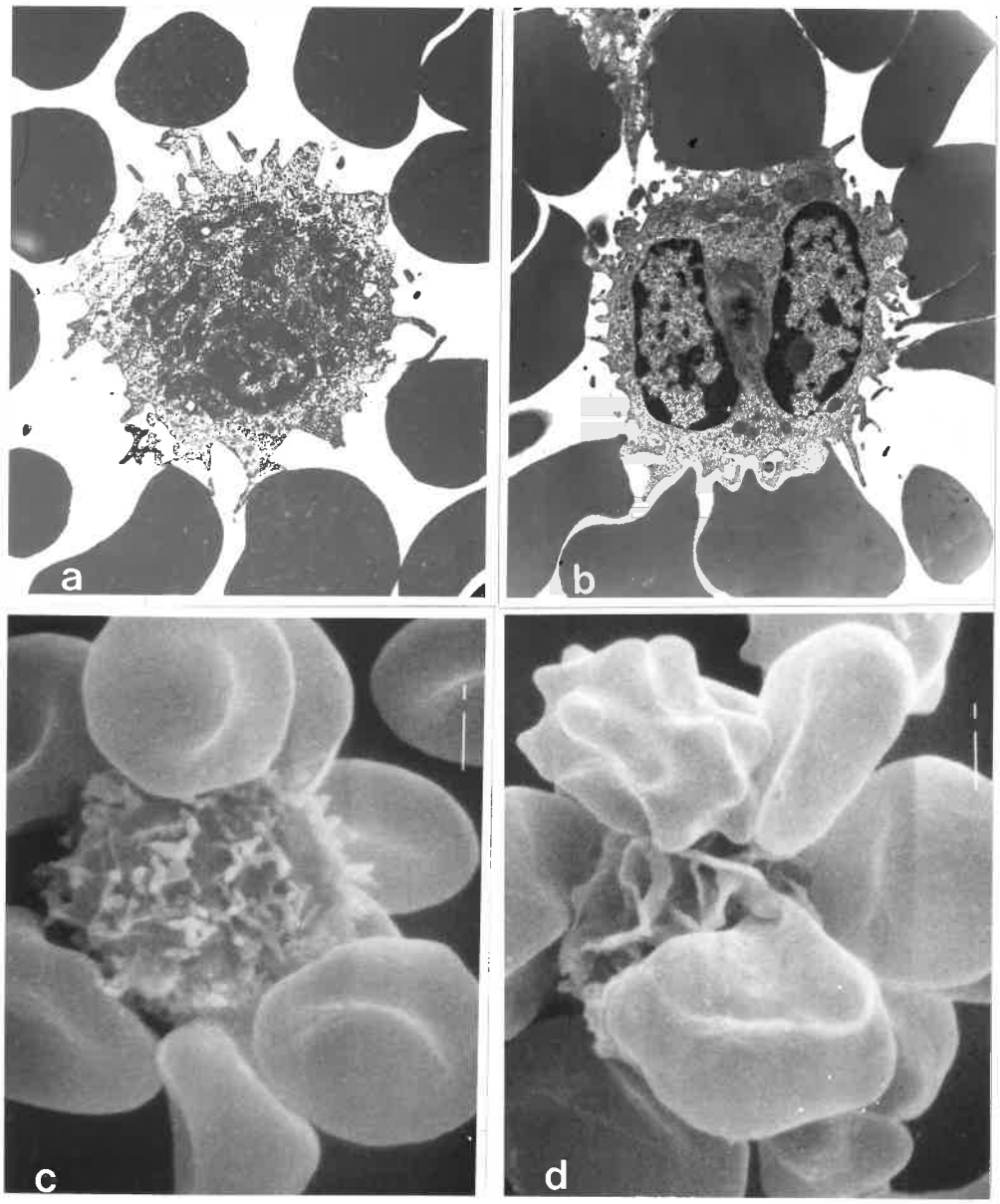

Figure 2.8

Figure 2.8 EA_M and EA_G rosette formation by HCs (a) TEM of EA_M rosette (x 7500), (b) TEM of EA_G rosette (x 6500) (c) SEM of EA_M rosette (x 7000) (d) SEM of EA_G rosette (x 7500). In the EA_M rosettes (a and c), indicator erythrocytes made contact with the HC mainly at the ends of the fine surface projections. Furthermore, relatively fewer indicator cells were involved in most rosettes and the erythrocytes usually preserved their regular biconcave shape. In contrast, in the EA_G (b and d), the indicator erythrocytes made contact with extensive areas of the HC surface including areas of well-developed ruffles. In addition, many erythrocytes seemed to be distorted by the rosetting process since control EA_G indicator cells had a regular biconcave shape.

preparation at the dilution used to make EA_M. After 10 min incubation the cells were washed and rosette tested with unsensitized ox erythrocytes and with EA_M indicator cells. The treated HCs did not form rosettes with unsensitized ox erythrocytes. The percentage of cells forming EA_M rosettes, compared with a control treated in the same way with normal rabbit serum, was not reduced by the above treatment.

Complement components In order to investigate whether complement components, in particular CIq, could be causing apparent EA_M rosette formation by HCs, 2 experiments were conducted. In the first, the IgM used to prepare EA_M indicator cells was heat treated at 56°C for 20 min before reacting with ox erythrocytes. In addition, IgM was reacted with ox erythrocytes in HBSS containing 10 mM EDTA. The EA_M indicator erythrocytes thus prepared were thoroughly washed in HBSS before being used in the rosette test. In the second experiment, isolated mononuclear cells from a patient with HCL were reacted with serial dilutions of an anti-CIq serum for 10 min at room temperature. The leucocytes were then washed and rosette tested with EA_M in the usual way. The results of these experiments, which are given in Table 2.IV, clearly exclude the possibility of CIq binding causing EA_M rosette formation.

Blocking experiments In order to confirm that the rosettes formed between EA_M indicator cells and hairy cells were the result of antibody binding by a receptor for IgM (μFcR) on the HCs, attempts were made to block rosette formation by the addition of whole antibody or antibody fragments. The results of these experiments are shown in Table 2.V and Figure 2.9. The data confirms that the EA_M reagent was detecting a receptor for IgM and that IgG was not involved in EA_M rosette formation. It is also clear that IgM in its monomerized form blocked much more

Table 2.IV Experiments to exclude CIq as a cause of EA_M rosette formation

Treatment of indicator erythrocytes	Treatment of leucocytes	EA_M rosette formation (%)
	Patient LS	
nil	nil	55
heat-treated IgM	nil	56
EA_M formed in 10mM EDTA	nil	59
	Patient MB	
nil	no serum	73
	normal rabbit serum $^1/_{10}$	82
	anti-CIq serum $^1/_{10}$	86
	anti-CIq serum $^1/_{20}$	74
	anti-CIq serum $^1/_{40}$	62
	anti-CIq serum $^1/_{80}$	74

efficiently than in the pentameric form; some preparations of pentameric IgM blocked quite efficiently while others completely failed to block rosette formation even at 1 mg/ml. IgM complexed to its antigen was a more effective blocking reagent than unbound IgM, and the Fc piece rather than the Fab region is probably involved in μFcR binding.

3.3 *Conditions affecting EA_M rosette formation*

Various physical factors were found to effect EA_M rosette formation by hairy cells, and these were taken into account before adopting the routine procedure described above in Methods.

Indicator erythrocyte concentration The maximum number of EA_M rosettes were formed when an equal volume of leucocytes at 2 x 10^6/ml were mixed with EA_M indicator cells at a concentration of 1% (v/v). When the concentration of indicator erythrocytes was lowered to 0.5%, the % rosette formation fell by about 50%. At a concentration of 2% EA_M, no increase in rosette formation by HCs was observed but the preparations were more difficult to score because of the relative paucity of leucocytes.

Time and temperature The effects of time and temperature on EA_M rosette formation are considered in some detail in Section II of this chapter. In brief, however, it was observed in most cases studied that rosette formation was possible at 0°C, room temperature, and 37°C. However, the percentage of cells forming rosettes at 0°C was always much lower than at the higher temperatures. In general, but by no means always, rosette formation was higher at 37°C than at room temperature. At both temperatures the percentage of cells forming rosettes reached a maximum after a short period of incubation (generally about 15 min) and then fell rapidly. With some patients, on some occasions, this time was slightly shorter and an incubation time of 10 min was adopted as standard.

Table 2.V The blocking effect of different immunoglobulins and immunoglobulin fragments on EA_M rosette formation by hairy cells

Inhibitor	Concentration	% inhibition of rosette formation†	
		mean	range
Human cord serum	-	0	-
Monomeric IgG	10 mg/ml	0	-
Aggregated IgG	10 mg/ml	0	-
Pentameric IgM	1 mg/ml	20	0-81
Aggregated IgM	1 mg/ml	0	-
$Fc_5\mu$	0.5 mg/ml	72	65-79
$F(ab')_2\mu$	0.5 mg/ml	0	-
Toxoplasma antigen	-	6	-
Anti-toxoplasma IgM	-	17	-
IgM-antigen complex	-	80	-

† The result given is the mean and range of 2 or more experiments on freshly isolated PB or splenic HCs and on cells from the HC culture. The % inhibition is calculated in terms of a control incubated for the same time to allow for receptor shedding (Section II).

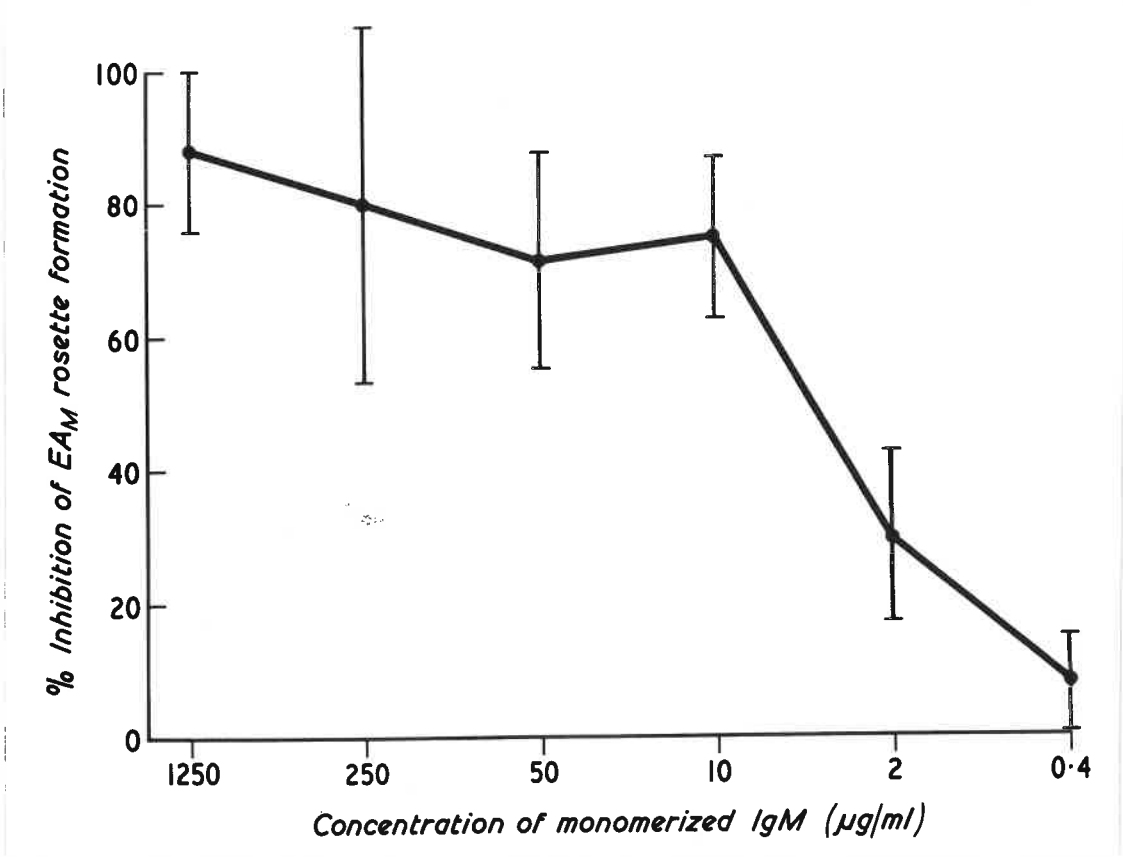

Figure 2.9 Inhibition of EA_M rosette formation by hairy cells by monomerized IgM. ⊕ , mean and standard deviation of experiments conducted on 3 different PB samples from HCL patients and using 2 sources of monomerized IgM.

Table 2.VI records selected, but representative, results obtained from the peripheral blood mononuclear cells of 20 HCL patients tested for EA_M rosette formation for 10 min at the two temperatures. Table 2.VII records the results obtained from the 5 spleens tested at both temperatures, and it can be seen that a higher percentage of splenic cells form rosettes at 37°C.

pH When cells were being tested for the effect of EDTA on EA_M rosette formation, it was thought possible that the drop in % rosette formation observed might be due to the acid conditions of the EDTA solution. Cells from the peripheral blood of a patient with HCL were therefore tested at the routine pH of 7.0 - 7.2, and the % EA_M rosette formation compared with that obtained at pH 5.6. At the acid pH there was a 65% reduction in rosette formation, and all subsequent EA_M rosette tests (including blocking with EDTA) were carried out at neutral pH.

Stabilization on ice If EA_M rosettes were allowed to form, centrifuged, and read immediately, the score obtained did not differ from preparations kept on ice for a period before reading. However, cytocentrifuge preparations from the samples which had not been stabilized on ice were poor. Furthermore, if the preparation was kept at room temperature after rosette formation and centrifugation, but before scoring, the percentage of rosettes obtained was much lower than preparations scored immediately. This last result can be attributed to continued shedding of μFcR by hairy cells (Section II of this chapter). Therefore when more than one test was being carried out it was found convenient to place the tube containing formed rosettes in ice until all tests were ready to be read. In cytocentrifuge preparations, the morphology of rosettes was much better preserved when the samples had been on ice for an hour or more before centrifugation. The results

Table 2.VI The effect of temperature on EA_M rosette formation by PB hairy cells†

Patient	% rosette formation at: 37°C	24°C*
KD	62	66
MB	84	70
PS	28	30
	20	2
	15	31
HD	16	1
LS	55	71
DC	59	64
	24	12
FB	37	28
	48	55
AD	65	42
LP	78	66
LH	66	51
FW	41	52
	45	34
BN	15	37
NW	18	18
	12	1
RR	36	55
MR	42	54
	32	18
AB	59	35
	39	56
	26	15
GH	50	50
TN	18	12
LK	49	26
JC	38	74

† Peripheral blood mononuclear cells obtained at the different times of testing were incubated with EA_M indicator erythrocytes for 10 min at the temperature indicated. The mixtures were then centrifuged and placed on ice before reading as described in Methods.

* Approximate room temperature.

Table 2.VII The effect of temperature on EA_M rosette formation by splenic hairy cells†

Patient	% rosette formation at: 37°C	24°C*
NW	37	5
LP	55	49
AD	47	37
HR	35	3
AB	46	18

† Splenic mononuclear cells were incubated with EA_M indicator erythrocytes for 10 min at the temperature indicated. The mixtures were then centrifuged and placed on ice before reading as described in Methods.

* Approximate room temperature.

suggest that a period on ice after rosette formation stabilizes formed rosettes. Consequently, a routine stabilization period of 1 h on ice was adopted for all test.

3.4 *Density of the receptor for IgM on hairy cells*

Various experiments were carried out to examine the density of the receptor for IgM (μFcR) on hairy cells.

EA_M rosette titration Figure 2.10 shows the dose response curves obtained when EA_M prepared from progressive dilutions of sensitizing IgM antibody were rosette tested with HCs. After a plateau phase, weaker sensitization is accompanied by progressively lower percentages of EA_M rosette formation. The quantity of sensitizing IgM required for detection of μFcR appears to parallel that required for complement fixation; for if the same progressive dilutions of EA_M reacted with a fixed dilution (1/10) of AKR mouse complement, and then titrated with normal mononuclear PB cells for EAC rosette formation, the dilution of IgM marking the end of the plateau phase of rosette formation is similar in both tests.

Sensitization with human IgM Sera from 2 cases of infectious mononucleosis were employed to sensitize ox erythrocytes, and the sensitized EA_M thus obtained were used in rosette tests with PB hairy cells from 2 cases of HCL. The results obtained are presented in Table 2.VIII; the given dilutions of antibody had to be used to avoid agglutination of sensitized ox erythrocytes. The % rosette formation obtained with these human EA_M is very low. Previous blocking experiments (Table 2.V) demonstrated that the μFcR on hairy cells recognised human IgM.

Binding of fluorescein-labelled, monomerized, human IgM Attempts were made to detect the μFcR on PB hairy cells employing the fluoresceinated,

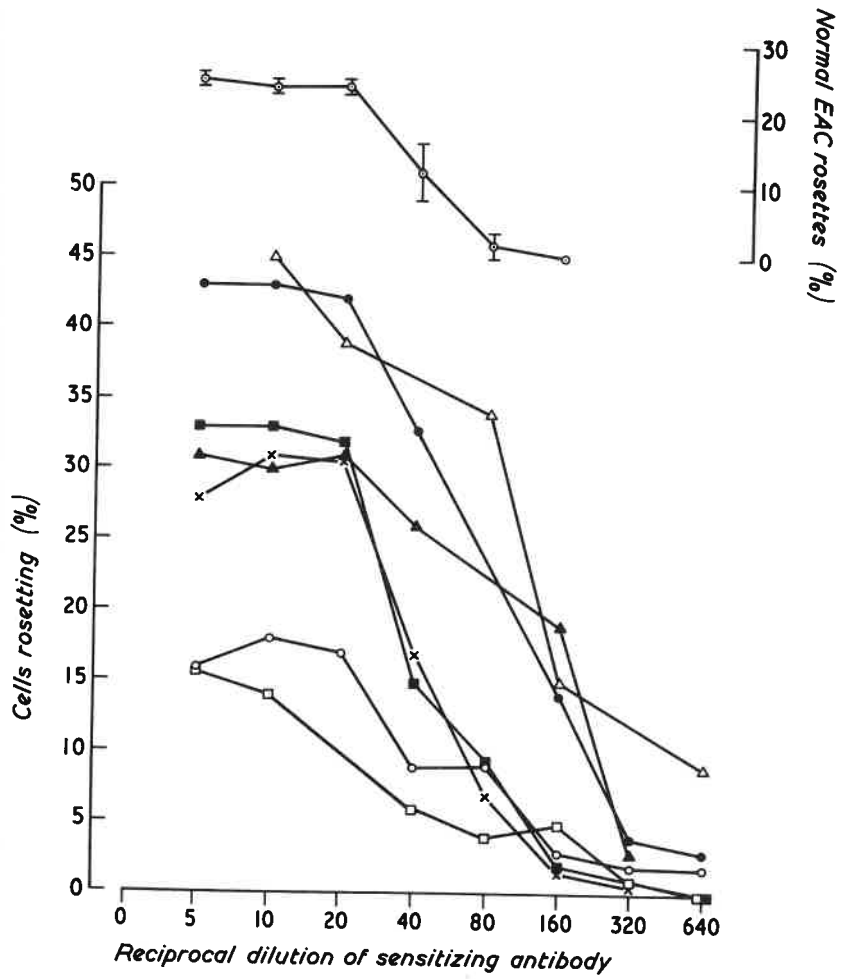

Figure 2.10 EA_M rosette titration. Percentage rosette formation by hairy cells from the peripheral blood of 7 individuals with HCL when rosetted with ox erythrocytes treated with serial dilutions of IgM antibody. Upper figure is the mean ± ISD of mononuclear cells from the PB of 3 normal persons rosetted with the same EA_M indicator cells reacted with a fixed concentration of AKR mouse complement.

Table 2.VIII Rosette formation with ox erythrocytes sensitized with Paul-Bunnell-positive sera

Patient	% cells rosetting with: EA_M hu(1)†	EA_M hu(2)‡	EA_M rabbit‡‡
MB	4	0	52
RH	3	0	17

† EA_M hu(1). Ox erythrocytes sensitized with serum from a patient with infectious mononucleosis. Dilution 1/40.

‡ EA_M hu(2). Ox erythrocytes sensitized with serum from a second patient with infectious mononucleosis. Dilution 1/80.

‡‡ EA_M rabbit. Standard EA_M reagent (Methods) Dilution 1/10.

monomerized human IgM preparation in a manner analogous to the detection of γFcR on B cells with aggregated IgG (Dickler and Kunkel, 1972; Dickler, 1976a). The labelled IgM was employed at about 3 mg/ml at room temperature, as recommended for aggregated IgG binding (Dickler, 1976a).

The experiment was attempted on the freshly isolated mononuclear cells from 6 patients with HCL, all tested at a time when the % of rosette formation with EA_M was high. Cells from 3 patients (RH, LS, MB) completely failed to stain. The HCs from patients HD and FW showed faint, but definite, cap fluorescence; and, at a different time of testing, the HCs from PS stained with a very faint diffuse fluorescence.

3.5 Blocking of EA_M rosette formation

The results of specific blocking experiments with immunoglobulin fragments have already been given in 3.2 of this section. Here, the effect of various chemicals and heteroantisera on EA_M rosette formation are considered.

Chemical inhibitors Table 2.IX illustrates that rosette formation requires the divalent cations of Ca^{++} and/or Mg^{++} together with active metabolic activity by the hairy cell involved. Possible involvement of microfilaments is suggested by the results obtained using cytochalasin B, but as will be shown in the next section (Section II), the presence of either cytochalasin B or puromycin prevents re-expression of the μFcR after it has been shed. The result obtained with papain possibly shows that there is only partial proteolysis of the μFcR by this enzyme.

Heteroantisera EA_M rosette formation by HCs was found to be exquisitely sensitive to blocking by the anti-hairy cell serum (AHS).

Table 2.IX The effect of various inhibitory reagents on EA_M rosette formation by hairy cells

Inhibitor	Concentration	% inhibition of rosette formation*	
		Mean	Range
EDTA, pH 7.0	10 mM	85	75-100
sodium azide	100 mM	90	80-100
cytochalasin B	20 µg/ml	34	26-34
puromycin	2.5 µg/ml	6	0-12
papain	1%	34	-

* The result given is the mean of two or more experiments and the % inhibition is calculated in terms of a control incubated for the same time to allow for shedding.

The PB mononuclear cells from 7 cases, and splenic cells from 3 cases, of HCL were treated with dilutions of AHS from 1/20 to 1/400 and tested for EA_M rosette formation. In very case 100% inhibition of rosette formation was obtained. At dilutions of AHS greater than 1/400, inhibition of rosette formation started to fall off as is shown for 2 cases (LH and DC) in Figure 2.11. This blocking of the HC μFcR by AHS at very high dilutions was peculiar to this particular receptor. Thus, as will be discussed in succeeding chapters, rosettes formed between HCs and EA_G, mouse erythrocytes, anti-immunoglobulin-coated erythrocytes, or sheep erythrocytes (1 case, Chapter 4), were either unaltered by treatment with AHS or blocked only by high concentrations of the serum. Similarly, the μFcR possessed by the B cells of chronic lymphocytic leukaemia (Section III) was only partially blocked by the AHS at a dilution of 1/20, and the μFcR expressed by T cells following culture (Section III) was not at all blocked by AHS when tested at a dilution of 1/100.

Cells from the HC culture, 95% of which formed EA_M rosettes, were incubated with serum against the p29,34 Ia-like antigen, at a dilution of 1/50. The cells were then tested for EA_M rosette formation and compared with a control incubated with normal rabbit serum for the same time. The anti-p29,34 serum completely abolished rosette formation. At this dilution of anti-p29,34 serum, EA_G rosette formation was reduced by only 4%.

4. DISCUSSION

In this section, the hairy cells from PB, spleen, bone marrow and lymph node of patients with HCL are shown to possess a receptor

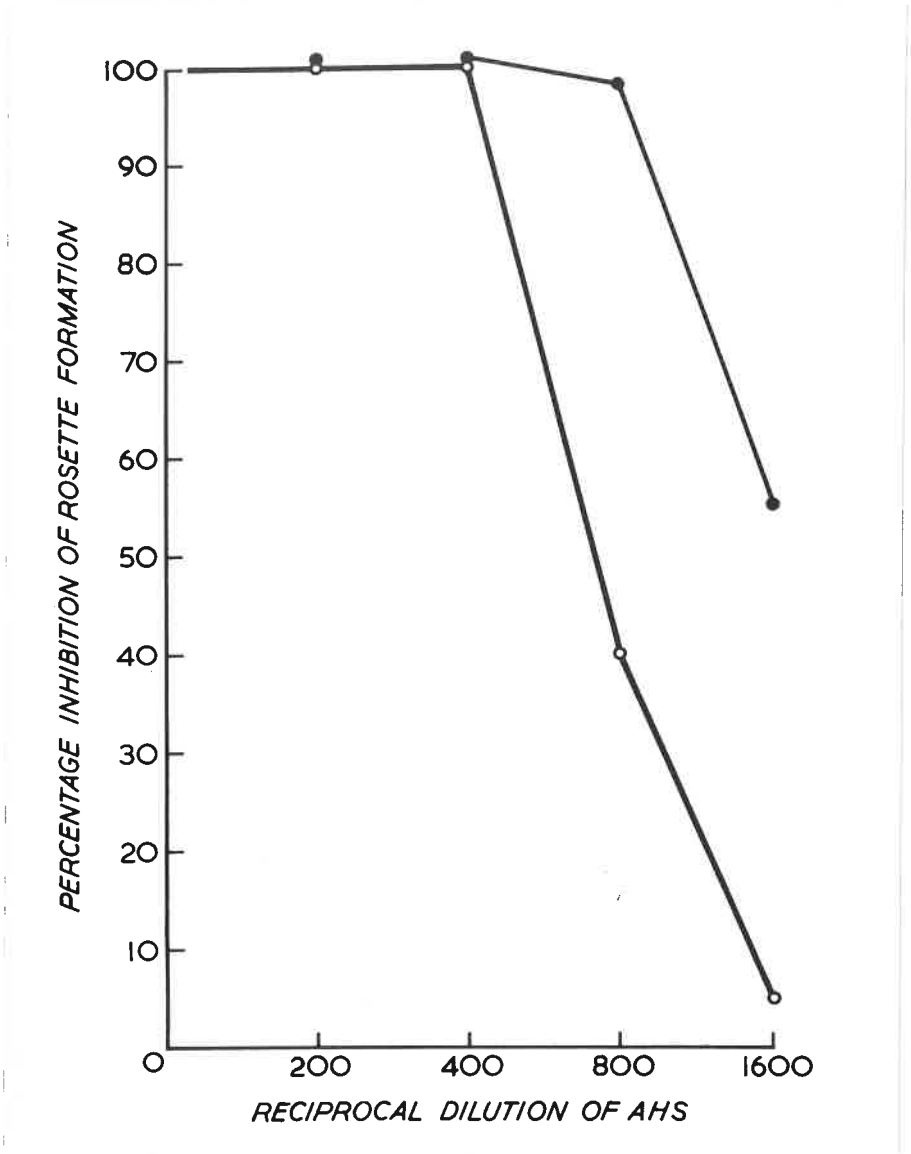

Figure 2.11 Blocking of EA_M rosette formation by AHS. The isolated mononuclear cells from the peripheral blood of 2 patients with HCL were treated with various dilutions of AHS for 10 min at room temperature before testing for EA_M rosette formation. The percentage rosette formation obtained was compared with that of a control incubated with normal rabbit serum at the same time, and the percentage inhibition thus obtained is plotted against the dilution of AHS serum employed for blocking.

for homologous and heterologous IgM. The EA_M rosette method used to detect the receptor, and the conditions required for rosette formation, are described. Morphological evidence clearly demonstrates that it is the pathological hairy cells which form EA_M rosettes, and this is confirmed by receptor expression by the cells from a cultured hairy-cell line. The receptor is expressed without culture in serum-enriched medium, and cells bearing μFcR simultaneously express γFcR. This original observation of μFcR on HCs has subsequently been confirmed by 2 other groups (Habeshaw, Dewar and Ramsy, personal communication; Ferrarini et al, 1977).

The simultaneous presence of γFcR on the cells forming EA_M rosettes makes the purity of the indicator system crucial. The possibility of CIq acting as a cementing substance for both IgM and IgG (Gmelig-Meyling et al, 1976) was excluded. No IgG was detectable in the IgM preparation used to prepare EA_M when tested by immunoprecipitation, and the IgG fraction from this same early bleed appeared to have no anti-ox erythrocyte activity. Further, neither normal nor pathological monocytes, both of which have strong receptors for the Fc of IgG (Koziner et al, 1977), formed rosettes with the EA_M reagent. Thus HCs would have to possess an extra-avid γFcR to detect trace amounts of IgG in the EA_M reagent, and this was shown not to be the case. The possibility was also excluded of EA_M rosette formation being a mixed agglutination reaction between antigens shared by HCs and ox erythrocytes.

The characteristically different morphology of EA_M and EA_G rosettes when seen by EM is further evidence that the EA_M rosettes are not the result of trace IgG contamination. It is possible that the point attachments seen in EA_M rosettes are the result of low receptor density

on the HC surface, and that, conversely, the extensive areas of contact and erythrocyte deformation seen in EA_G rosettes reflect a high receptor density. However, other evidence suggests that the different morphology of the two types of rosette is the result of a different kind of ligand binding. The SEM pictures of HCs in this section confirm previous studies showing both microvilli and broad-based ruffled membranes or ridge-like structures on the cell surface (Golomb et al, 1975; Deegan et al, 1976; Matre et al, 1977). This characteristic surface topography is one of the hybrid features of HCs. Ridges and ruffles are features associated with normal and leukaemic monocytes,(Polliack et al, 1974; Golomb et al, 1975), while the villous surface is a feature of normal and leukaemic lymphocytes (Lin et al, 1973; Polliack et al, 1973). The EA_M rosettes are mostly formed by the microvilli of the HC, and this may be a B-lymphocyte feature of HCs (Section III). Figure 2.8 illustrates that EA_G rosettes, on the other hand, are formed by attachment of the indicator erythrocytes over much broader areas of the HC surface, including the broad-based ruffles. This type of rosette formation is typical of the rosettes formed by monocytes (LoBuglio et al, 1967; Parker and Stuart, 1978), and the deformation of the attached erythrocytes is also a feature of monocyte rosettes (LoBuglio et al, 1967; Parker and Stuart, 1978). Other evidence pointing towards the monocytic nature of the γFcR of HCs will be given in Chapters 3 and 4.

Blocking of EA_M rosette formation by IgM, but not by IgG, further confirmed the specificity of the EA_M reagent. Whole pentameric IgM, however, did not always block rosette formation; some preparations blocked efficiently, while others failed to block. When monomerized

IgM was used, blocking of a dose-dependent type was obtained. These results were attributed to the exposure of a binding site on the IgM molecule either by monomerization of the pentamer, or by the 'stapling' rearrangement undergone by IgM when it binds to antigen (Feinstein, 1976). This interpretation was confirmed by using an antigen-antibody system employing the large toxoplasma antigen: in this system, the complexed IgM-antigen blocked much more efficiently than free IgM alone. Similar results have recently been obtained by Pichler and Knapp (1978), who used ovalbumin-IgM anti-ovalbumin complexes in their investigation of the μFcR on normal human B lymphocytes. This requirement for IgM monomerization, or 'stapling', may account for the rather inefficient blocking obtained by Ferrarini et al, (1977) when they attempted to block the μFcR on normal human B cells with whole IgM, since this receptor may be similar to the μFcR on both HCs and the cells of chronic lymphocytic leukaemia (Section III). The blocking of EA_M by whole IgM obtained by these authors may have been the result of IgM monomers in their reagent. For blocking, they used IgM isolated from the serum of a patient with Waldenstrom's macroglobulinaemia, and such sera often have a high proportion of monomeric IgM (Stobo and Tomasi, 1967; Damacco et al, 1970).

Aggregated IgM did not block EA_M rosette formation. In this regard the μFcR on HCs differs from the γFcR on both lymphocytes and macrophages. Whereas normal lymphocytes and macrophages can bind monomeric IgG (Phillips-Quagliata et al, 1971; Ramasamy et al, 1976), efficient binding requires the IgG to be complexed to its antigen, or heat aggregated (Phillips-Quagliata et al, 1971; Basten et al, 1972; Dickler and Kunkel, 1972; Shevach et al, 1974; Ramasamy et al, 1976). It has been suggested that the enhanced binding of complexed or

aggregated IgG is due to increased energy of binding resulting from summation of individual binding sites, rather than to the occurrence of allosteric change (Phillips-Quagliata et al, 1971; Ramasamy et al, 1976). However, a recent report of separate γFcR on murine leukaemia cells (Cooper et al, 1977), one for native IgG and one for IgG complexed to antigen, suggests that the situation might be more complex.

The Fc region of the antibody was required for IgM receptor binding, and this requirement is similar to that for IgG binding by B cells (Paraskevas et al, 1972; Dickler, 1974). Confirmation that the Fc region of IgM is involved in binding was obtained by the ability of $Fc_5\mu$ fragments to block EA_M rosette formation, and by the failure of $F(ab)_2\mu$ fragments to do so; normal human T cells (Gmelig-Meyling et al, 1976; Conradie and Bubb, 1977) and B cells (Ferrarini et al, 1977) also bind IgM through the Fc region.

The density of μFcR on HCs appears to be low. For example, the EA_M rosette titration experiment demonstrated that the indicator erythrocytes had to be coated with quite a high density of IgM for EA_M rosette formation to take place. Coating of ox erythrocytes with natural, rather than hyperimmune, antibody to form EA_M resulted in the virtual failure of HCs to form rosettes. The analogous situation of using natural anti-erythrocyte antibodies (such as anti-Rhesus or anti-Forssman) to identify γFcR detects only lymphocytes with a high density of receptor (Shevach et al, 1973; Natvig and Frøland, 1976). Attempts to detect fluorescein-labelled, monomerized, IgM bound by HCs were only of limited success, and this might be attributed to a low receptor density making the labelled cells difficult to visualize. It is probable, however, that labelled IgM-antigen complex would provide

a better experimental system. Nevertheless, when hyperimmune heteroantisera are used in the rosette method, a higher number of γFcR-bearing normal lymphocytes are identified than when FITC-labelled immune complexes are used (Winchester et al, 1976), and the former method is probably slightly more sensitive. Kinetic experiments following shedding of μFcR by HCs (Burns et al, 1977b) provided further evidence for the rather low density of μFcR. Shedding and re-expression of the μFcR was monitored by rosette formation with the standard EA_M indicator erythrocyte but in order to detect a similar shedding γFcR by HCs, the indicator EA_G erythrocytes had to be coated with very small amounts of IgG (Burns et al, 1978a).

The ability of the AHS to block EA_M rosette formation by HCs, even at very high dilutions, might suggest that the μFcR is particularly immunogenic and that much of the AHS antibody is directed against this receptor. However, previous studies of blocking of rosette formation by antisera require a more cautious interpretation. Anti-macrophage serum blocks γFcR binding of EA_G by mouse macrophages (Holland et al, 1972), but in this system the Fc as well as the $F(ab')_2$ region of the antiserum is involved in blocking and the authors conclude that the antibody is not directed toward the γFcR *per se* but to an adjacent antigen. Similarly, steric hindrance has been suggested as a reason for the blocking of E-rosette formation following anti-human T-cell serum treatment of human T cells (Galili et al, 1977; Gattringer and Wick, 1977). It may be that the μFcR on HCs is closely adjacent to an antigen detected by the AHS, or even that IgM binding by this receptor is weak and therefore particularly susceptible to steric hindrance. Blocking of EA_M rosette formation by serum to the p29,34, anti-Ia-like antigen might be interpreted in

the same way. EA_M rosette formation by the cultured HCs was completely blocked by this antiserum whereas EA_G rosette formation was almost unaffected. This result differs from that of Ferrarini et al, (1977) who reported no blocking of EA_M rosette formation by pretreatment of normal B cells with anti-Ia-like antiserum. It is possible that these conflicting results are due to differences between normal B cells and hairy cells, since Ferrarini et al did not attempt prestaining of HCL cells. However, similar conflicting results have been obtained concerning the specific blocking of the γFcR by anti-Ia serum both in mouse and man (Dickler and Sachs, 1974; Schirrmacher and Halloran, 1975; Solheim et al, 1976), and such blocking has been attributed to either steric hindrance by the antiserum (Schirrmacher and Halloran, 1975) or to ligand binding of the Ia antigen altering the cell membrane and affecting the function of the γFcR (Dickler et al, 1977). Others have failed to demonstrate any relationship between the two membrane components in either mouse or man (Woofsy et al, 1977; Chess et al, 1976). Such conflicting results could be attributed to steric hindrance which might vary in different studies. Three factors could cause variations in the sensitivity of the test and thus the ease of blocking by steric hindrance: (a) Both receptors for Fc on B cells, μFc and γFc, are cyclically shed and re-expressed (Section II; Burns et al, 1977a; Sarmay et al, 1978). Thus, although there is probably always some receptor present on the membrane, its density of expression fluctuates. (b) The methods used for receptor detection vary as described above, and these different methods have varying degrees of sensitivity. (c) The amount of anti-Ia antibody required to sterically hinder Fc-ligand binding is possibly not present in all the antisera used. The antibody concentration is rarely cited, but in any case the antibody

is not affinity purified since it takes 100 kg of cells to prepare a few micrograms of pure antigen (Schlossman, 1976). When blocking of the γFcR by anti-Ia antibody was quantified (Dickler et al, 1975), a higher concentration of $F(ab)_2$ fragments, compared with whole antibody, was required. The failure to block EA_G rosette formation by HCs with anti-p29,34 serum reported here may be due to the same factors, particularly since γFcR expression by HCs is high. However, it has been reported that anti-Ia serum has no effect on the binding of complexed IgG by the γFcR of macrophages, (Dickler, 1976b), and the γFcR of HCs bears many resemblances to the γFcR of macrophages (Chapter 3). It may be that this γFcR is a different structure from that of B cells.

II Kinetic studies on the turnover of the receptor for IgM

1. INTRODUCTION

Shedding is a term employed to describe the release by the cell of plasma membrane antigens (Loor, 1977). In studies of the μFcR on HCs it was observed that the percentage of HCs forming EA_M rosettes fell rapidly after the final wash. Evidence is presented here that the cells were shedding the μFcR.

By following the rate of shedding and subsequent re-expression of receptors, an estimate of the membrane turnover time of the receptor-bearing cells can be obtained. Previous estimates of membrane turnover in haemic cells have employed a variety of methods: these have included measurements of the incorporation into the plasma membrane of radiolabelled aminoacids and the subsequent disappearance of label after the removal and chase of the radioactive precursor (Nachman et al, 1971); of the release of surface immunoglobulin (SIg) labelled either externally by lactoperoxidase-catalysed radio-iodination or following the biosynthetic incorporation of radiolabelled amino acid (Melchers et al, 1975); of the release of radiolabelled antibodies against surface HLA antigens (Menne and Fladd, 1973; Lewis et al, 1976); and of the half-life of SIg in terms of both its release and re-expression after proteolysis (Ferrarini et al, 1976a) or antibody stripping (Loor et al, 1972; Wilson et al, 1972). Measurements of membrane turnover made by most of the above techniques involve antigenic modulation (Bretscher and Raff, 1975) - the artificial removal of membrane protein - and must be treated with caution since such methods may alter the rate of replacement of the protein (Loor, 1977).

Membrane kinetic studies of the chronic leukaemias have been confined to

lymphocytic leukaemia (CLL) (Menne and Fladd, 1973; Lewis et al, 1976; Lewis and Pegrum, 1977) in which the cells have been shown to have a markedly reduced turnover rate. It has been suggested both that this is a manifestation of the leukaemic state and that this change in membrane function may account for the reduced immunological responsiveness of the cells of CLL (Lewis et al, 1976).

In this section, the kinetics of spontaneous μFcR shedding and resynthesis are used to provide a measure of membrane turnover in HCL. In addition, since CLL cells also bear a μFcR (Section III), the same method is used to study the membrane kinetics of CLL cells. The data presented provide new information concerning the relationship between HCs and CLL cells and also demonstrate that reduced membrane turnover rates are not a necessary feature of chronic leukaemia.

2. MATERIALS AND METHODS

2.1 *Patients*

The peripheral blood from seven patients with HCL and four patients with typical CLL (with a high peripheral leucocyte count) was examined. In one of the cases of HCL splenic HCs were also studied. All the HCL cases belong to the series studied in Section I.

2.2 *Mononuclear cell preparation*

Leukaemic cells from PB and spleen were isolated over Ficoll-Isopaque, washed and resuspended in HBSSA as described in Section I. Cells isolated from the HCL spleen comprised >90% HCs, and those from all 7 cases of HCL >50% HCs. All four cases of CLL studied had PB leucocyte counts of >100,00/mm^3, and the isolated mononuclear cells were >90% leukaemic B lymphocytes both morphologically and by light chain restriction

of SIg.

2.3 *Indicator cells*

Ox erythrocytes coated with rabbit IgM antibody were prepared as described in the previous section.

2.4 *Kinetics of rosette formation*

Leucocytes at 2 x 10^6 cells/ml were mixed with an equal volume of EA_M (1%) in HBSSA. The mixture was incubated at 37°C in a water bath and samples were removed for reading at times 0, 10 min, 30 min, 1 h, and at hourly intervals thereafter until the end of each experiment. Mixing was only carried out as each sample was removed. In some experiments incubation was at room temperature as stated in Results. Also, in some tests, leucocytes were incubated for the various time periods in the absence of EA_M, indicator cells only being added at the time of removal of samples.

At the times of sampling, 3 drops were removed from the mixture with a pasteur pipette and the sample was immediately centrifuged at 150 x g for 1 min. The pellet was placed on ice for an hour or more to stabilise formed rosettes before gently resuspending. The percentage of rosetting cells was assessed by using a fluorescein diacetate method (Ramasamy, 1974) in which only viable fluorescing cells were scored under combined U.V. and phase contrast illumination; no loss of viability was noted during the period of each experiment. The morphological nature of the rosetting cells was confirmed by cytocentrifuging the samples and subsequently staining with Giemsa.

2.5 *Concentrated μFcR material*

The supernatants from PB HCs, cultured for 3 h in HBSSA (but in the absence of indicator erythrocytes), was concentrated (x 10) by positive pressure filtration. The resulting concentrate was used as a blocking

reagent in tests designed to demonstrate shedding of μFcR material.

3. RESULTS

3.1 *Shedding of the μFcR*

Reduction in EA_M rosette formation It was observed that the percentage of HCL cells or CLL cells forming EA_M rosettes fell rapidly after the final wash, whether maintained at room temperature or at 37°C. Thus, for example, cells from the HC culture formed 92% EA_M rosettes when tested immediately after washing in HBSSA. After 30 min on the bench, the same washed cell preparation formed only 48% EA_M rosettes. If, however, cultured cells which had remained on the bench in culture medium (RPMI-1640 medium + 10% foetal calf serum (FCS)) were washed in HBSSA and rosette-tested immediately, 92% of cells formed EA_M rosettes. No cells were ever seen to form rosettes with the unsensitized ox erythrocyte control.

Freshly isolated, and washed, HCs also rapidly lost the ability to form EA_M rosettes, whether maintained at 37°C or at room temperature. This is illustrated in Figure 2.12 where the freshly isolated cells were incubated in HBSS in the absence of EA_M indicator particles: the subsequent re-expression of the μFcR will be considered below.

The rate of fall in EA_M rosette formation with time was very similar whether EA_M indicator cells were present or not during incubation of the HCs. This is shown in Figure 2.13 where it is also shown that when the cells were incubated in HBSS containing 10% FCS there is no loss of μFcR material as measured by EA_M rosette-forming ability. Thus it would appear that the μFcR on HCs is shed spontaneously, rather than as the result of antigenic modulation, or stripping, by the EA_M indicator cells. Also,

since there was no loss of viability in the absence of FCS and only viable cells were counted, the presence of serum in the incubation medium probably stabilises this loosely bound receptor.

Capping The manner in which μFcR was shed in the absence of EA_M cells is not clear. Under phase contrast there appeared to be particles of plasma membrane which may have been released with the receptor. In the presence of EA_M indicator particles, cells were seen to be capping and occasional clusters of indicator erythrocytes without a central leucocyte (as identified by fluorescent positivity) could be seen. Capping of EA_M is illustrated in Figure 2.14.

Supernatant blocking Cells from the HC culture were EA_M rosette tested in the presence of concentrated supernatant from cultured PB HCs. In two experiments, 66% and 90% inhibition was obtained. This result demonstrates that the μFcR is actually shed from the cell membrane and not simply obscured or reprocessed by the cell.

3.2 Receptor synthesis and turnover by HCs

μFcR regeneration Freshly isolated, and washed, PB HCs from one patient were mixed with EA_M and incubated at 37°C. Aliquots from the mixture were tested for rosette formation after various time intervals, and shedding of receptor as described above was seen to have occurred. Thus at time 0, EA_M rosette formation was 65% falling to 10% after 1 h, and to 4% after 2 h. After 3 h and 4 h the % rosette formation was 5 and 14 respectively. The mixture of leucocytes and indicator cells was now divided and incubated overnight at both 4°C and at 37°C. After this time, the two samples were tested for rosette formation. The sample maintained at 4°C did not form any EA_M rosettes whereas the sample incubated at 37°C yielded 35% EA_M rosettes.

Figures 2.12 and 2.13 both show that receptor regeneration by HCs

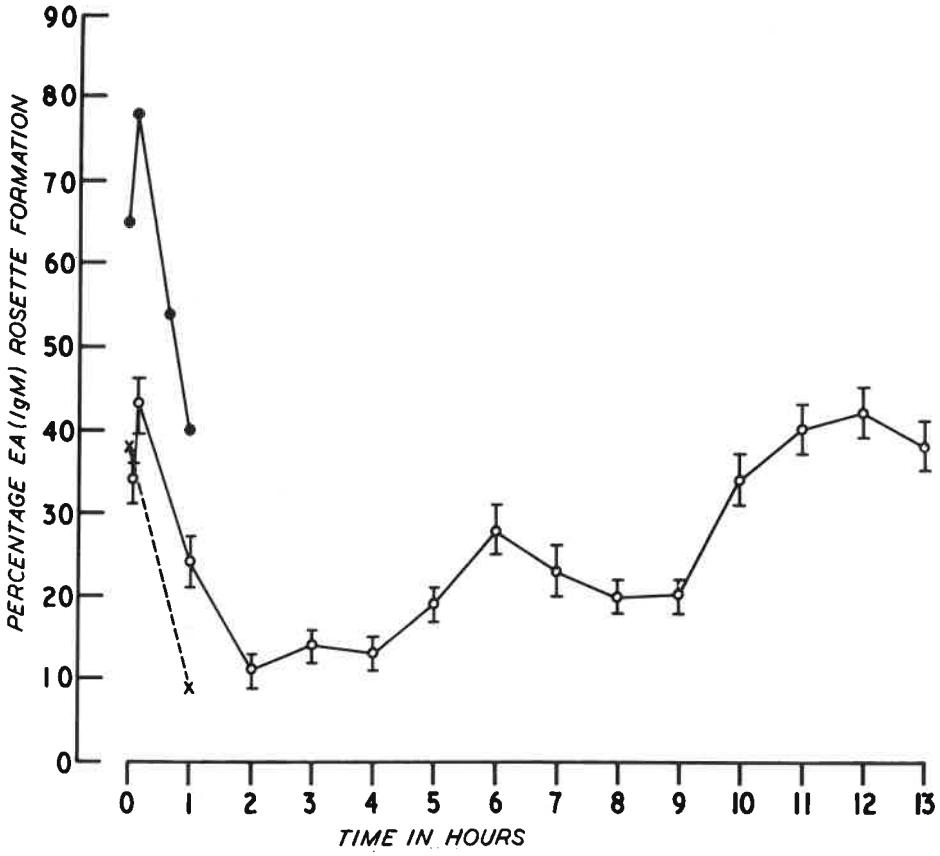

Figure 2.12 Mononuclear cells from three different cases of HCL incubated at 37°C (○,●) and at room temperature (x) without added EA_M. At the time intervals indicated, cells were removed and the % EA_M rosettes obtained are graphed. The rate of fall in the absence of EA_M is comparable to that observed in the presence of EA_M indicator cells (Figure 2.13), and the secondary rise in receptor positivity is also seen to occur. The vertical bars represent 1 SEM.

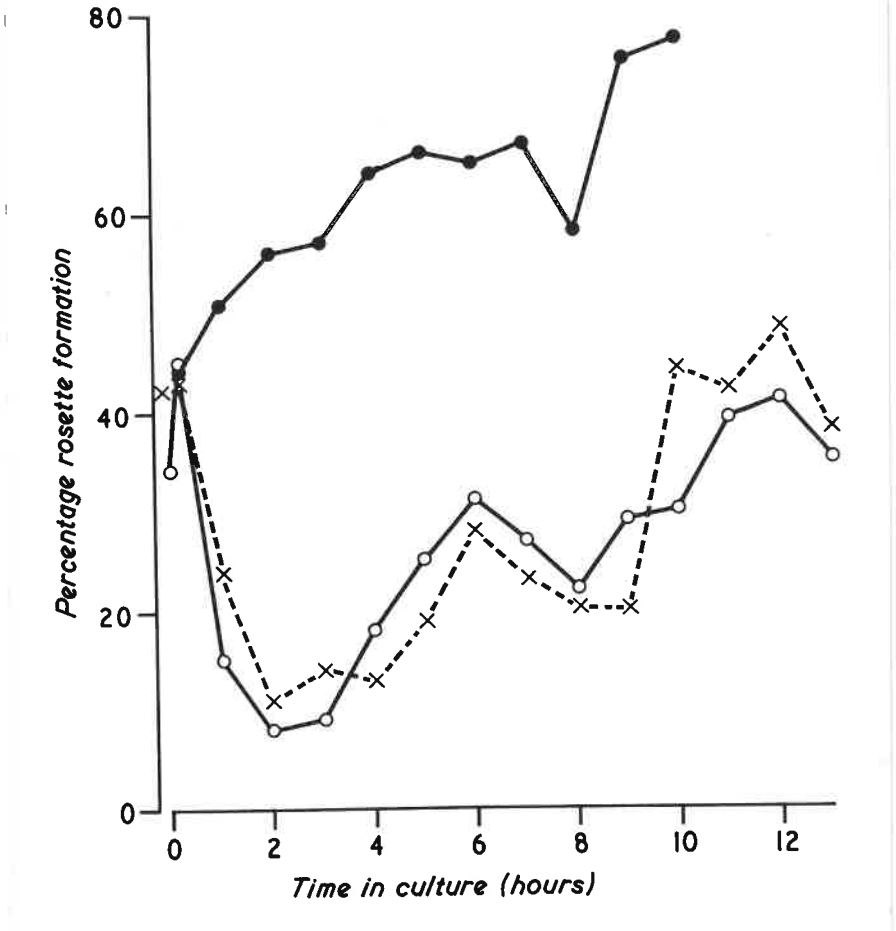

Figure 2.13 Receptor shedding and re-expression by the mononuclear cells from a case of HCL. Cells were cultured in either HBSSA (○,X) or HBSS + 10% FCS (●) in the presence (○;●) or absence (x) of EA_M indicator cells. Aliquots were removed for rosette testing at the times indicated and more than 200 cells were counted in each determination.

also took place in the absence of EA_M indicator cells, and that the shedding and regeneration of receptor is a cyclical process.

Membrane turnover The percentage EA_M rosette formation obtained after incubating HCs for various periods of incubation at 37°C was monitored in a further 6 patients with HCL, and the results are shown in Figure 2.15. The cyclical pattern observed whereby rosettes are formed, shed and reformed is clearly illustrated. In general, few weak rosettes were seen; cells either formed strong rosettes with more than 10 adherent erythrocytes or were completely negative. The rate of fall, both initially and after each regeneration peak, was rapid in all cases, and the percentage EA_M rosette formation by HCs in the re-expression peaks was always lower than the peak intially present.

Four, and possibly five, of the HCL patients illustrated in Figure 2.15, and the case in Figure 2.13, had a peak-to-peak time of around 6 h. One patient's cells (KD; Figure 2.15) had a peak-to-peak expression time of 8 h, and it is of interest to note that HCs from this case appeared morphologically more mature in possessing more heavily condensed peripheral nuclear chromatin than most typical HCs (Figure 2.16).

Since the surface of splenic HCs has been reported to differ from that of PB HCs in the same case (Catovsky, 1977), it was of interest to examine the relative rates of re-expression of μFcR in splenic compared with PB HCs. Figure 2.17, which compares the rate of turnover of splenic HCs and PB cells from the same patient taken at the time of splenectomy, shows that cells from the two sites have a comparable rate of re-expression of the receptor.

In the presence of 10% FCS, the percentage of cells forming EA_M rosettes continued to increase to 77% after 10 h of culture (Figure 2.13). The receptor profile of this patient's cells (SIg (IgDλ) 67%; E 34%; EA_G 74%)

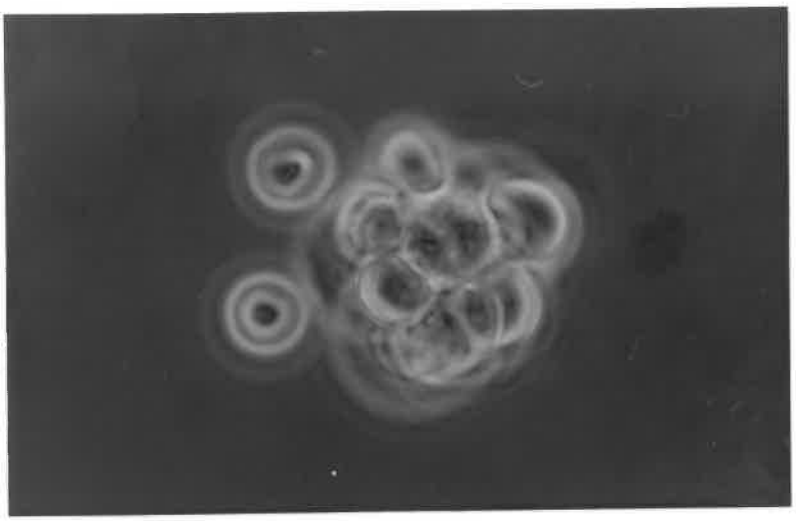

Figure 2.14 A

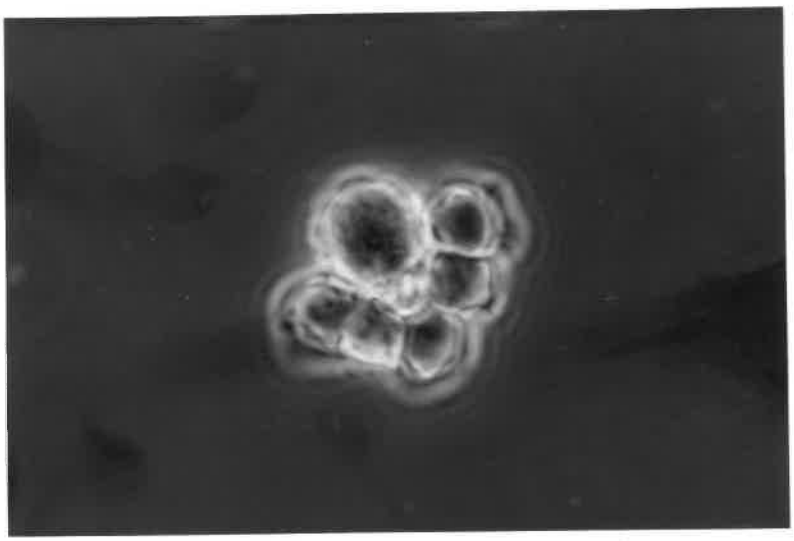

Figure 2.14 B

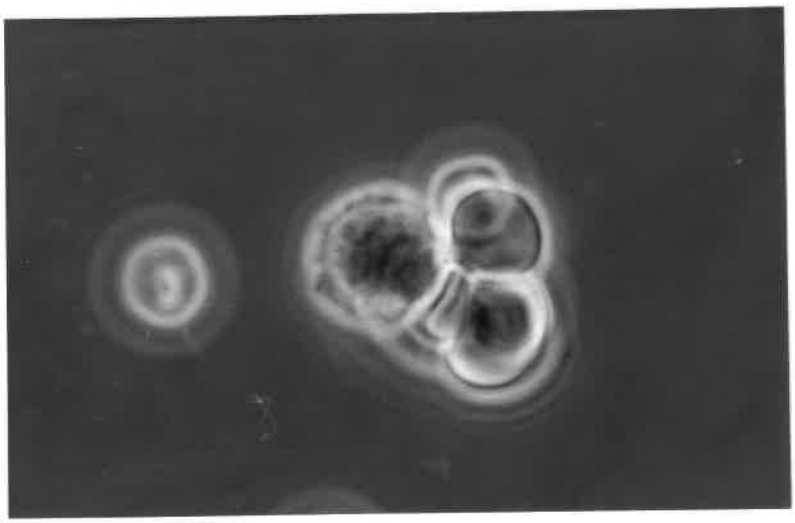

Figure 2.14 C

Figure 2.14 Phase contrast micrographs of EA_M rosette-formation by HCs. A, The morula-like pattern of rosetting indicator cells is shown. B, The indicator cells are clearly beginning to cap. C, After a further period of incubation at 37°C the indicator cells are completely capped at one pole of the hairy cell.

suggested that only approximately 67% of the cells belonged to the monoclonal HC proliferation, while the remaining 34% of cells were T cells. Thus, after some 5 h culture in 10% FCS (a time corresponding to the first re-expression peak of HC μFcR), a percentage of the EA_M rosette-forming cells were probably μFcR-bearing T cells. Also, since the sum of percent E^+ and EA_G^+ cells exceeded 100%, and since there is a virtual absence of both normal monocytes and normal B lymphocytes in HCL (Chapter 3), the overlap probably represents a small population of γFcR-bearing T cells. This is a general feature of HCL, where the proportion of γFcR-bearing T cells is often high. A consideration of the T-cell populations in HCL will be given in Chapter 3.

Requirement for protein synthesis It has been stated above that HCs which were maintained at 4^oC overnight after shedding their μFcR did not regenerate receptors. This suggested that receptor re-expression was an energy-dependent process. In two experiments, the kinetic test in the presence of indicator cells was carried out with and without added puromycin (final concentration 2.5 μg/ml) as an inhibitor of protein synthesis. As is illustrated in Figure 2.18, the puromycin had no effect on the initial rosette formation, but rapidly inhibited re-expression of the receptor even when added shortly before the re-expression peak. These experiments show that re-expression of the μFcR requires continuous protein synthesis and does not simply represent passive uptake of previously shed receptor.

Cytochalasin B, which only partially inhibits EA_M rosette formation at a concentration of 20 μg/ml (Section I), prevented receptor re-expression, almost completely, at a concentration of 10 μg/ml. Hence intact microfilament function is probably also required for μFcR regeneration.

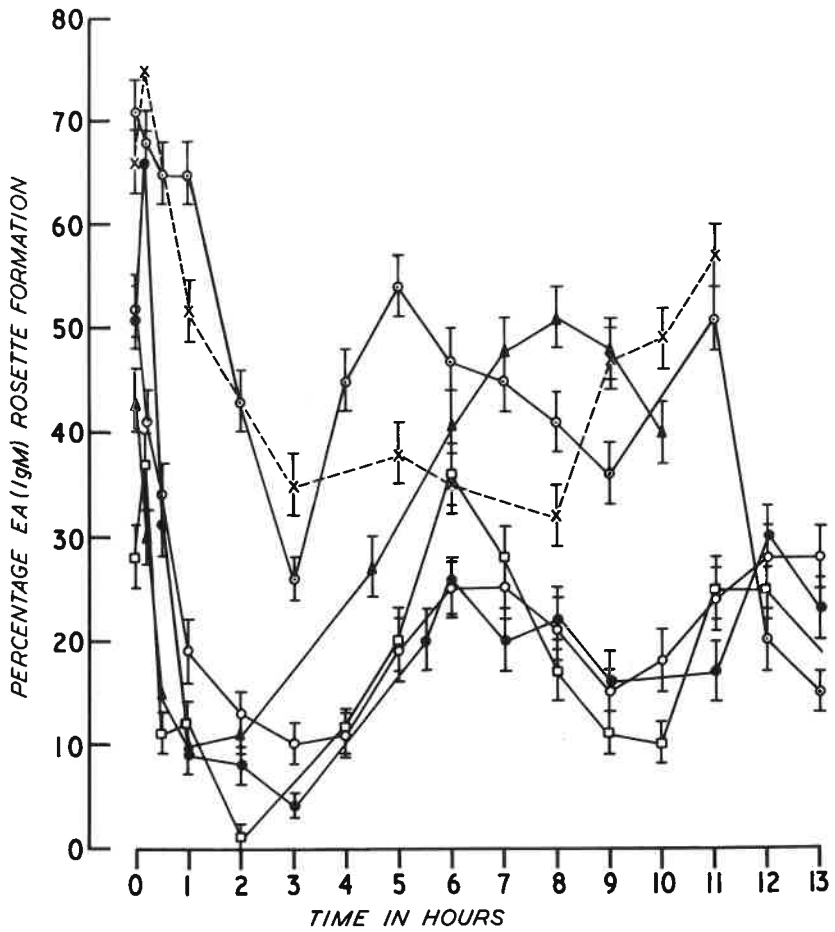
PERCENTAGE EA (IgM) ROSETTE FORMATION
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13
TIME IN HOURS

Figure 2.15

Figure 2.15 Kinetics of EA_M rosette formation by HCs. The percentage of total mononuclear cells forming EA_M rosettes obtained from counts of >200 cells after different periods of continuous incubation at 37°C, in the presence of EA_M, is shown. Cytological examination of cytocentrifuge preparations at each time of sampling showed that only HCs were forming rosettes. The symbols represent the data obtained on a single occasion from each of the 6 patients with HCl tested, mean ± SEM. The experiment was repeated on 4 patients with exactly comparable results for each individual. (▲, patient KD).

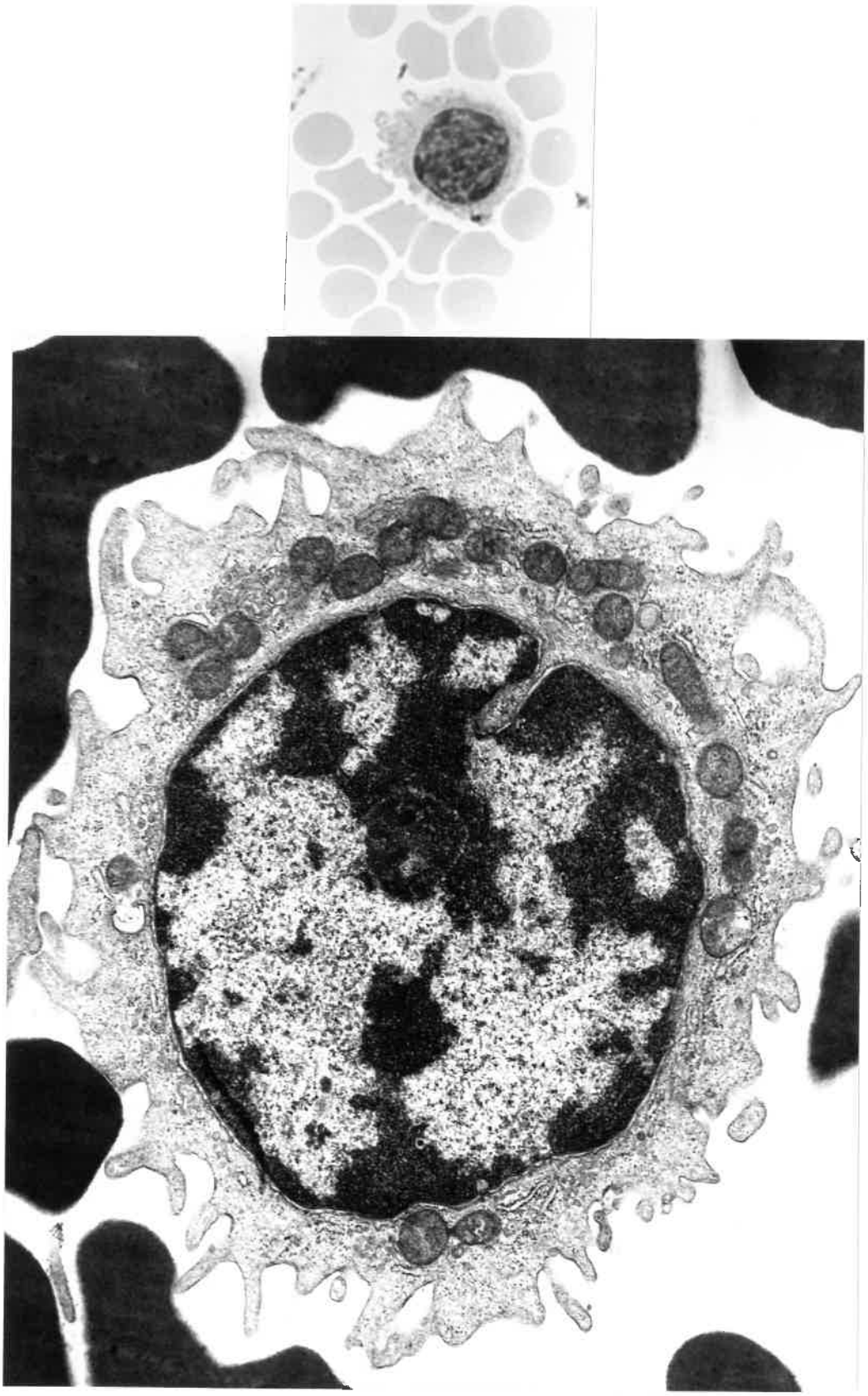

Figure 2.16

Figure 2.16 Cells from patient KD. Cytocentrifuge preparation stained with Giemsa, and EM (x 10,000) of EA_M rosette formation. The nucleus is more central and spherical than in most HCs from typical cases, and the rather heavy peripheral chromatin condensation is well illustrated.

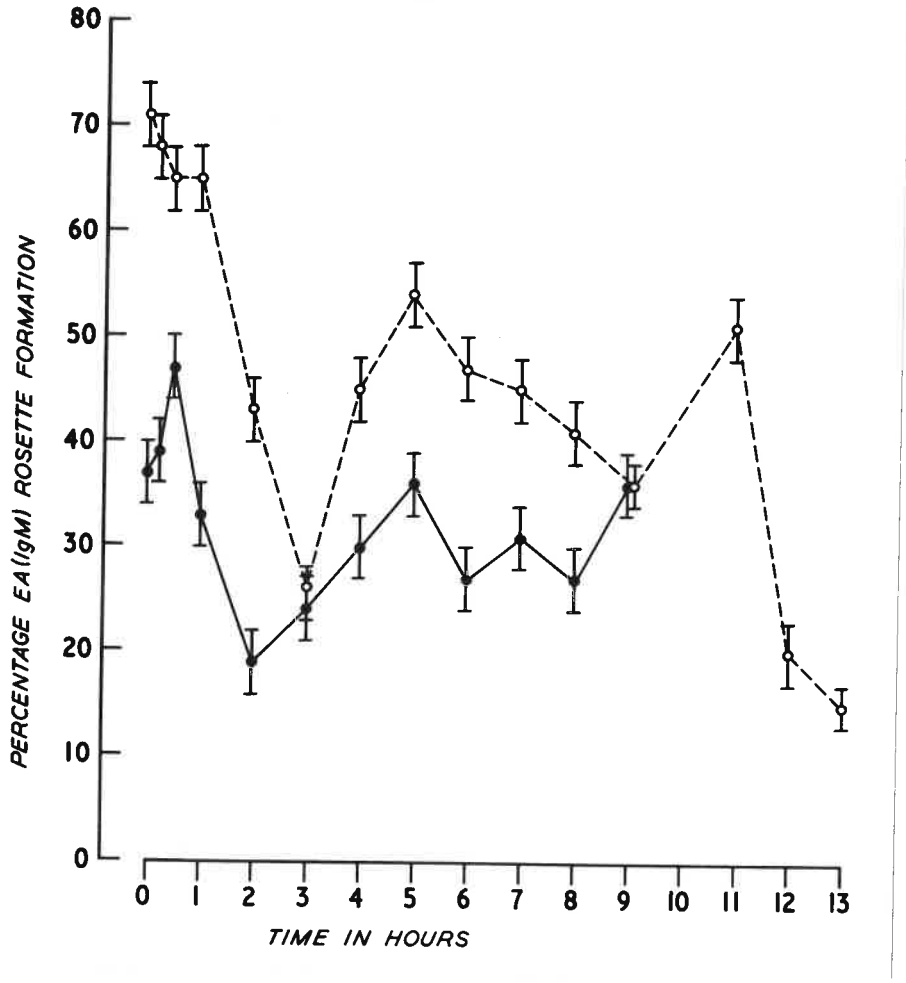

Figure 2.17 Comparison of the kinetics of EA_M rosette formation by HCs from the spleen (○) and PB (●) from the same case of HCL taken at the time of splenectomy. HCs from both sources show similar rates of shedding and re-expression. Vertical bars represent 1 SEM.

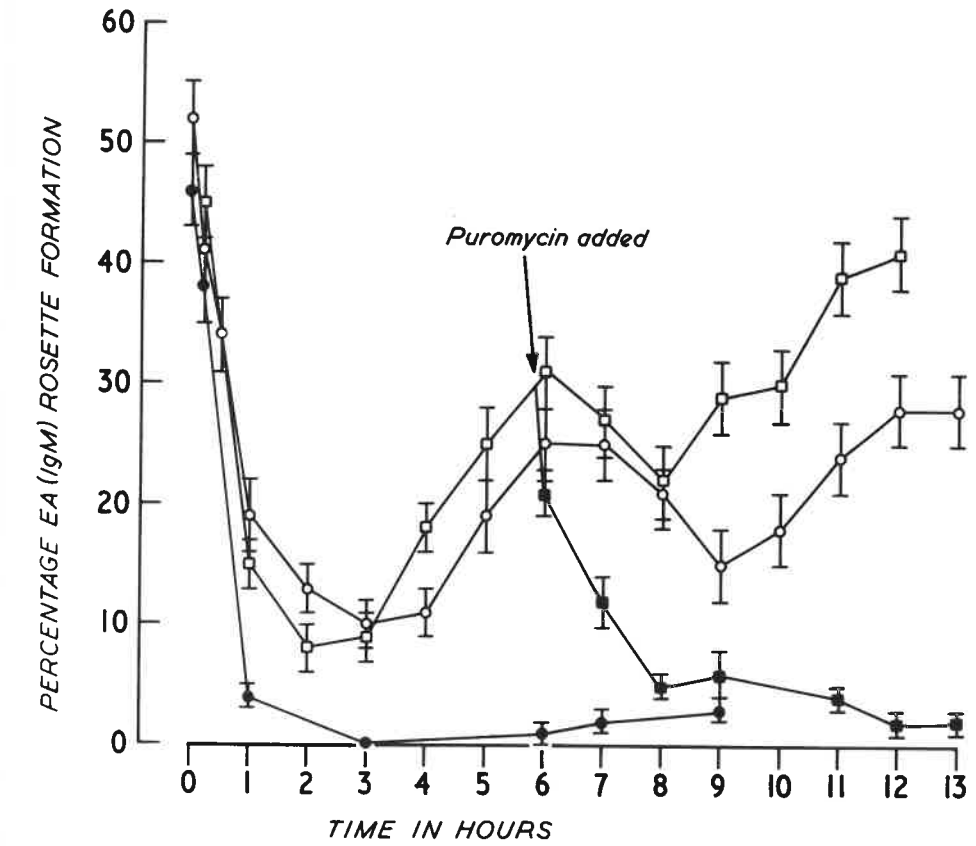

Figure 2.18 Kinetics of EA_M rosette formation in the presence (●;■) and absence (○;□) of puromycin (2.5 µg/ml). The failure of HCs to re-express µFcR in the presence of puromycin is clearly shown. In one experiment (○;●) puromycin was added at time zero, and in the other experiment (□;■) puromycin was added at the time illustrated. Error bars = 1 SEM.

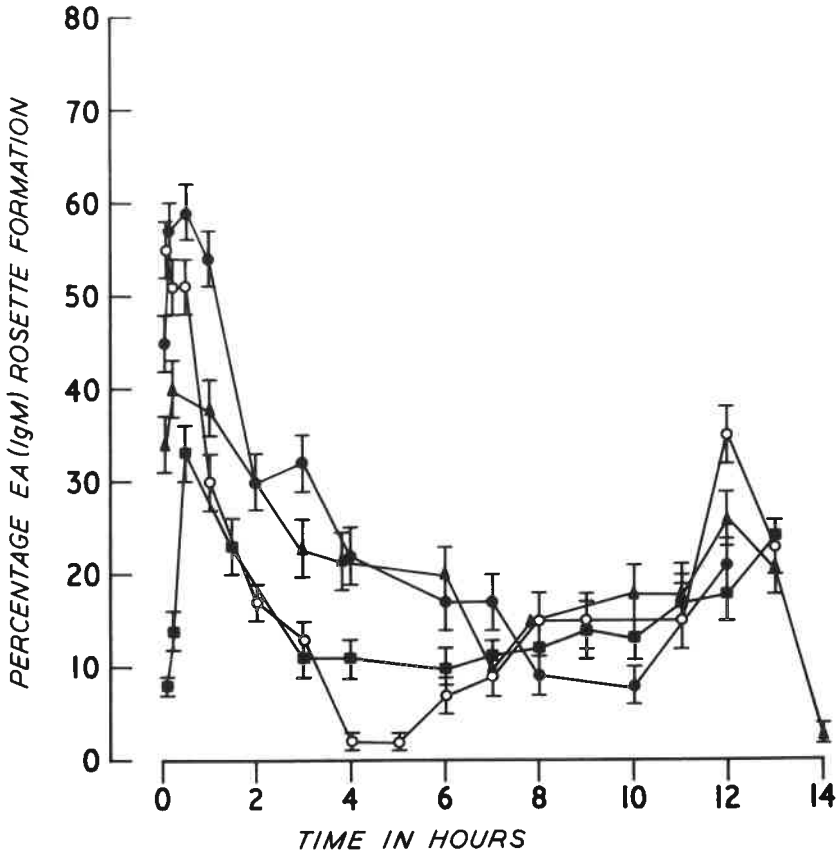

Figure 2.19 Kinetics of EA_M rosette formation by CLL lymphocytes. Vertical bars represent 1 SEM.

3.3 *Membrane turnover in CLL*

The same kinetic experiment carried out on four cases of CLL is illustrated in Figure 2.19. In these cases the re-expression times were 12, 12, >12, and >13 h.

4. DISCUSSION

In this section the shedding and the kinetics of re-expression of the receptor for IgM on the HCs of HCL and the lymphocytes of CLL are described.

Receptor shedding and re-expression is not a feature peculiar either to the μFcR or to pathological cells. Very recently Sarmay et al, (1978) described a similar process for the γFcR, complement receptors, and sheep erythrocyte receptors in both normal human and CLL cells. Early experiments failed to demonstrate γFcR shedding by HCs (Burns et al, 1977b), but by reducing to very low levels the amount of sensitising rabbit IgG used to form EA_G it was possible to do so (Chapter 3). Sarmay et al used anti-Rhesus antibody on human erythrocytes as their indicator system for γFcR, and this relatively insensitive system probably requires high receptor density for rosette formation to occur. When any of these receptors are shed, some receptor probably remains on the membrane; however, the density of residual receptor is much reduced and requires more sensitive techniques for its detection. Indirect evidence of γFcR shedding has also come from the isolation of γFcR material from cell culture supernatants (Molenaar et al, 1977), and these authors were probably also detecting shed μFcR material since their isolated receptor bound IgM and since it has recently been demonstrated that human lymphoblastoid cell lines possess μFcR (Pichler et al, 1978).

The nature of shedding remains uncertain (reviewed by Loor, 1977). It is generally stated that shedding depends on capping, and, like the μFcR shedding described in this section, shedding of the complement receptor on lymphocytes is accompanied by capping (Gormus and Shands, 1975). Cap formation is an energy-requiring process (Taylor et al,1971; Loor et al, 1972), whereas the process of shedding is not inhibited by either azide (Sarmay et al, 1978) or by puromycin (Figure 2.18). Recently, Yefenof et al, (1978) have presented evidence that there is no direct quantitative relationship between capping and shedding. In this study, it was also observed that in the EA_M rosette preparation immediately preceeding the μFcR regeneration peak, many of the cells had attached clusters of indicator erythrocytes at one, or sometimes two, poles. Further, in this same preparation, many of the indicator EA_M cells appeared to be 'sticky' since frequent pairs or even triplets of erythrocytes were seen. Thus it would appear that μFcR regeneration begins at one pole and the receptors then spread over the surface of the cell. This is similar to SIg re-expression where re-expression also starts at one pole (Loor et al, 1972). The indicator cell 'stickiness' observed can possibly be attributed to secretion of receptor material during the re-expression process: but there is no clear operational difference between shedding and secretion (Loor, 1977), hence the observed phenomena are probably a continuum of shedding, secretion, and integration of new receptor material into the cell membrane.

The kinetics of EA_M rosette formation and re-expression by HCs follow a cyclical pattern with a modal peak-to-peak re-expression time of 6 h. The turnover of the receptor was followed through three cycles and it is of interest to note that each re-expression peak was lower than

that preceeding. It is difficult to offer any definitive explanation for this observation, but it may reflect a consumption of available mRNA, since continued protein synthesis was shown to be required for re-expression of the receptor. However, since the HCs and indicator EA_M cells were incubated together as a mixture in most tests, it is also possible that the indicator cells became progressively less sensitive due to binding free μFcR released by the HCs. Some support is lent to this interpretation by the higher re-expression peaks observed when the HCs were incubated without indicator erythrocytes and then rosette tested at the various time intervals.

The requirement for protein synthesis together with the other general features of the receptor such as its inhibition by azide, its temperature dependence, and its inhibition by cytochalasin B make it likely that the receptor is an integral membrane component. Therefore the receptor kinetics probably reflect plasma membrane turnover. Another possibility is that this expression of membrane receptor is dependent on the cell cycle. However, this proposition is less attractive since no mitotic figures were seen through three cycles of re-expression, and cell cycle synchrony of the major population of cells throughout this time seems unlikely.

It is of interest that in six of the seven HCL patients the peak-to-peak re-expression time was 5-8 h, a time directly comparable with the replacement of other membrane components in normal human (Ferrarini et al, 1976a; Lewis and Pegrum, 1977) and mouse (Wilson et al, 1972; Vitetta and Uhr, 1972; Loor et al, 1972) B lymphocytes reported by others. This turnover time is considerably shorter than that reported for membrane protein turnover in rabbit alveolar macrophages

(Nachman et al, 1971), and it is tempting to speculate that this substantiates other recent immunological evidence favouring a B lymphocyte, rather than monocyte, lineage for the HCs of HCL (Catovsky, 1977). However, it is possible that the difference in membrane turnover times is attributable to a species effect rather than to a true difference between lymphocytes and monocytes, or indeed to the population of lymphocytes under study since Melchers et al, (1975) have shown that memory B cells have a slow membrane turnover time.

Since the cells of some cases of CLL also have a receptor for bound IgM it was possible to study the re-expression of this receptor using the same method. Whilst confirming previous reports of a reduced rate of membrane turnover in CLL cells (Minty and Sachs, 1975; Lewis et al, 1977), this was also quantitated to around 12 h in four cases. One case of HCL with a membrane turnover time of 8 h appeared cytologically more mature than the 6 h cases and the range of turnover times reported here therefore strengthens suggestions that HCL encompasses a range of clinical and cytological features (Burns et al, 1973; Burns et al, 1977a) and suggests that cells from the more mature end of the spectrum closely resemble CLL cells. On the other hand, the pattern of turnover seen in the majority of cases of HCL indicates a clear difference between HCL and CLL and also shows that substantially reduced rates of membrane turnover are not an essential feature of chronic leukaemias.

III Distribution of the μFcR on haemic cells and the relationship of the μFcR on HCs to that on other cell types

1. INTRODUCTION

Receptors for IgM have been described on rabbit and guinea-pig macrophages and neutrophils (Henson, 1969), mouse peritoneal macrophages (Lay and Nussenzweig, 1969) and guinea-pig splenic macrophages (Rhodes, 1973).

It is only recently that μFcR have been described on human haemic cells, and such receptors on human lymphocytes are currently the subject of considerable interest. A receptor for IgM was first described on normal peripheral T cells after a period of *in vitro* incubation in IgM-free medium containing FCS (Moretta et al, 1975; McConnell and Hurd, 1976). Later, an IgM receptor which did not require a period of preincubation for its detection was described in some patients with Hodgkin's disease (Habeshaw et al, 1976), on a small but uncharacterized population of normal lymphocytes (Barker et al, 1976), and on the pathological cells of both HCL (Burns et al, 1977a) and CLL (Pichler and Knapp, 1977; Cawley and Burns, 1977; Burns et al, 1977b). Recently, the presence of a μFcR on the cells of HCL and CLL has been confirmed (Ferrarini et al, 1977), and the same study has revealed that the receptor is also present on a population of normal human B lymphocytes. Very recently, independent confirmation of a receptor for IgM on human B cells has come from two other groups (Pichler and Knapp, 1978; Romagnani et al, 1978).

The recognition of a μFcR on some T cells, and the subdivision of normal T cells into T_M and T_G populations, depending on whether they have

a receptor for IgM or igG (Moretta et al, 1976), has found application in the study of lymphoproliferative disorders. Thus some cases of T-cell acute lymphoblastic leukaemia have been shown to be derived from the T_M population (Moretta et al, 1977a), as have two cases of Sezary's syndrome (Worman et al, 1978). It has been shown that normal T_M cells possess a helper function, while the mutually exclusive population of T_G cells fulfills a suppressor function in the immune response (Moretta et al, 1977b), and since cells from the Sezary syndrome can also act as helper T cells (Broder et al, 1976) this function of T_M cells probably remains true for pathological T-cell proliferations.

The μFcR on B cells may also prove to have nosological and functional significance, and it is therefore of importance to establish the distribution of this receptor in populations of human haemic cells. The present section investigates a wide variety of lymphoproliferative disorders for the presence of IgM receptors and also considers differences in receptor expression between T cells and B cells.

2. MATERIALS AND METHODS

2.1 *Patients*

Clinical material from patients with a wide variety of haematological diseases was obtained, and details of the tissues studied are presented in the Results. The patients were diagnosed on clinical and haematological criteria, but where immunological marker studies assisted in the diagnosis these data are included in the relevant tables. In addition, PB samples obtained from 18 normal volunteers and 2 'normal' spleens were studied. The spleens, collected immediately after surgical removal, were from (a) a patient with hereditary spherocytosis and (b) a patient undergoing

radical surgery for stomach cancer.

2.2 *Cultured haemic cells*

Cells were obtained from established cell lines as a kind gift from Dr. A. Karpas, Department of Haematological Medicine, University of Cambridge. Two of the lines studied were EBNA-positive B-cell lines (Lines 117 and 284) and details of these cells have been given by Karpas et al, (1977). The ENBA-positive B-cell line, Raji, was obtained as a kind gift from Dr. I. McConnell, MRC Group on Mechanisms in Tumour Immunity, Cambridge. Cells from a monoblastoid-cell line (230) (Karpas et al, 1978a), and cells from a cell line established from a patient with myelomonocytic leukaemia which had possessed T-cell features (241) (Karpas et al, 1978b) but which was now either transforming to, or being replaced by, cells with B-cell characteristics were also studied.

2.3 *Detection of the* μFcR

The indicator cells and the methods used for detection of the μFcR was exactly as described in Section I of this chapter. It should be emphasized that, unless otherwise stated, the receptor was detected on cells washed and resuspended in HBSSA without serum and that, apart from the routine 10 min incubation, no period of culture was involved.

3. RESULTS

3.1 *Normal mononuclear cells*

Peripheral blood At different times, the PB from 18 normal individuals was collected and the mononuclear cells isolated over Ficoll-Isopaque were rapidly tested for EA_M rosette formation. At a time when the unsensitized ox erythrocyte control was negative, either 0% (4), 1% (3), 2% (4), 3% (1), 5% (4) or 6% (1) formed EA_M rosettes.

Spleen The results obtained from 2 'normal' spleens are shown in Table 2.X.

Nature of the cell forming EA_M rosettes Stained cytocentrifuge preparations revealed that the cells from normal PB which formed EA_M rosettes were nearly all large esterase-negative lymphocytes.

The very small percentages of cells forming rosettes in normal PB made further characterization difficult, however preliminary experiments were carried out in attempts to identify further these cells.

PB mononuclear cells were allowed to adhere to a glass petri dish in the presence of 10% serum. After incubation for 15 min at 37°C, the dish was inverted and non-adherent cells collected. Weakly adherent cells were then washed from the dish with phosphate-buffered saline and glass-adherent cells were removed by treating them with 10 mM EDTA. The cells from each group were then washed and tested for EA_M rosette formation. No rosettes were observed in the glass-adherent group, 1% was seen in the weakly adherent group, and 2% in the non-adherent cell group.

In another experiment, T cells were removed from the PB mononuclear cells of 2 volunteers by E rosetting the cells and centrifuging the rosetted population through a Ficoll-Isopaque mixture. Non-T cells were collected from the interface and tested for EA_M rosette formation. In one sample 6% of cells formed rosettes, but in the other no rosette-forming cells were seen.

3.2 Acute lymphoblastic leukaemia (ALL)

The surface marker characteristics of PB mononuclear cells from 5 cases of ALL are given in Table 2.XI. It is clear that very few, if any, ALL cells form EA_M rosettes.

In one case (patient 5), mononuclear cells were incubated at 37°C

Table 2.X Immunological marker studies on the mononuclear cells from two 'normal' spleens

Spleen	γFc %	μFc %	E %
a) hereditary spherocytosis	35	4	45
b) stomach cancer	34	5	51

overnight in culture medium (RPMI) containing 10% FCS. After this time, 3% of cells formed EA_M rosettes.

3.3 B-cell lymphomas and leukaemias

Non-Hodgkin's lymphoma The mononuclear cells from the PB of 4 patients and from lymph nodes of 2 different patients with non-Hodgkin's lymphoma were examined for EA_M rosette formation. No patient had >3% rosette formation by either PB or node mononuclear cells.

Hodgkin's disease (HD) Mononuclear cells from the PB of 3 patients with HD were examined. One of the patients was in the terminal stage IV phase of the disease, and another had the rare leukaemic form of HD with some 39% of the pathognomonic Reed-Sternberg cells in the PB. In none of these patients were any EA_M rosette-forming cells seen.

The spleen of another patient with HD was also obtained for study; only 4% of splenic mononuclear cells formed EA_M rosettes.

In one case of HD, the PB mononuclear cells were cultured overnight in medium (TC 199) containing 10% FCS. After this time, the cells were washed and marker studies were repeated. The results are shown in Table 2.XII. It can be seen that the T cells in this case of HD required culture before forming E rosettes and that most T cells belong to the T_G population with only some 20% cells forming EA_M rosettes after culture (T_M cells).

Prolymphocytic leukaemia (PL) The PB from one patient with PL was obtained for study. The marker profile of mononuclear cells from this patient is given in Table 2.XIII. It can be seen that very few, if any, PL cells possess the μFcR.

Chronic lymphocytic leukaemia (CLL) Mononuclear cells were obtained from the PB of 45 patients with CLL and tested for EA_M rosette formation. In 15 cases, repeat blood samples were obtained on one or more occasions.

Table 2.XI Immunological markers in 5 cases of ALL

Patient	% rosette formation		
	γFc	μFc	E
1	31	7	7
2	5	0	9
3	21	1	9
4	5	0	2
5	7	0	0

Table 2.XII Immunological markers in a case of HD

Material	% rosette formation		
	γFc	μFc	E
Freshly isolated mononuclear cells	76	0	36
Cultured cells*	63	20	66

* Cultured overnight in TC 199 + 10% FCS

Table 2.XIII Immunological markers of mononuclear cells from the PB of prolymphocytic leukaemia

% rosette formation				
γFc	μFc	EAC^{+}	Mo^{++}	E
58	4	15	1	1

$^{+}$ Receptor for fixed C3 (see Chapter 3)

$^{++}$ Receptor for mouse erythrocytes (see Chapter 3)

The results obtained for EA_M rosette formation in the 45 patients are presented in Table 2.XIV. From the table, it is clear that the majority of cases of CLL possess the μFcR. Also, with some exceptions, the receptor is consistently expressed on about the same proportion of cells when repeat testing is carried out on a single case.

Full surface marker phenotyping was carried out on 25 of the cases of CLL. Comparison of the surface receptors with μFcR expression in these cases did not reveal any definite pattern. However, of the 25 cases only 5 failed to express SIg (Chapter 4), and 4 of these cases also had very low μFcR expression (Table 2.XIV). On the other hand, the fifth SIg-negative case had a high percentage of cells expressing the μFcR, and at least 2 cases with low μFcR expression had a high percentage of SIg positive cells (Table 2.XIV). None of the 5 SIg negative cases had >5% T cells as assessed by E-rosette formation, and indeed none of the 45 cases of CLL were of the T-cell type by this criterion. All of the CLL cases tested invariably possessed a high percentage of cells which also bore a receptor for the Fc of IgG.

It was observed that the percentage EA_M rosette formation obtained in some cases of CLL was much higher under standard conditions (37^oC, 10 min) than when the test was performed at room temperature. This was confirmed in all but 4 of 21 cases of CLL tested, and the results shown in Table 2.XV illustrate that the difference in percentage rosette formation at the two temperatures, is much more pronounced than that in PB HCs (Table 2.VI, Section I) and is similar to that in splenic HCs (Table 2.VII, Section I). Incubation at 37^oC for longer than 10-15 min caused CLL lymphocytes, like HCs, to shed the μFcR (Figure 2.19, Section II).

Table 2.XIV EA_M rosette formation in CLL

Patient	% EA_M rosettes
1	61, 59, 62, 48
2	51, 77
3	55
4	16, 33, 3, 34, 14
5	40, 45, 27
6	11
7	43, 51
8	36
9	24
10	30, 31
11	10
12	79
13*	<1, 5, 3
14*	5, 2, <1
15	36
16	14
17	38, 74
18	45, 80, 26
19	3
20	72
21	26, 16
22	25
23	48

Table 2.XIV continued

Patient	% EA_M rosettes
24	53
25	20
26$^{\nabla}$	89, 78
27	79
28	54, 17
29^{+}	11
30*	8
31^{+}	0
32	25
33	53, 17
34*	8
35	54
36	20
37	0, 65
38	31
39	49
40	87
41	97
42	72
43	95
44	50
45	15

* Negative for surface immunoglobulin (SIg)

$^{\nabla}$ Negative for SIg

$^{+}$ The majority of cells possess monoclonal SIg

3.4 *Terminal B cells*

The pathological plasma cells from a total of 25 patients with a variety of immunoproliferative diseases was studied. These patients included: 8 with IgG myeloma (2 with plasma cell leukaemia); 6 with IgA myeloma; 1 with Bence-Jones myeloma and one with primary amyloidosis; 2 with IgD myeloma; 2 with IgM myeloma; and one with IgE myeloma. In addition, 4 patients with mixed lymphoid/plasmacytoid proliferations were studied: one of these presented the typical clinical and pathological features of Waldenström's macroglobulinaemia, while 2 others presented a similar picture, but with IgA paraproteinaemia; the final patient presented with substantial hepatosplenomegaly and widespread lymphadenopathy, and a marrow infiltration with 15% and 6% of lymphoid and plasmacytoid cells respectively (lymphoma with IgM paraproteinaemia).

PB and bone marrow was examined in all the cases, and in none did EA_M rosette formation exceed 6%. No plasma cells were ever seen to form EA_M rosettes.

3.5 *Monocytes and histiocytes*

Acute monocytic leukaemia (AMoL) The results obtained in 6 cases of AMoL are presented in Table 2.XVI. These results clearly show that the majority of leukaemic monocytes do not form EA_M rosettes and the high percentage of cells with (strong) γFcR incidentally confirms the purity of the EA_M reagent.

Chronic myelomonocytic leukaemia The PB from 3 patients, and a lymph node and bone marrow aspirate from one of these patients was examined for cells forming EA_M rosettes. In only one patient were the PB cells found to form any rosettes (6%), and these cells had a lymphoid morphology.

Table 2.XV The effect of temperature on EA_M rosette formation by CLL lymphocytes from PB

Patient	% rosette formation at: 37°C	24°C*
1	59	45
	48	5
	61	39
2	51	40
3	51	55
4	33	8
	16	1
5	40	34
6	11	3
7	52	26
8	36	20
9	24	21
10	30	18
11	10	21
12	79	22
13	5	0
14	5	2
15	36	12
16	14	20
17	38	27
18	45	19
19	3	0
20	72	15
21	26	32

* approximate room temperature

Table 2.XVI Immunological marker data on isolated mononuclear cells from the PB of 6 cases of AMoL

Patient	% monocytoid cells in cytocentrifuge preparations	% cells rosetting γFc	μFc	E
1	96	99	0	4
2	70	33	0	-†
3	90	85	11	3
4	40	36	0	2
5	70	66	0	<1
6	50	87	0	14

† not done

Histiocytic diseases Mature histiocytes; from the spleen of a case of Gaucher's disease, from the bone marrow of a case of sea-blue histiocytosis, and from the PB of a case of sinus histiocytosis were examined for EA_M rosette formation. The results given in Table 2.XVII show that histiocytes do not possess μFcR.

3.6 Granulocytes

Acute promyelocytic leukaemia (APL) Peripheral blood cells from one case of APL and bone marrow cells from another case were examined for EA_M rosette formation. The immunological markers obtained in these 2 cases of APL are given in Table 2.XVIII, and it is clear that the promyelocytes, or hypergranular myeloblasts, of this disease do not form EA_M rosettes.

Chronic granulocytic leukaemia (CGL) The PB cells from 4 cases of CGL, and BM cells from 2 of these cases, were examined for EA_M rosette formation. In no case was more than 1% of rosette-forming cells seen.

3.7 Pathological T cells

Two patients with Sezary's syndrome were examined. Mononuclear cell preparations from the PB of both patients contained over 90% of the typical pathological T cells. By the standard EA_M technique, freshly isolated mononuclear cells from the 2 patients formed 4% and 9% EA_M rosettes respectively.

Surface marker studies of cells from these 2 patients showed that whereas over 90% of cells formed E rosettes, cells from both patients lacked receptors for γFc and fixed C3 (Table 2.XIX). The patients' cells were then cultured overnight in either RPMI medium alone, or the same medium supplemented with 10% FCS or 10% autologous serum. After this time, the marker studies were repeated; these results are given in Table 2.XIX. Throughout the period of culture the cells retained

Table 2.XVII Immunological markers in histiocytic diseases

		% rosette formation			
Disease	tissue	γFc	μFc	EAC^{+}	E
Gaucher's	spleen	71	0	68	5
Sea-blue histiocytosis	bone marrow	80	0	50	24
Sinus histiocytosis	PB	84	0	4	0

$^{+}$ Receptor for fixed C3 (Chapter 3)

Table 2.XVIII Immunological surface markers in APL

Case	tissue	% rosette formation			
		γFc	μFc	EAC+	E
1	PB	70	0	2	25
2	BM	17	0	0	28

+ Receptor for fixed C3 (Chapter 3)

100% viability, as assessed by trypan blue dye exclusion. The percentage of cells forming E rosettes was consistently high and did not fall with culture in the one patient tested. Following culture in medium containing 10% serum, but not in medium alone, a large proportion of the Sezary cells in both cases were seen to form EA_M rosettes, but not EA_G rosettes. This observation of μFcR without γFcR incidentally confirms that contaminating IgG in the EA_M reagent is not the cause of EA_M rosette formation.

The time course of μFcR expression by Sezary cells was followed by removing aliquots from the cultured cells at various time intervals and testing for E and EA_M rosette formation. As is shown in Figure 2.20, there was a progressive rise in the percentage cells forming EA_M rosettes until about 6 h when a plateau was reached. At no time did more than 10% cells form EA_M rosettes in the absence of serum, and autologous serum was as effective as FCS in enhancing rosette formation. Cytocentrifuge preparations from the rosette populations confirmed that it was the pathological Sezary cells which were forming rosettes (Figure 2.21).

The effect of temperature on μFcR expression by Sezary cells was tested by maintaining leucocytes on ice in parallel with the time-course experiment described above. In no instance was more than 11% cells observed to form EA_M rosettes when removed from the ice and mixed with indicator erythrocytes for 10 min prior to scoring.

3.8 *Cultured cells*

The three B-cell lines examined for μFcR (Raji, Line 117 and Line 284) were all weakly positive. The percentage EA_M rosette formation was: Raji, 4%; 117, 6%; 284, 3%. The EBNA-negative monoblastoid-cell line was completely negative for EA_M and for all other surface markers

Table 2.XIX Surface marker profile of the mononuclear cells from two patients with Sezary's syndrome on initial isolation and following culture

Patient	% rosette formation												
	E				γFc				μFc				EAC
	Fresh	Cultured*			Fresh	Cultured			Fresh	Cultured			Fresh
		1	2	3		1	2	3		1	2	3	
1+	96	nt§	96	nt	2	2	2	2	4	9	66	66	<1
2++	93		nt		3		nt		9	6	43	nt	3

* Culture in 1. RPMI alone 2. RPMI + 10% FCS 3. RPMI + 10% autologous serum

\+ Culture for 12 h : mean of 2 occasions studied.

++ Culture for 9 h at 37°C.

§ Not tested

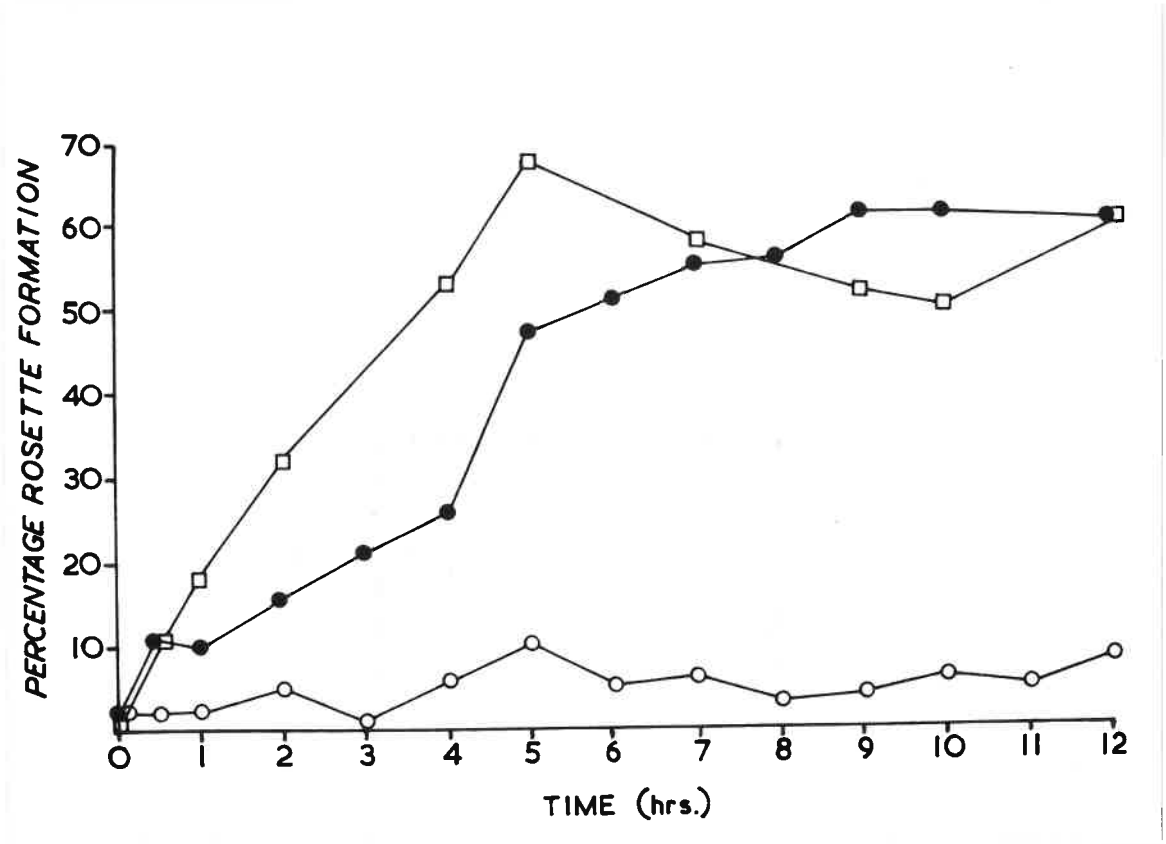

Figure 2.20 The effect of time and serum on the percentage EA_{Mo} rosette formation by Sezary cells from patient 1 cultured at 37°C : RPMI alone (○); RPMI supplemented with 10% FCS (●); RPMI supplemented with 10% autologous serum (□).

examined.

Cells from line 241 did not form EA_M rosettes on the first occasion of study (Table 2.XX). At this time, the majority of the cells formed E rosettes and were possibly T cells although there was a population of cells, possibly B cells, which formed spontaneous rosettes with mouse erythrocytes. A repeat marker study after a further 3 mo in culture (Table 2.XX) revealed the emergence of a B-cell clone which was SIg positive and E rosette negative. At this time, 20% of the cells formed EA_M rosettes.

4. DISCUSSION

This study has shown that the μFcR which is present on HCs is absent from the cells of a wide variety of human leukaemias, lymphomas and allied disorders when the same methods of detection are used. Of the diseases studied, only CLL had a significant proportion of cells which express this receptor.

Thus the lymphoblasts of non-T ALL, the prolymphocytes of PL (Galton et al, 1974), and the mature B cells from several patients with lymphoma, all lacked the μFcR.

Although the exact nature of the Hodgkin's infiltrate is unknown, it is assumed that the large Reed-Sternberg cell is involved in the neoplastic process (Siegal and Good, 1977). This cell may be of B-cell (Hayhoe et al, 1978) or of histiocytic lineage (Siegal and Good, 1977). Material from a case of Reed-Sternberg cell leukaemia (Hayhoe et al, 1978) afforded an opportunity to study the immunological markers present on such cells. Cells from this case, and morphologically normal cells from the PB and spleen of other cases of HD uniformly lacked

Table 2.XX Surface marker profile of cell line 241 on two occasions of study

Date of study	% rosette formation					
	γFc	μFc	EAC	Mo^{+}	E	SIg^{++}
16.3.77	0	0	0	15	90	0
27.6.77	13	20	23	0	0	D19, M15, K9

$^{+}$ Receptor for mouse erythrocytes (Chapter 3)

$^{++}$ Surface immunoglobulin (Chapter 4)

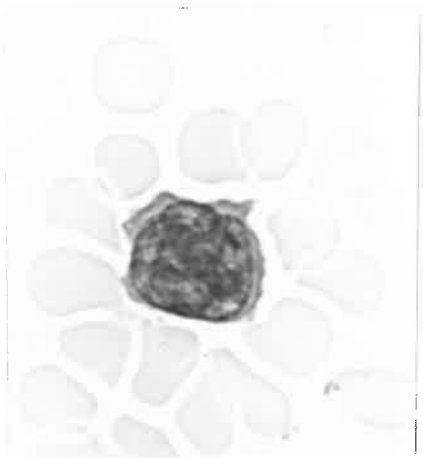

Figure 2.21 Cytocentrifuge preparation of a Sezary cell forming an EA_M rosette after 6 h in culture. The convoluted nucleus of this typical Sezary cell is well illustrated.

receptors for IgM. This result is at variance with a single report from Habeshaw et al, (1976) who obtained EA_M rosette formation in the morphologically normal PB of 5 of 32 patients with HD studied. Habeshaw et al, did not preincubate their cells but used the same procedure as is described in this chapter. It may be that the limited studies reported here have merely failed to encounter a case of μFcR-positive HD. However, Habeshaw et al, comment that they were perhaps detecting immature T cells, and their PB findings of only 34% T cells in the single case in which data are presented, together with only 29% EA_M rosette formation in the same case, suggests that this might be the case. A serum factor in the PB of HD patients often prevents a large proportion of T cells from forming E rosettes (Siegal, 1976), and these cells can be made to reattain normal rosette-forming ability by culturing overnight in medium containing FCS. Since these are the same requirements as for EA_M rosette formation by T cells (see below), it was of interest to examine PB cells from patients with HD for the proportions of T_M and T_G cells (Moretta et al, 1977b). This was done in a single case. Culture of the lymphocytes from HD patients restored the E-rosette forming-ability of T cells but also revealed a gross distortion in the proportions of T_M to T_G in this disease; T_M cells were markedly depressed and T_G cells correspondingly increased. This result may be of some interest since the T cells in HD respond poorly to PHA and since the PB of patients with HD has increased suppressor lymphocyte activity (Siegal, 1976): both of these features characterize the functional capacities of T_G cells (Moretta et al, 1977).

Terminal B cells, as seen in a variety of myelomas, universally lacked the μFcR. Human leukaemic monocytes, histiocytes and granulocytic cells also lacked the receptor and may thus differ from animal cells of

these types (Henson, 1969; Lay and Nussenzweig, 1969; Rhodes, 1973). Incidentally the results from two cases of APL recorded here, whilst showing the absence of the μFcR, substantiate studies of murine neutrophil maturation which show that the γFc receptors appear at an early phase of neutrophil differentiation that precedes the appearance of complement receptors (Rabellino et al, 1978).

The B lymphocytes of CLL and the T lymphocytes from two cases of the Sezary syndrome both expressed a receptor for IgM; and the μFcR was also present on a small population of normal PB and spleen cells. The different conditions required to demonstrate IgM receptor expression by B cells and T cells are well illustrated by the pathological cells from these two conditions.

Thus the B cells of CLL express the receptor without culture in medium containing serum, and cells bearing the μFcR simultaneously bear γFcR. This result agrees with the findings of Pichler and Knapp (1977), and Romagnani et al (1978) for CLL cells. The failure of Ferrarini et al, (1977) to detect μFcR on some of their cases of CLL without preincubation can almost certainly be attributed to receptor shedding during the cell isolation procedure. Whether these conditions are also true for normal B cells is difficult to determine. The results in this section suggest that the few normal PB cells which express μFcR without culture may be B lymphocytes. However, normal PB contains very few IgM receptor-bearing B cells (B_M cells), most B_M cells being found in the tonsil (Pichler and Knapp, 1978; Ferrarini et al, 1977). Also, the techniques required for B_M cell detection are prolonged (T cell depletion) and probably enable μFcR shedding to take place.

The expression of μFcR without incubation appears to be a feature of B_M cells as opposed to T_M cells. There have been reports of receptor

expression by T_M cells immediately after isolation (Gmelig-Meyling et al, 1976; Bolhuis and Nooyen, 1977), but this has not been the experience of most authors either for normal T_M cells (Moretta et al, 1975; McConnell and Hurd, 1976; Ferrarini et al, 1977; Conradie and Bubb, 1977; Preud'homme et al, 1977b; Romagnani et al, 1978), or for the pathological T_M cells of the Sezary syndrome recorded here. It is difficult to account for the findings of Gmelig-Meyling et al, and Bolhuis and Nooyen, but the latter workers do manipulate their cells in FCS-containing medium and, particularly in their mixed rosette tests, their washing and incubation steps can take as long as 40 min: the data presented in this section shows that this time is probably long enough for μFcR expression by some T_M cells. The observation that autologous serum was as effective as FCS in the culture medium contradicts the findings of Moretta et al (1975). This is not due to a peculiarity of Sezary cells since Romagnani et al (1978) have recently recorded the same findings for normal human T cells. It is possible that there is a serum factor present in only some sera, analogous to the blocking factor for E rosette formation present in some HD sera but not others (Siegal, 1976).

Receptors for IgM and IgG on both pathological and normal human T cells appear to be mutually exclusive (McConnell and Hurd, 1976; Moretta et al, 1976, 1977b), and this therefore is another major difference between T_M cells and (pathological) B_M cells.

Recent reports have agreed in showing that detection of the μFcR on B_M cells requires the EA_M indicator cells to be coated with a higher concentration of IgM than is required for T_M receptor detection (Ferrarini et al, 1977; Pichler and Knapp, 1978; Romagnani et al, 1978). These same reports also emphasise the difficulty encountered in blocking

EA_M rosette formation by B cells when using whole IgM. Pichler and Knapp (1978) have demonstrated that the μFcR of CLL cells is much more effectively blocked when IgM is complexed to its antigen, and this is also true for HCL cells (Section I). These findings may reflect a difference between the receptors on T_M and B_M cells since the T_M receptor is very efficiently blocked by native IgM (Moretta et al, 1975; McConnell and Hurd, 1976; Ferrarini et al, 1977; Romagneni et al, 1978). Since the IgG receptors for native and complexed IgG on murine leukaemia cells may be separate (Cooper et al, 1977), the difference in T_M and B_M μFcR binding may have a molecular basis, although there is one report of more efficient monomeric IgM binding by T cells (Preud'homme et al, 1977b).

The finding of the μFcR on a small number of cultured human B-cell lines agrees with the recent findings of Pichler et al (1978). Pichler et al, established that the EA_M indicator cells had to be coated with a high density of IgM, that all of the 4 B-cell lines tested, but not the MOLT 4 T-cell line, expressed μFcR and that receptor expression did not necessarily indicate a particular class of SIg expression or secretion. In this section it is shown that B-cell lines, but not cultured monoblastoid cells or T cells, express the receptor. However, it is also shown that receptor expression is not dependent on either EB virus infection or on any SIg expression since the HC line (Section I) was EBNA negative and line 117 is SIg negative (Karpas et al, 1977).

Finally, mention should be made of the possible significance of the B cell μFcR both in T-B cell co-operation and in B-cell ontogeny. The important functional differences between T_M and T_G cells are established (Moretta et al, 1977), and there is mounting evidence that under some conditions γFc can activate both human (Moller, 1969; Cooper

Seligmann, 1977) and mouse (Berman and Weigle, 1977) lymphocytes. The role of the B_M cell awaits further study, but it is shown here that of a wide variety of B-cell disorders, the μFcR is restricted to the cells of HCL and CLL. Several pieces of evidence, including a slow SIg replacement time and the presence of SIgG in many cases (Chapter 4) make it possible that both HCL and CLL represent clonal expansions of memory B cells. The intriguing possibility exists that binding of IgM-antigen complexes to memory B cells is one of the signals inducing them to secrete IgG.

CHAPTER 3

OTHER IMMUNOLOGICAL MARKERS IN HAIRY-CELL LEUKAEMIA

I The γFc receptor and the Ia-like antigen

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2.2 *Indicator cells and EA_G rosette formation*

2.3 *Immunofluorescent staining with anti-Ia-like serum*

2.4 *Blocking by anti-Ia-like serum*

3. RESULTS

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3.2 *γFcR density, shedding and morphology*

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II Receptors for fixed C3 and the Epstein-Barr virus

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2.2 *Cultured cells*

2.3 *Complement-sensitized red cells (EAC)*

2.4 *Complement-sensitized yeast cells (Yeast C3b)*

2.5 *Rosette tests*

2.6 *Blocking with monomerized IgM*

2.7 *Epstein-Barr nuclear antigen (EBNA)*

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3.2 EAC3b and EAC3d rosettes

3.3 Blocking with monomeric IgM

3.4 Yeast C3b rosette test

3.5 EBNA and cultured cells

4. DISCUSSION

III Receptors for mouse erythrocytes and for monkey erythrocytes

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2.2 Mk rosettes

2.3 Antisera to C3 and C4

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3.2 Mo rosettes in leukaemia and lymphoma

3.3 Cultured cells

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4. DISCUSSION

IV T lymphocytes and B lymphocytes in HCL

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2. MATERIALS AND METHODS

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2.2 T_M and T_G cells

2.3 Anti-hairy-cell serum

2.4 Surface markers and electron microscopy

2.5 Esterase cytochemistry

3. RESULTS

3.1 T_M and T_G cells in HCL

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4. DISCUSSION

OTHER IMMUNOLOGICAL MARKERS IN HAIRY-CELL LEUKAEMIA

I The γFc receptor and the Ia-like antigen

1. INTRODUCTION

Most of the major studies of HCL have agreed that HCs have receptors for the Fc of IgG (γFcR) (Fu et al, 1974; Haegert et al, 1974; Jaffe et al, 1974; Utsinger et al, 1977; Catovsky, 1977; Cawley et al, 1978, 1978a; Jansen et al, 1978). However, the methods used to detect the receptor have varied, and some workers have concluded that the nature of the γFcR on HCs is evidence for these cells belonging to the monocyte-histiocyte series (Jaffe et al, 1974; King et al, 1975).

Various human haemic cells can be shown to possess γFcR and subpopulations of most cell types probably express the receptor at some stage of development, thus: T cells (Ferrarini et al, 1975); B cells (Dichler and Kunkel, 1972); monocytes (Huber et al, 1968); and neutrophils (Messner and Jelinek, 1970) have all been demonstrated to possess surface receptors for IgG. Unclassified cells variously described as third population cells (Frøland and Natvig, 1973), null cells (Chess et al, 1975), K cells (Perlmann et al, 1976) and L cells (Horwitz and Lobo, 1975) also carry γFcR. Although these unclassified cells are probably heterogeneous in terms of both their surface marker phenotypes and their potential function (Horwitz and Garrett, 1977; Schlossman and Chess, 1976), a characteristic finding among studies of the various cell types within this group is the possession of a strong receptor for IgG. The γFcR on this unclassified cell group (UCG) bears many similarities to the γFcR on monocytes and macrophages, and differs in many respects from the γFcR

present on SIg-positive B lymphocytes; these differences are set out and compared with the γFcR on HCs in Table 3.I.

A differentiation antigen has been identified in man by absorbing alloantigens of the HLA-A, HLA-B and HLA-C groups from pregnancy sera with platelets and studying the properties of the residual serum (Winchester et al, 1975). At the same time, a heteroantiserum raised to purified glycoprotein from the HLA-D linked B-cell antigen complex (p 29,34) (Humphreys et al, 1976; Springer et al, 1976, 1977), has been tested and found to be identifying the same antigen as the allosera. The alloantigens thus identified have been provisionally designated HLA-DR (for D-related) (WHO Report, 1977), although the name of, Ia-like antigen, is in general use because of its relationship to the Ia antigen of the murine H-2 system.

The Ia-like antigen is present on normal and leukaemic human B cells (Arbeit et al, 1975; Schlossman et al, 1976; Billing et al, 1976), on subsets of the UCG and monocytes (Schlossman et al, 1976; Chess and Schlossman, 1977) and on the cells from non-T acute lymphoblastic leukaemia (Schlossman et al, 1976). Human T cells bearing a receptor for the Fc of IgG (T_G cells) probably also possess the Ia antigen but at low density (Samarut et al, 1977), and the antigen is also to be found on human myeloid stem cells (Cline and Billing, 1977), and on the neoplastic cells of both acute myeloid (Schlossman et al, 1976) and some cases of chronic myeloid (Janossy et al, 1977; Barnard et al, 1978) leukaemias. However, since the Ia-like antigen is not found on mature granulocytes or on the plasma cells of multiple myeloma of any class (Schlossman et al, 1976; Kadin and Billing, 1978; Halper et al, 1978; Burns et al, 1978d), the Ia-like antigen is probably an early differentiation antigen which is lost in the final maturation steps of B cells and myeloid cells (Burns et al,

Table 3.I Comparison of the γFcR on monocytes and UCG[+], on B lymphocytes, and on HCs

Test	Monocytes and UCG	B lymphocytes	HCs	Reference
EA_G rosette formation with erythrocytes coated with <5,000 IgG molecules (e.g. with anti-Rh or anti-Forssman antibodies)	+	-	+	Shevach et al, 1973 Jaffe et al, 1974 King et al, 1975 Natvig and Froland, 1976 Lobo and Horwitz, 1976
Deformation of sensitized erythrocytes forming rosettes	+	-	+	Lo Buglio et al, 1967 Parker and Stuart, 1978 Burns et al, 1978a
Binding of EA_G by frozen sections of tissue	+	-	+	Shevach et al, 1973 King et al, 1975 Deegan et al, 1976 Matre et al, 1977
Receptor sensitive to protease	-	+	-	Dickler, 1974, 1977a Matre et al, 1977
Receptor blocked by anti-Ia-like antigen[++]	-	+	-	Dickler, 1976a, 1976b Schlossman and Chess, 1976 Solheim et al, 1976 Burns et al, 1978a

[+] Unclassified cell group includes null cells, K cells, third population cells and L cells (see text).

[++] Blocking of EA_G rosette formation, but not antibody-mediated cell cytotoxicity.

1978d; Ross et al, 1978).

The Ia-like antigen is probably interrelated with but not identical to, the γFcR (Solheim et al, 1976; Chess et al, 1976; Dickler et al, 1977; Woofsy et al, 1977). The nature of this relationship, whilst not fully understood, appears to be different with different cell types. Thus, anti-Ia-like serum blocks IgG binding by B cells (Dickler 1976a, 1976b; Solheim et al, 1976; Dickler et al, 1977) and T_G cells (Samarat et al, 1977), but not binding by the UCG or monocytes (Chess et al, 1976; Dickler, 1976b).

This section sets out to determine the nature of the γFcR on HCs, and while confirming previous reports that HCs possess the Ia-like antigen (Naeim et al, 1977; Burns et al, 1977d) establishes that IgG binding by HCs is not blocked by anti-Ia-like serum.

2. MATERIALS AND METHODS

2.1 Patients, tissues and cell preparation

The details of all the patients with HCL studied, and details of the tissues and mononuclear cell isolation have been given in Chapter 2. On every occasion that cells from a case of HCl were obtained, the cells were tested for the presence of the γFcR.

2.2 Indicator cells and EA_G rosette formation

The preparation of EA_G indicator cells was exactly as described in Chapter 2. On occasions when indicator erythrocytes coated with a low density of IgG were required, this was done by sensitizing the ox erythrocytes with serial dilutions of IgG antiserum as described previously (Chapter 2.I).

Rosette formation with EA_G was performed in 3 ways:

a) Suspension The formation and scoring of EA_G rosettes has been described in Chapter 2. After the suspension scores had been read the morphology of rosette-forming cells was checked by staining cytocentrifuge preparations with Giemsa.

b) Monolayers The ability of HCs to adhere to glass (Rieber et al, 1977) was utilised. Lab-Tek (Miles Laboratories Inc., Illinois) tissue culture slides were employed as suitable chambers to hold 1 ml washed mononuclear cell preparations at 1 x 10^6/ml in 20% homologous serum over the clean glass slides. The cultures were incubated at 37°C for 80 min before thoroughly washing in HBSSA to remove non-adherent cells. As a control both for glass adherence and for EA_G rosette formation, monolayers of monocytes from normal PB were prepared and tested in the same way.

The chambers holding the monolayers were then flooded with 1 ml EA_G at 1% in HBSS, and indicator cells of different degress of sensitization were tested for EA_G rosette formation. After 30 min at room temperature, the chambers were inverted and gently rinsed with HBSSA before reading for rosette formation by the monolayer of cells. These monolayers were occasionally stained with Giemsa, although red cell attachment could be seen without staining.

c) Frozen sections Cryostat sections were cut from pieces of spleen from one patient (HR) with HCL. The sections were cut immediately after the spleen was removed, and allowed to attach to glass slides. Cytological and cytochemical staining of the sections demonstrated that most of the cells were HCs. The glass slides with the sections attached were transported to the laboratory in containers filled with HBSSA. The slides were then placed in petri dishes and flooded with

indicator cells at 1% (EA_G, EA_M, EAC, E) for 30 min at room temperature, or 15 min at 37°C followed by 1 h at 4°C (for EA_M and E). After this time the sections were washed gently with HBSSA, then stained with Giemsa and read for erythrocyte attachment.

2.3 Immunofluorescent staining with anti-Ia-like serum

The rabbit antiserum raised against purified p29,34 Ia-like antigen (Springer et al, 1976) has been described in Chapter 2. Very little of the antiserum was available and therefore $F(ab)_2$ fractions were not prepared. Because of this, staining was usually carried out on cytocentrifuge preparations of cells which had been acetone fixed at -20°C overnight in order to prevent Fc binding of the antisera. An indirect staining technique was employed; the cells were first stained with the anti-Ia-like serum at a dilution of 1/50, washed with HBSS, and then stained with a goat anti-rabbit serum at appropriate dilution.

Under these conditions, control preparations of freshly-isolated CLL cells, and cells from several B-cell lines, showed strong membrane fluorescence, whereas freshly isolated cells from a case of T-cell Sezary Syndrome and cells from an established T-cell line (Line 45) (Karpas et al, 1977) did not stain at all. Preparations examined with this serum were particularly clean, and it is interesting to note that the bone marrow cells from several cases of myeloma were also negative (Burns et al, 1978d) thus confirming the results of others for plasma cells (Kadin and Billing, 1978; Halper et al, 1978).

2.4 Blocking by anti-Ia-like serum

The procedure used for blocking by antisera has been described in Chapter 2. For blocking, the anti-Ia-like serum was diluted to 1/50 in HBSS, and a normal rabbit serum at the same dilution was employed as a control.

3. RESULTS

3.1 EA_G rosette formation

It was shown in Chapter 2 that HCs formed rosettes with the EA_G reagent. The standard EA_G reagent is very sensitive and in all the cases of HCL studied, most, or all, HCs formed rosettes. Percentage EA_G rosette scores obtained from the PB of HCL cases reflect mostly the varying HC involvement. The spleens obtained from HCL patients, however, comprise of >80% HCs, and the point of γFcR expression by HCs is better made by examination of splenic EA_G scores. The results of EA_G rosette formation by the splenic mononuclear cells from 11 patients with HCL are given in Table 3.II. Cultured HCs continue to express the γFcR; after 18 days in culture 99% of cells formed EA_G rosettes and after 66 days 93% continued to do so.

HCs which had adhered to glass as a monolayer, and HCs *in situ* in frozen sections of spleen, bound EA_G. Monolayers of HCs bound ox erythrocytes coated with dilutions of IgG at 1/50, 1/100, 1/200 and 1/300 just as effectively as the monolayers of normal human monocytes used as a control. In neither the monolayer preparations, nor the sections of spleen, were any HCs observed to bind unsensitized ox erythrocytes, erythrocytes sensitized with IgM, or with IgM + mouse complement (EAC).

3.2 γFcR density, shedding and morphology

Percentage rosette titrations were carried out with EA_G indicator cells coated with serial dilutions of IgG. The results of these experiments were given in Chapter 2 (Figure 2.7), and it was shown that rosette formation between PB HCs and normal PB monocytes started to fall off at around the same dilution of sensitizing IgG antibody.

Table 3.II EA_G rosette formation by the mononuclear cells obtained from the spleens of 11 patients with HCL

Patient	% EA_G rosette formation
RH	80
MB	78
PS	91
NW	82
LH	96
DC	90
LP	94
AD	93
HR	91
AB	91
AF	95

In order to determine whether the γFcR, like the μFcR, is shed and regenerated, it was necessary to reduce the sensitivity of the indicator EA_G cells to a minimum. A repeat EA_G rosette titration was carried out with the PB HCs from 1 patient (HR). The highest plateau dilution of coating IgG was found to be 1/600. Figure 3.1, which plots the % rosette formation with time by PB HCs when held as a mixture with 1/600 EA_G at 37°C in the presence and absence of puromycin, demonstrates that the receptor for IgG is shed and that the regeneration process requires protein synthesis.

Electron microscopy revealed that the EA_G indicator cells were deformed by rosette formation with HCs. This has previously been illustrated in Chapter 2 (Figure 2.8), and a further example is shown in Figure 3.2 Such deformation of the indicator erythrocytes was a general feature of EA_G rosette formation but was not seen with EA_M rosettes.

3.3 Ia-like antigen and γFcR blocking

Staining for the Ia-like antigen was performed on splenic HCs from 2 patients (LP and LH) and the PB mononuclear cells from 3 patients. The results are given in Table 3.III, and it is clear that when allowance is made for T cells (which are negative by this method of identification) almost all HCs are positive for the Ia-like antigen. Over 90% of cultured HCs also stained with this anti-p29,34 antiserum.

Blocking of EA_G rosette formation in the presence of anti-Ia-like serum was attempted, on two occasions, with cultured HCs. On one occasion 3%, and on the other 4%, blocking was obtained from an initial EA_G rosette formation of 93%. This concentration of serum both stained the HCs and totally blocked EA_M rosette formation by them.

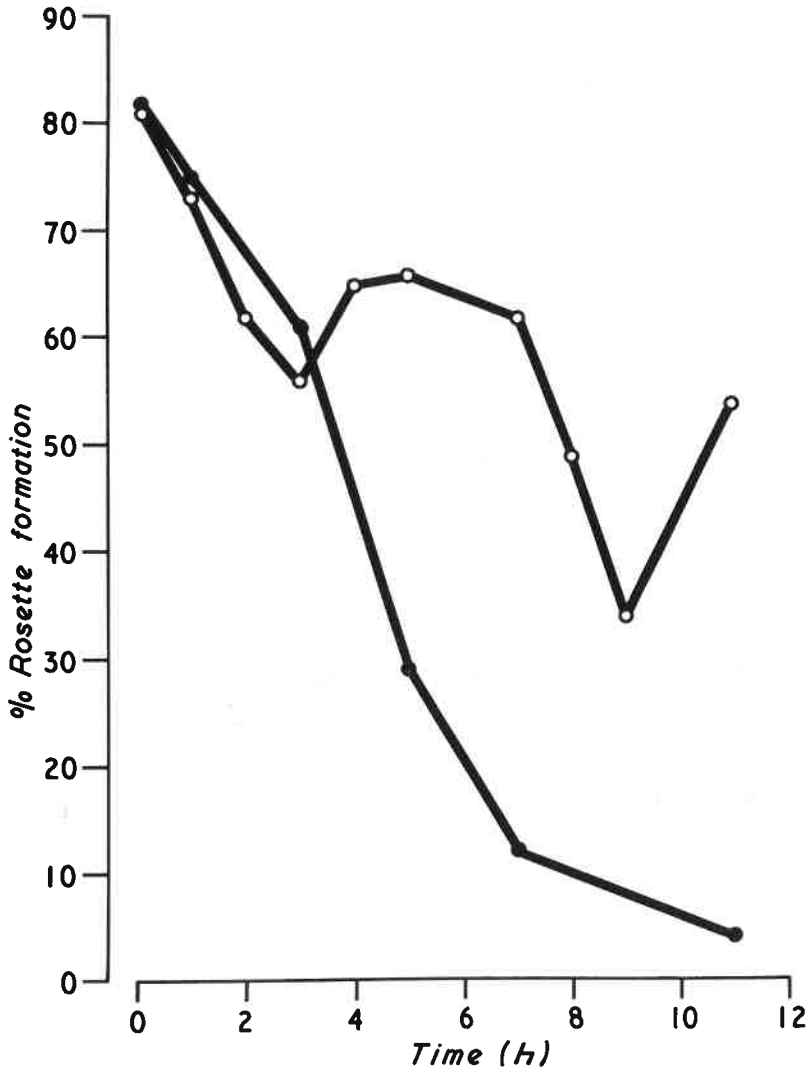

Figure 3.1 Kinetics of EA_G rosette formation by HCs. Mononuclear cells were incubated with EA_G at 37°C in the presence (●) and absence (○) of puromycin (2.5 µg/ml). Aliquots were removed for rosette testing at the times indicated and more than 200 cells were counted in each determination.

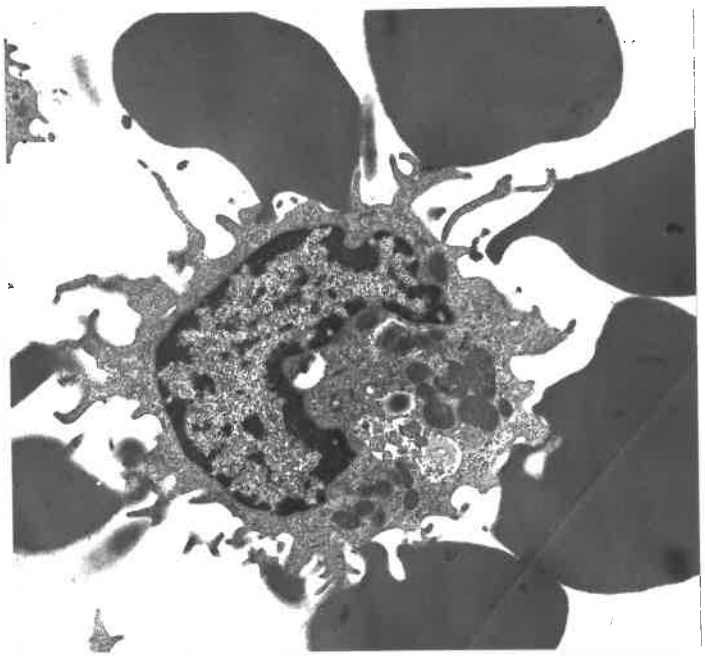

Figure 3.2 EA_G rosette formation by HCs.

Table 3.III Anti Ia-like, p29,34 antigen staining by HCs

Patient	Material	T cells (%)	% cells staining Ia antigen
LH	spleen	5	90
LP	spleen	18	75
LH	PB	50	50
LH	PB	23	79
DC	PB	86	16
BN	PB	44	50

4. DISCUSSION

In this section the presence of a receptor for IgG on HCs is confirmed, and it is shown that the heteroantiserum-erythrocyte complex used for detection identifies almost all HCs as γFcR positive.

Various features suggest that the γFcR on HCs is different from the γFcR present on a population of human B lymphocytes and that it bears resemblances to the μFcR on monocytes and the UCG (Table 3.I).

Thus, in agreement with others (Jaffe et al, 1974; King et al, 1975), it is demonstrated here that HCs form rosettes with indicator erythrocytes coated with low levels of IgG. It is of interest that EA_G made with such low levels of sensitizing IgG were required to show that the γFcR on HCs is shed, thus confirming the observation of Sarmay et al, (1978) for normal and CLL cells. The time taken for shedding and re-expression of the γFcR by HCs is also seen to resemble the time for μFcR re-expression (Chapter 2; Burns et al, 1977b) by HCs.

HCs, like monocytes, adhere strongly to glass in the presence of high levels of serum (Rieber et al, 1977), and in this section it is shown that they will bind EA_G when tested in such monolayers. Similarly, macrophages in frozen sections of tissue bind EA_G but lymphocytes do not (Shevach et al, 1973). The observation that HCs in frozen sections also bind EA_G (Jaffe et al, 1974; King et al, 1975; Deegan et al, 1976; Matre et al, 1977) is confirmed here. The deformation of erythrocytes caused by HCs binding to EA_G has been commented upon in Chapter 2, and this feature is also common to monocyte, but not lymphocyte, EA_G rosette formation (Lo Buglio et al, 1967; Parker and Stuart, 1978).

The finding of the Ia-like antigen on HCs (Naeim et al, 1977; Burns et al, 1977d), confirmed here, is not a specific feature of either

lymphocytes or monocytes. Nor is this an unexpected finding since it has been reported that HCs have the capacity to stimulate in allogeneic mixed lymphocyte cultures (MLC) (Kitani, 1976; Boldt et al, 1977) - a feature coded for by the HLA-D region of the HLA chromosomal complex. It has been suggested that in man, as for some Ia antigens in the mouse, the Ia-like antigens may be the major MLC-activating determinants (Arbeit et al, 1975; Zeijlemaker et al, 1976; Solheim et al, 1976). In this regard, it is of interest that only some 20% of the UCG are positive for the Ia-like antigen (Schlossman and Chess, 1976), and that antibody to this antigen, as with HCs reported here, does not block EA_G binding by the γFcR of these cells whereas the γFcR of B cells is blocked by anti-Ia-like serum (Arbeit et al, 1975; Solheim et al, 1976; Dickler et al, 1977).

Within the UCG, an Ia-negative population (the L cells of Lobo and Horwitz (1976)) is very poor at stimulating in MLC (Horwitz and Garrett, 1977) as are human monocytes (Zeijlemaker et al, 1976). If, therefore, HCs are related to the UCG by virtue of the nature of the γFcR, it would most likely be to the Ia-positive subpopulation. This subpopulation has the capacity to secrete immunoglobulin and probably has low density SIg on the cell membrane (see Chapter 6).

II Receptors for fixed C3 and the Epstein-Barr virus

1. INTRODUCTION

There have been conflicting reports concerning the presence of a receptor for C3 on HCs, and this controversy is, as yet, unresolved (Catovsky, 1977). Some authors have reported the presence of a receptor for C3 on HCs when employing a rosetting system using ox cells coated with IgM and complement (Haegert et al, 1974; Catovsky et al, 1974a, 1975). Others (Shevach et al, 1973; Jaffe et al, 1974; King et al, 1975; Jansen et al, 1978) have failed to find a receptor for C3 on HCs.

The present section reports observations relating to this controversy and suggests that technical difficulties may account for previous reports showing the presence of a C3 receptor on HCs. Also, since there is a close relationship between C3 receptors on human B cells and receptors for the Epstein-Barr virus (EBV) (Jondal et al, 1976; Yefenof and Klein, 1977), a limited study was undertaken to investigate the presence, or absence, of receptors for EBV on HCs.

2. MATERIALS AND METHODS

2.1 Patients and mononuclear cell isolation

Mononuclear cells were obtained from patients with HCL, CLL and normal individuals. The criteria for diagnosing the patients have been described in previous chapters, as have the cell isolation techniques. Briefly, a Ficoll-Isopaque mixture was employed and no particular precaution was taken to avoid monocyte contamination since monocytes

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are virtually absent in HCL (Seshadri et al, 1976; Higgy et al, 1978; Burns et al, 1978b).

2.2 *Cultured cells*

Cultured hairy cells The establishment of the HC culture has been described in Chapter 2. After several weeks in culture the cells continued to display the typical morphological appearance of HCs and contained the isoenzyme 5 (tartrate resistant) of acid phosphatase characteristic of HCs (Yam et al, 1971) (Figure 3.3).

The cells of this culture did not form the large clusters typically seen with lymphoblastoid cell lines, but grew very slowly as small clusters of a few cells over a monolayer of cells (possibly fibroblasts) from the original splenic material. Throughout the period of culture, the great majority of cells resembled HCs morphologically although, after 60 days, increasing numbers of larger cells (up to 10%) with a transformed appearance began to appear. Virtually all the leucocytes, including the larger cells, were positive for tartrate-resistant acid phosphatase.

After 2 weeks of culture, attempts were made to infect the HCs with Epstein-Barr virus (the EBV was obtained from a producer lymphoblastoid cell line and was a gift from Professor Epstein, Department of Pathology, University of Bristol). The cell line resulting from EBV infection in no way resembled HCs morphologically or immunologically. More than 2 weeks elapsed before cluster formation was observed and then the EBV infected cells grew rapidly in very large clusters typical of cultured lymphoblastoid cell lines.

Raji cell line This line is an EBV-carrying non-producer cell line derived from a patient with African Burkitt Lymphoma (Epstein et al, 1966). This established lymphoblastoid cell line will form strong EAC3d rosettes

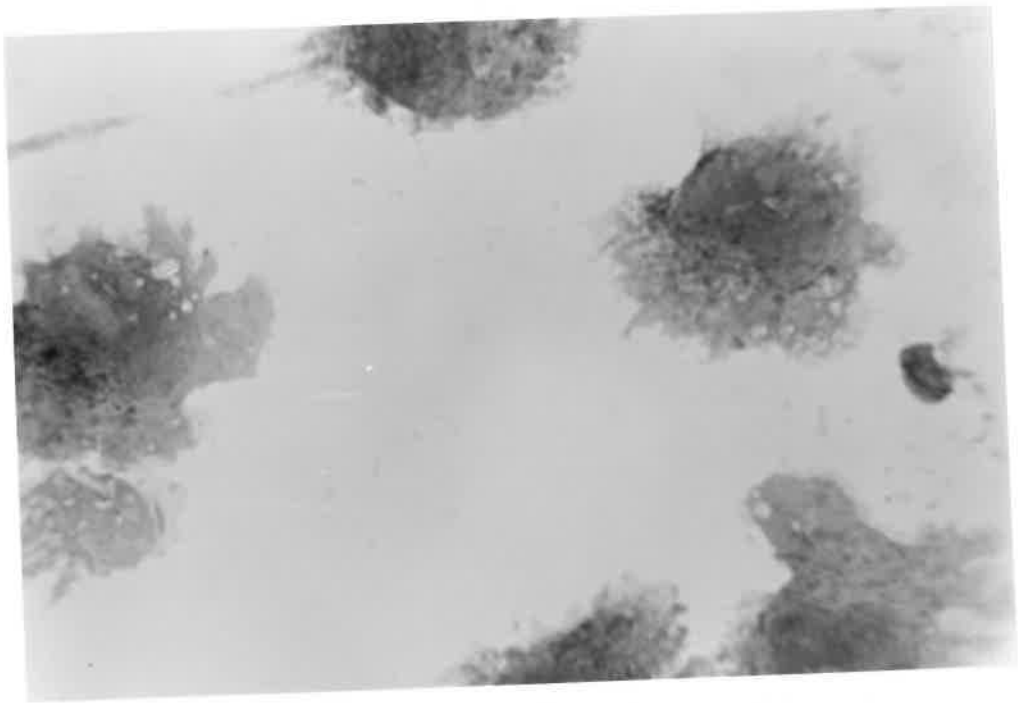

Figure 3.3 Hairy cells after 6 weeks in culture. The kidney shaped nucleus with nucleolus and the cytoplasmic 'hairs' with vacuolation are all typical features of HCs. In addition, this preparation demonstrates the tartrate-resistant acid phosphatase isoenzyme. Acid phosphatase staining was performed by the method of Goldberg and Barka (1962) in the presence of L(+) tartaric acid at a concentration of 0.05M (Li et al, 1970).

but few cells, if any, form EAC4 rosettes (Bokisch and Sobel, 1974). However, although Raji cells do not possess immune adherance receptors (C4, C3b), they will form rosettes with EAC3b due to the strong C3d receptors binding the C3d of the EAC3b reagent (Ross and Polley, 1976). These cells were a kind gift from Dr. I. McConnell.

2.3 Complement sensitized red cells (EAC)

Routine EAC EA_M prepared with ox erythrocytes and rabbit IgM were prepared as described in Chapter 2. Complement sensitized red cells (EAC) were prepared from the EA_M cells by the method of Ross et al, (1973b). In this method the EA_M cells are incubated at 37°C for 15 min with AKR (C5-deficient) mouse serum (diluted 1/10 in CFT buffer) as a source of complement. The resultant EAC were washed and suspended to 1% in HBSSA. EAC indicator cells prepared in this way from whole mouse serum are probably coated with some C3b but mostly with the C3d fragment that remains after cleavage by the C4b-C3b inactivator (KAF) (Ross et al, 1973a; Rabellino and Metcalf, 1975).

EAC3b and EAC3d Indicator cells coated with a preponderance of C3b were prepared with the use of suramin (Antrypol ^{R}) which inhibits the serum of C4b-C3b inactivator, probably by binding to C3b, so that most of the fixed C3 remains in the C3b form (Tamura and Nelson, 1967; Lachman et al, 1973). The EAC3b indicator cells were prepared from EA_M by incubation with zymosan-absorbed human serum (R3 reagent) which contains little C5 and C6 and is non lytic for EA_M (Lachmann et al, 1973). The R3 serum was diluted 1/10 in CFT buffer and mixed with an equal volume of EA_M (at 2% in CFT buffer) prewarmed to 37°C. The complement activation was allowed to proceed for 70 sec at 37°C before adding 5 µl of Antrypol (50 µg/ml) to the reaction mixture and thoroughly mixing for 2 min. EAC3d cells were prepared in a similar manner, but incubation

was for 30 min at 37°C without added Antrypol to enable KAF to inactivate the fixed C3b. The indicator cells thus prepared were thoroughly washed and resuspended to 1% in HBSSA.

2.4 Complement sensitized yeast cells (Yeast C3b)

Yeast cell walls spontaneously trigger the alternative pathway of complement activation and as a result become coated with C3b. This alternative pathway-derived C3b is bound to the yeast cell wall in such a way that it is relatively resistant to cleavage by KAF (Fearon and Austen, 1977).

For the preparation of Yeast C3b particles the method of Mendes et al, (1974), as modified by Nash (1976) was initially employed. Briefly, yeast cells were fixed with glutaraldehyde and sonicated to remove clumps. These were then washed in CFT buffer and adjusted to 2 x 10^8 cells/ml. Fresh human serum, diluted 1/5 in CFT buffer, was mixed with an equal volume of the yeast suspension and incubated for 30 min at 37°C. The complement-coated yeast cells were then washed four times with cold CFT buffer and adjusted to a concentration of 10^8 cells/ml. As control cells, yeast particles were incubated with heat-inactivated serum.

In later tests, the modification described by McConnell and Hurd (1976) was used in order to reduce non-specific protein adherence. In these tests washed, glutaraldehyde-fixed, and sonicated yeast cells were incubated in fresh human serum (0.5 ml undiluted) for 5 min at 37°C. The particles were then washed six times in 2M NaCl, filtered through a cotton-wool plug and resuspended to 1% in HBSSA. Control particles were obtained by incubating yeast cells in serum containing 10 mM EDTA which prevents complement activation.

2.5 Rosette tests

Rosette tests with the EAC reagent were performed as previously

described for EA_G in Section I. Briefly, two drops of indicator cells were mixed with two drops of leucocytes (2 x 10^6/ml) and centrifuged at 150 g for 1 min. The pellets were kept on ice before scoring by the method of Ramasamy (1974). Control indicator cells coated with IgM alone (EA_M) were included with every test.

The yeast C3b test was performed in exactly the same way with the Yeast C3b cell suspension (and appropriate control) replacing EAC. Some difficulty was encountered in scoring because of the autofluorescence of the glutaraldehyde-fixed yeast cells, and the unavoidable presence of small aggregations of these cells which appear as brightly fluorescing rosettes. This was overcome by omitting the white light and changing the u.v. filter to the Zeiss UG1 exciter, whereby the yeast cells fluoresced a blue-green and the leucocytes a brighter apple-green. In this way pseudo-rosettes were readily distinguished from true rosettes in which the central cells could be clearly seen.

2.6 *Blocking with monomerized IgM*

HCs were incubated for 10 min at room temperature with different concentrations of IgM obtained from a case of Waldenstrom's macroglobulinaemia and monomerized by dithiothreitol reduction and iodoacetamide reduction (Rhodes, 1973) as described in Chapter 2. These cells were then tested for EAC rosette formation. As a control, the test was performed using lymphocytes from four normal volunteers.

2.7 *Epstein-Barr nuclear antigen (EBNA)*

The EBNA antigen was sought using the indirect fluorescent method of Reedman and Klein (1973) on acetone-fixed leucocytes. Cells to be tested were cytocentrifuged and fixed, in acetone, at -20°C overnight. Fresh serum from a Paul-Bunnell-positive donor was diluted 1/10 in HBSS and incubated over the fixed cells for 30 min at 37°C. The slides were

thoroughly washed and then stained with an FITC-coupled rabbit anti-human C3 serum (kindly supplied by Dr. A. Karpas) at room temperature, and rewashed. Positive controls were several established B-cell lines and a negative control was an established T-cell line (Karpas et al, 1977). Positive cells were identified by the characteristic strong nuclear staining and almost completely unstained cytoplasm; cells displaying spotty cytoplasmic staining were scored as negative.

3. RESULTS

All of the EAC indicator cells formed 100% rosettes with the Raji cell line. In addition, the Yeast C3b particles formed >50% rosettes with the monocytes from a case of acute monocytic leukaemia. Results of rosette formation with normal PB leucocytes are given in the relevant tables.

The results of the various rosette tests with PB HCs are given in Tables 3.IV and 3.V.

3.1 EA_M and EAC rosettes

As is shown in Table 3.IV, the percentage of HCs forming EAC rosettes was consistently lower than the percentage forming EA_M rosettes (at the same temperature). In addition, it should be noted that, in general, when EA_M rosette formation was low EAC rosette formation was very low or absent; this was particularly noticeable with splenic HCs which generally showed <5% EAC rosette formation and low EA_M at room temperature.

3.2 EAC3b and EAC3d rosettes

As is seen from Table 3.V, a similar percentage of mononuclear cells from all 6 cases of HCL tested formed EAC rosettes whether the indicator erythrocytes were coated with C3b or C3d. In all cases this percentage

Table 3.IV Percentage of PB mononuclear cells from HCL rosetting with different indicator particles

Patient	Indicator particle			
	EA_M +	EAC	Yeast C3b	Yeast control
KD	42	31	0	0
MB	50	32	0	0
MH	23	0	-*	-
HD	33	10	3	2
LS	55	28	3	0
DC	49	41++	-	-
FB	55	33	-	-
AD	47	26	-	-
LP	66	-	0	0
LH	21	13	-	-
BN	37	13	-	-
RR	55	29	-	-
HR	54	19	-	-
AB	24	4	-	-
GH	50	19	-	-
TN	12	4	-	-
LK	11	-	0	0
	26	22	-	-
Normal Δ	3±1	21.5±2	20±3	4±2

+ EA_M carried out at room temperature as control for EAC

* Not done

++ Blocking with monomeric IgM carried out on this occasion (Figure 3.4)

Δ Average results from 4 volunteers (±1 SD)

Table 3.V Percentage of rosettes formed with different C3 reagents by PB mononuclear cells from HCL patients

Patient	Indicator particle			
	EA_M [+]	EAC3b	EAC3d	Yeast C3b
MB	33	10	18	4
RH	20	9	11	- [++]
LS	71	34	22	3
DC	44	20	16	<1
LH	20	16	12	0
NW	37	3	2	-
Normal [Δ]	3±1	33±3	26±2	18±2

[+] EA_M at room temperature as EAC control

[++] Not done

[Δ] Mean ±SD of 3 normal individuals

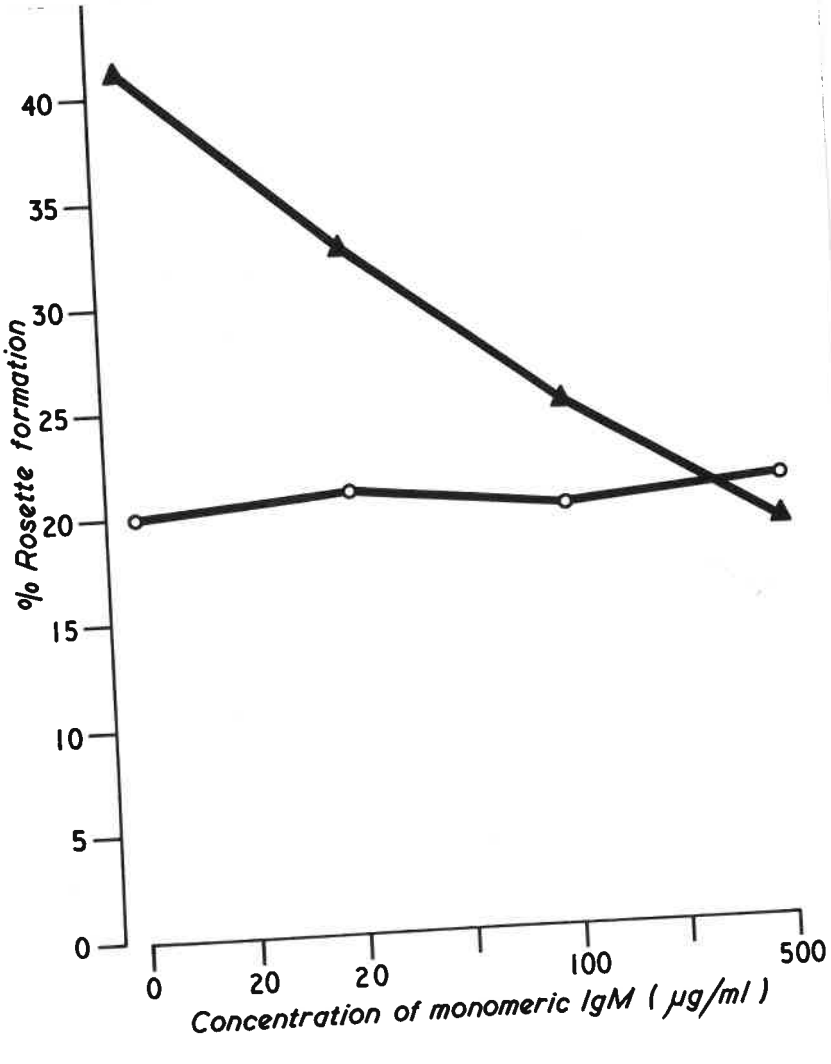

Figure 3.4 Inhibition by monomeric IgM of apparent EAC rosette formation by HCs (▲) and a normal lymphocyte control (o).

Table 3.VI Complement receptors on CLL cells

Patient	% rosette formation		
	Yeast C3b	EAC3b	EAC3d
1	24	7	39
2	8	6	21
3	17	19	73
4	5	1	22
5	-[+]	15	37
6	-	4	22

[+] Not done

was lower than that obtained with EA_M.

These results are in contrast to EAC rosette formation by normal PB mononuclear cells (Table 3.V) where EAC3b rosette formation was higher than EAC3d rosette formation. In CLL, on the other hand, rosette formation with EAC3d was higher than that with EAC3b and the results for 6 cases of CLL are given in Table 3.VI.

3.3 *Blocking with monomeric IgM*

As is shown in Figure 3.4, monomeric IgM caused substantial inhibition of apparent EAC rosette formation by the HCs of patient DC, chosen because of the high percentage of EAC rosettes observed (Table 3.IV). It has been previously shown that EA_M rosette formation by HCs is specifically inhibited to a similar degree by monomeric IgM (Chapter 2).

EAC rosette formation by lymphocytes from normal PB was not reduced by this treatment even at an IgM concentration of 500 µl/ml.

3.4 *Yeast C3b rosette test*

All the HCL patients tested with the Yeast C3b reagent showed a very low percentage of rosette formation (Tables 3.IV and 3.V), while in normal individuals a similar percentage of lymphocytes formed EAC and Yeast C3b rosettes.

3.5 *EBNA and cultured cells*

Cells from the spleen of 2 patients (LH and LP) and from the PB of 1 patient (DC) with HCL were tested for EBNA at the same time as positive controls from established B-cell lines. The cells from all of the HCL patients were EBNA negative.

Cells from the cultured HCs were tested for EBNA after 18 days and after 66 days of culture. These cells were completely negative. After 18 days a portion of this HC culture was 'infected' with EBV and an aliquot tested for EBNA the next day. These cells were also EBNA

Table 3.VII Marker data of culture established from splenic HCs and on transformed cells following EBV 'infection'

Time in culture (days)	Untreated cell line (% rosettes) E	γFc	μFc	SIg	EBV 'infected' cell line (% rosettes) E	γFc	EAC	SIg
18	4	99	92	36	EBV added at this time			
29	-[+]	-	95	75	-	20	-	2
46	0	92	93	82	0	0	85	0

[+] Not done

negative at this time. Unfortunately the 'infected' line became contaminated and lost for study before the EBNA test was repeated, although the transformed, blastic, appearance of these cells some 4 weeks after infection suggested that they would have been positive. The rather incomplete marker studies carried out on the HC culture and the transformed line are given in Table 3.VII, and it can be seen that cells from these 2 cultures bear no apparent relation to one another. Also, although cells from the non-infected HC culture were not tested for EAC because of the high percentage of cells bearing the μFc receptor, it should be noted that a high proportion of the 'infected' cells bore an EAC receptor.

4. DISCUSSION

The results presented in this section suggest that EAC rosettes formed by HCs are the result of binding of IgM used to form the EAC indicator cells by the μFcR of HCs.

By the use of yeast cells coated with C3b activated by the alternative pathway it is shown here that HCs do not have a receptor for complement. The fact that the percentage of EAC rosettes was consistently lower than that of EA_M rosettes supports the contention of IgM binding, and this was confirmed by the blocking data in which EAC rosette formation was blocked by monomeric IgM in a manner analogous to μFcR blocking (Chapter 2). It is also of interest that the apparent EAC receptor was, like the μFcR, exquisitely sensitive to blocking by high dilutions of the anti-hairy cell serum. The variable difference between the percentage of EA_M and EAC rosette formation is probably, at least in part, attributable to varying degress of steric hindrance of the bound IgM by the complement,

but may also be the result of imperfect mixing when complement is bound to the EA_M. It is interesting to note that by reducing the dilution of complement (serum) used to sensitize the EA_M from 1/10 to 1/5, EAC rosette formation in one case of HCL fell by over 30%. It is known that EAC made from IgG and complement will bind to monocytes via the γFcR (Shevach et al, 1973), and that a proportion of cultured T cells can bind IgM which has been bound with complement (McConnell and Hurd, 1976), therefore probably the complement coat does not totally obscure the Fc piece of bound Ig. In this regard, it is of interest that the figure given by Catovsky (1977) illustrating a 'hairy cell forming a rosette with ox cells coated with IgM and C3 (EAC rosette) electronmicrograph' is identical to the TEM pictures of EA_M rosette formation shown in Chapter 2, and this is in contrast to the broad areas of contact described and illustrated for typical B cell EAC rosette formation (Bentwich et al, 1973).

The failure of some workers (Shevach et al, 1973; Jaffe et al, 1974; King et al, 1975; Deegan et al, 1976; Matre et al, 1977; Jansen et al, 1978) to detect an apparent C3 receptor with a rosette test involving IgM is probably attributable to their use of sheep, rather than ox, indicator erythrocytes. Ox erythrocytes are able to absorb much higher levels of IgM before agglutinating than are sheep erythrocytes (Uhlenbruck et al, 1967) and it was previously shown that high concentrations of sensitizing IgM are necessary for EA_M rosette formation (Chapter 2). When ox erythrocytes coated with IgM and complement have been employed in a rosetting system, some authors have concluded that HCs do have a receptor for EAC (Haegert et al, 1974; Catovsky et al, 1975; Catovsky, 1977).

The simple tests used in this report to distinguish between EAC3b

and EAC3d rosettes were performed because HCL has been regarded as a variant of CLL, and because CLL cells have been reported to possess a receptor for C3d, but not C3b (Ross et al, 1973a). On the other had, the EAC3b reagent detects a higher percentage of complement receptors than the EAC3d reagent in both normal lymphocytes (Ross and Polley, 1976) and in blood from patients with Waldenstrom's macroglobulinaemia (Ross and Polley, 1975). Therefore the test described was performed on HCs in order to discount the possibility that EAC rosette results were spuriously low, not because of incomplete hindrance of EA_M, but because of a lack of either C3b or C3d. In fact, while previous results for CLL and normal lymphocytes were confirmed with these reagents, there was no difference in percentage rosette formation by HCs using either indicator particle. Also, whether EAC3b or EAC3d indicator cells were used the percentage rosette formation was consistently lower than in the EA_M test.

The conclusion of a lack of receptors for fixed C3 is supported by other information. Thus, monolayers of glass-adherent HCs do not bind EAC under conditions permitting monocytes to do so (Zidar et al, 1977), and cryostat sections of HC spleens fail to bind EAC whereas the B-lymphocyte areas of spleens from patients with nodular lymphomas firmly bind this reagent (Shevach et al, 1973; Jaffe et al, 1974; Matre et al, 1977): both of these findings showing the failure of HCs to bind EAC were confirmed in the previous section. Recently, Catovsky (1977) has utilised human erythrocytes coated with C3b prepared in serum in a low ionic strength solution (thus avoiding the presence of IgM) to show that nine cases of HCL lacked a receptor for C3b.

Further, indirect, evidence for the lack of C3 receptors on HCs comes from cultural studies. Despite numerous attempts, HC lines have proved

and EAC3d rosettes were performed because HCL has been regarded as a variant of CLL, and because CLL cells have been reported to possess a receptor for C3d, but not C3b (Ross et al, 1973a). On the other had, the EAC3b reagent detects a higher percentage of complement receptors than the EAC3d reagent in both normal lymphocytes (Ross and Polley, 1976) and in blood from patients with Waldenstrom's macroglobulinaemia (Ross and Polley, 1975). Therefore the test described was performed on HCs in order to discount the possibility that EAC rosette results were spuriously low, not because of incomplete hindrance of EA_M, but because of a lack of either C3b or C3d. In fact, while previous results for CLL and normal lymphocytes were confirmed with these reagents, there was no difference in percentage rosette formation by HCs using either indicator particle. Also, whether EAC3b or EAC3d indicator cells were used the percentage rosette formation was consistently lower than in the EA_M test.

The conclusion of a lack of receptors for fixed C3 is supported by other information. Thus, monolayers of glass-adherent HCs do not bind EAC under conditions permitting monocytes to do so (Zidar et al, 1977), and cryostat sections of HC spleens fail to bind EAC whereas the B-lymphocyte areas of spleens from patients with nodular lymphomas firmly bind this reagent (Shevach et al, 1973; Jaffe et al, 1974; Matre et al, 1977): both of these findings showing the failure of HCs to bind EAC were confirmed in the previous section. Recently, Catovsky (1977) has utilised human erythrocytes coated with C3b prepared in serum in a low ionic strength solution (thus avoiding the presence of IgM) to show that nine cases of HCL lacked a receptor for C3b.

Further, indirect, evidence for the lack of C3 receptors on HCs comes from cultural studies. Despite numerous attempts, HC lines have proved

exceedingly difficult to establish. Reports of established HC lines are few (Sinkovics et al, 1976; Golde et al, 1977b; Saxon et al, 1978b), and one of these lines (Golde et al, 1977b; Saxon et al, 1978b) was established from a very unusual case of HCL, the diagnosis of which has been questioned (Katayama, 1976; Banerjee et al, 1978). Moreover, cells from this line were EAC positive (by the Yeast C3b method). Of the other two lines established in culture from HCL patients by Golde and colleagues, one has changed the isotype of immunoglobulin being secreted during the period of culture and the other is a T-cell line. The two lines established by Sinkovics et al (1976) were reported to have intranuclear Herpes-type virus particles in most of the cells, and the authors suggested that these viral particles may be related to EBV. However, serological tests for EBV infection were not performed and there has been no subsequent paper following this brief report. There has also been a recent report demonstrating replication of type 1 Herpes simplex virus replication in freshly isolated and infected HCs, including the demonstration of intranuclear virus particles (Pozner et al, 1978), and it is possible therefore that Sinkovics et al were observing Herpes simplex particles. It is of passing interest that the ability to support herpesvirus replication was observed in cells from all four cases of HCL tested, but not in cells from any of nine cases of CLL tested (Pozner et al, 1978). Receptors for EBV in man are the exclusive property of B lymphocytes (Jondal and Klein, 1973; Greaves et al, 1975), and most established cell lines are both B lymphoblasts (Gordon et al, 1977; Karpas et al, 1977) and EBNA positive (Reedman and Klein, 1973). Generally the ability to 'immortalise' cells in a feature of EBV infection, and non-B, EBNA negative, cell lines are both rare and difficult to establish (Kaplan et al, 1974; Karpas et al, 1978a). The cultured HCs described

in this section were EBNA negative even after 66 days of culture when a typical B-cell line would be EBNA positive. Also, the time taken for the EBV 'infected' line to grow out and the major morphological and marker differences from HCs, suggested that this line was an outgrowth of a minor subpopulation of EBV-permissive B cells rather than an EBV infection of HCs; most cell lines are of this type no matter what the initial disorder or the original majority population of cells being cultured (Gordon et al, 1977; Karpas et al, 1977). These results might suggest a monocytic origin for the HC since cultured monoblast cell lines are EBNA negative (Karpas et al, 1978a), however it cannot be excluded that the HCs were infected by EBV and totally transformed. In addition, if the absence of a receptor for EBV on HCs is correct, this provides strong evidence for the lack of a C3 receptor on these cells since there is a strong association between these two receptors (Jondal et al, 1976; Yefenof and Klein, 1977). More work investigating the presence or absence of a receptor for EBV on HCs is clearly required.

Finally, two suggestions have been made to account for the absence of C3 receptors on HCs. Shevach and colleagues (Shevach et al, 1973; Jaffe et al, 1974) have shown that splenic macrophages, in contrast to macrophages from other sites, do not express receptors for C3, and they suggest that HCs derive from the same cell type as splenic macrophages. Some support is perhaps given to this theory by Ross et al (1978) who propose that during neutrophil maturation not all neutrophils pass through a complement receptor-positive stage. Jansen et al (1978) draw attention to murine studies indicating that the environment of the spleen is required for B cells to acquire the C3 receptor (Ryser and Vassalli, 1974) and suggest that HCs are not properly handled by the grossly deranged spleen of HCL and therefore do not acquire C3 receptors.

A third suggestion is possible. Some animal and human lymphoid cells can activate complement by the alternative pathway in both autologous and heterologous sera (reviewed by McConnell and Lachmann, 1976). It is possible that HCs activate the alternative pathway *in vivo* and thus block their own C3 receptors. Alternatively, activation of complement may be mediated by low affinity antibodies directed against the neoplastic HCs; specific autoantibody activity has been described in both CLL and chronic myeloid leukaemia (Metzgar et al, 1975) and has possibly been demonstrated in a case of HCL (Banerjee et al, 1978). Some evidence possibly supporting this hypothesis accounting for the lack of receptors for fixed C3 on HCs will be given in the next section.

III Receptors for mouse erythrocytes and for monkey erythrocytes

1. INTRODUCTION

Spontaneous rosette formation between human lymphocytes and sheep erythrocytes (Bach et al, 1969; Brain et al, 1970; Coombs et al, 1970) was established as a feature of T cells very shortly after the first descriptions of this phenomenon (Frøland, 1972; Jondal et al, 1972). This discovery has been vigorously pursued in many laboratories and spontaneous rosette formation is now a major tool for the classification and fractionation of human T lymphocytes. At the same time, many investigators looked for spontaneous rosette formation between human lymphocytes and erythrocytes of many other animal species, from the rhinoceros to the warthog (Bach, 1973). Only two such reactions have proved of much value in clinical research.

Spontaneous mouse erythrocyte (Mo) rosettes have been shown to be formed by a subpopulation of human B cells (Stathopoulos and Elliott, 1974; Gupta et al, 1976; Dobozy et al, 1976) which is cytochemically unremarkable (Higgy et al, 1977) but seems to be related to the clonal expansion of CLL (Stathopoulos and Elliott, 1974; Millard, 1976; Forbes and Zalewski, 1976). A report of Mo rosette formation in HCL (Catovsky et al, 1975) is often cited as evidence for the B-lymphoid nature of HCs although few reports have confirmed the presence of this receptor on HCs.

The literature on spontaneous monkey erythrocyte (MK) rosette formation with human leucocytes is confusing, and it has been suggested that the species of monkey used may determine the specificity of the

rosette (Siegal and Good, 1977). With rhesus monkey erythrocytes, Lohrmann and Novikovs (1974) reported that most normal human PB T lymphocytes, and the cells from some patients with CLL, formed rosettes. In contrast, Pellegrino et al, (1975a) reported that rosette formation with Mk was a B-cell marker when employing erythrocytes from the Japanese ape (M. speciosa). Rosette formation was only observed with PB B cells and with B-cell lymphoidblastoid cell lines. The same authors (Pellegrino et al, 1975b) subsequently reported linkage between a receptor for Mk on B cells and HLA antigen expression, and this report was confirmed by another group (Fellows et al, 1977) but employing erythrocytes from the marmoset (C. jacchus).

This section examines Mo and Mk rosette formation in HCL and provides some information bearing on the nature of Mk rosette formation by HCs.

2. MATERIALS AND METHODS

A description of mononuclear cell isolation from normal individuals and patients with HCL and other disorders has been given in the last section and, in more detail, in Chapter 2. The characteristics of the various cultured cells examined have also been given elsewhere. These were washed in serum-free HBSS before testing.

2.1 Mo rosettes

Blood was collected from the orbital venous plexus of young male CBA mice into Alsever's solution. Erythrocytes thus obtained were used for a maximum of two days. After collection the Mo were washed 4 times in physiological saline and resuspended in HBSS with bovine serum albumin (BSA). However, because of the technique recommended by Gupta et al, (1976), 5% BSA, rather than the usual 0.2% HBSSA used in other

rosette tests, was employed.

Rosette testing was carried out, with only some modification, after the method of Gupta et al, (1976). Leucocytes at 2 x 10^6/ml in HBSSA were mixed with 1% Mo and incubated at room temperature for 5 min. The mixture was centrifuged (150 g for 1 min) and the pellet placed on ice until reading of the percentage rosette formation was carried out. In this test and in the Mk rosette test below, scoring of viable rosette-forming cells was performed by the method of Ramasamy (1974) previously described.

2.2 *Mk rosettes*

Venous blood was obtained from either rhesus or cynomorgus monkeys which were under investigation for the effect of lithium on platelet function. However, no difference in Mk rosette formation was observed either between the two species of monkey, or between Mk obtained from animals which had recently received lithium and control animals. Erythrocytes were collected into Alsever's solution and used in rosette tests for up to 1 week after collection. It was also found that red cells stored at -20°C in 3M glycerol were suitable for use.

Before testing, Mk were thoroughly washed in physiological saline and resuspended to 1% (v/v) in Hanks' balanced salt solution. Preliminary investigation showed that there was no difference in the percentage rosettes formed when testing was carried out in phosphate-buffered saline over a pH range 6-10, or whether testing was at room temperature or at 4°C. Centrifugation (1 min at 150 g) was required for optimal rosette formation between the mixture of leucocytes and erythrocytes; if leucocytes and Mk were mixed and incubated at room temperature, or in an ice bucket, for 10 min and then read for percentage rosette formation, rosettes were seen to have formed but the score was <50% of that observed after centrifugation. The formed rosettes were very stable

and resistant to disruption, and a period on ice following centrifugation was not required. Incubation of formed rosettes for 10 min at 37°C did, however, reduce the percentage rosettes.

Scoring of rosettes was carried out by counting viable cells under combined u.v. and phase contrast microscopy after the addition of fluorescein diacetate (Ramasamy, 1974) and more than 200 cells were counted in each determination.

2.4 *Antisera to C3 and C4*

The specific antisera to human C3 and C4 were kindly supplied by Professor P.J. Lachmann, M.R.C. Group on Mechanisms on Tumour Immunity, The Medical School, Cambridge. The anti-C3 reagent was whole rabbit antiserum which was heat decomplemented before use, and the anti-C4 reagent was the IgG fraction of a hyperimmune sheep serum separated by DEAE chromatography.

3. RESULTS

3.1 *Mo rosettes formed by normal mononuclear cells*

The PB mononuclear cells from 6 normal individuals were examined for Mo rosette formation. The results were 0 (2), 2 (1), 4 (2) and 5 (1)% rosette formation. In two cases, T cells were removed by centrifuging E rosette-forming cells through a Ficoll-Isopaque gradient and the residual non-T cells tested for Mo rosette formation. In both cases the percentage rosette formation of the T-depleted cells exceeded that of the whole mononuclear cell preparation (0% to 6% and 4% to 10% respectively). In another 2 cases, Mo rosette-forming cells were isolated by allowing bulk preparations of mononuclear cells to form Mo rosettes and isolating the rosettes through a Ficoll-Isopaque gradient. Cytochemistry confirmed the relative T-cell depletion in such preparations,

but the Mo-rosette positive cells were themselves cytochemically unremarkable and, after lysis of the Mo, they did not reform rosettes with fresh Mo.

The mononuclear cells from a normal spleen were tested for Mo rosette formation: 2% of cells formed Mo rosettes.

3.2 *Mo rosettes in leukaemia and lymphoma*

PB mononuclear cells from a variety of disorders were examined for Mo rosette formation. No increase, above normal, was noted in non-Hodgkin's or Hodgkin's lymphoma, prolymphocytic leukaemia, acute monocytic or acute meylomonocytic leukaemia, or in acute lymphoblastic leukaemia.

One unexpected, and unexplained, result was obtained in a study of Gaucher's disease. Gaucher's disease is one of a group of hereditary metabolic disorders in which reticuloendothelial cells accumulate glucocerebroside due to an enzyme defect (Goldstone et al, 1976). The disease has an autosomal recessive mode of inheritance with minimal manifestations in the carrier heterocygote. The large storage cells are mainly found in the bone marrow and spleen and are assumed to be of monocytic origin. When splenic mononuclear cells were examined from a patient with Gaucher's disease, 53% of the large Gaucher's cells were seen to form Mo rosettes. Ox erythrocytes did not adhere to these cells.

Thirty five days after splenectomy, PB from the patient (a girl of seven), her sister (who also has Gaucher's disease but has not had splenectomy), both parents, and a seven-year-old girl as a control was examined for a variety of surface receptors. The results are shown in Table 3.VIII, and it can be seen that the whole family, including the apparently unaffected heterozygous parents, had a marked decrease in T cells, and an accompanying increase in the percentage of cells forming Mo rosettes.

Table 3.VIII Peripheral blood markers in Gaucher's disease

Patient	% rosette formation by mononuclear cells			Esterase staining[+]	
	Mo	E	γFc	T cell	B cell
Propositus post splenectomy	14	40	58	45	34
Sister with Gaucher's disease	22	53	45	54	35
Father	22	11	69	ND	ND
Mother	25	27	59	ND	ND
[++]Normal 7-year-old girl	4	71	24	ND	ND

[+] Staining and interpretation by the method of Higgy et al, (1977)

[++] Tested at the same time with the same reagents

ND Not done

The lymphocytes from 14 cases of CLL were examined for Mo rosette formation. In all cases some rosettes were observed, but the percentage varied from case to case: mean 30%, range 4-63%, s.d. 18%. No particular surface marker particularly correlated with a high or low Mo rosette formation.

Sixteen cases of HCL were tested for Mo rosette formation, several on more than one occasion. Of these cases, 8 never expressed >5% rosette formation by PB mononuclear cells on any occasion of testing. Of the remaining cases, 3 were generally <5% but occasionally yielded Mo rosette formation up to 17%. More often than not, however, PB cells from these 3 cases were virtually Mo negative. Five cases yielded Mo rosettes as follows: KD, 20%; HD, 15%, 12%; LS, 17%; FW, 10%; RR, 11%.

Splenic cells from 6 cases of HCL were also tested for Mo rosette formation. The % scores were as follows: DC, 4; LP, 19; LH, 47; NW, 30; HR, 39; AB, 8. Thus the capacity for HCs to form Mo rosettes appears to be a feature of splenic, rather than PB, HCs. However, another feature of Mo rosette formation by HCs appears to be a correlation with the SIg phenotype. As will be presented in Chapter 4, SIg expression in HCL can be divided into types, arbitrarily numbered I, II and III. All but one of the patients never expressing Mo rosettes in the PB belong to SIg type I (IgG only), whereas all but one of the Mo-positive patients tested for SIg belong to SIg type II (multiple heavy chain isotype). The two patients with low Mo rosette expression by splenic HCs (DC and AB) belong to SIg type I, and the other patients with high splenic Mo rosette scores belong to SIg type II. The single exception to type I by PB cells never expressing Mo is patient LP, and it can be seen above that this SIg type II patient, although above normal, had rather poorer Mo rosette formation by splenic cells than the other three type II patients.

3.3 Cultured cells

After two weeks of culture, cells from the primary HC culture established from the spleen of patient HR (Mo rosettes, 39%) did not form any Mo rosettes. At this time, the cultured cells continued to express SIg, γFc, μFc and Mk receptors.

The established monoblastoid cell line (L230, Karpas et al, 1978a) formed 3%, weak, Mo rosettes. A B-cell line tested did not form Mo rosettes.

3.4 Cells forming Mk rosettes

Normal mononuclear cells Mononuclear cells from the PB from 7 normal volunteers was tested for Mk rosettes. A mean of 14% (range 2-22, SD 7%) rosette formation was obtained. The rosette-forming cells, in each case, resembled monocytes when cytocentrifuge preparations were stained with Giemsa.

B-cell leukaemias The results obtained for Mk rosette formation in a variety of B-cell leukaemias are given in Table 3.IX. The case of acute lymphoblastic leukaemia was of the non-T variety, and it is clear that some blasts form Mk rosettes. Most cases of CLL studied had a small percentage of leukaemic cells which rosetted with Mk, but only 1 case had a major proportion of cells forming Mk rosettes (42%), and this case is considered in further detail below. The case of Waldenstrom's-type mixed lymphoid-plasmacytoid proliferation, and the case of plasma-cell leukaemia given in Table 2.X, both make it clear that mature, immunoglobulin-secreting, lymphocytes and plasma cells can form Mk rosettes. However, although only these two cases are presented to illustrate this point because of the high proportion of pathological cells in the PB in both cases, a high percentage of Mk rosette-formation was observed in the PB and marrow of all of a wide

variety of myelomas studied for this marker. In some of these cases the plasma cell involvement was not high, and it was clear that in myeloma, myeloid precursors and monocytes, as well as a high proportion of PB lymphocytes, formed Mk rosettes.

Myeloid leukaemias Leukaemic myeloid cells at all stages of maturation from myeloblasts to neutrophils formed Mk rosettes. These results are shown in Table 3.X. The results indicate that in each of the conditions studied, the vast majority of leukaemic myelocytes present form Mk rosettes.

Leukaemias in remission Table 3.XI illustrates that in patients undergoing treatment for leukaemia and lymphoma, even when the PB appears to be returning to more normal cell proportions, the percentage of cells forming Mk rosettes remains high. In these cases, Mk rosettes are formed by morphologically normal T lymphocytes. In addition, a case of HD without morphological PB involvement was examined (Table 3.XI), and this too showed Mk rosette formation by T cells greatly in excess of the normal range.

Cultured cell lines Seven cell lines were examined for Mk rosette formation (Table 3.XII). It is clear that cultured B lymphoblasts, T lymphoblasts (although to a lesser extent), monoblasts and HCs all form Mk rosettes. Further, Mk rosette formation does not depend upon the Ia-like antigen, SIg, complement receptors or Epstein-Barr virus transformation.

HCL The cells from 20 patients with HCL were examined for Mk rosette formation, many on several occasions. The results of some of these studies are shown in Table 3.XIII. It can be seen that most HCs invariably formed Mk rosettes and that in some cases, but not always, a proportion of the T lymphocytes in HCL formed Mk rosettes. The firm

Table 3.IX Mk rosette formation by leukaemic B cells

case	Disease	Material[++]	Predominant cell type (%)	Mk rosettes (%)
1	Acute lymphoblastic leukaemia	PB	lymphoblast (>95)	20
		BM	lymphoblast (>95)	22
2	Prolymphocytic leukaemia	PB	prolymphocyte (>95)	3
3-14	Chronic lymphocytic leukaemia	PB	lymphocyte (>80)	Mean 17 (0-42; SD 13)
15	Mixed (Waldenstrom-type) proliferation with IgA paraprotein and leukaemic involvement	PB	lymphoid-plasmacytoid cells (80)	73
16	IgG plasma cell leukaemia	PB	plasma cell (70)	85

[++] PB = peripheral blood

BM = bone marrow

Table 3.X Mk rosette formation by leukaemic myeloid cells

Case	Disease	Material	Predominant cell type (%)	Mk rosettes (%)
1	Acute myeloid leukaemia	PB	myeloblast (>90)	70
		BM	myeloblast (>90)	95
2	Acute promyelocytic leukaemia	BM	promyelocyte (70)	73
3	Acute promyelocytic leukaemia	PB	promyelocyte (60)	72
4+	Acute monocytic leukaemia	PB	monoblast (70)	69
5	Chronic myelomonocytic leukaemia	PB	monocyte (60)	84
6	Chronic myelomonocytic leukaemia	PB	neutrophils and metamyelocytes (60), monocytes (20)	90
		BM	metamyelocytes and neutrophils (80)	85

+ Following 1 day of treatment

Table 3.XI Mk rosette formation by peripheral blood T cells in patients with leukaemia[++] and Hodgkin's disease

Case	Disease being treated	% rosette formation E[Δ]	% rosette formation Mk
1	Waldenstrom's macroglobulinaemia	48	72
2	Acute myelomonocytic leukaemia	88	42
3	IgM myeloma a) in remission	70	85
	b) in relapse[o]	42	92
4	Hodgkin's disease	36 → 66[*]	53

++ In each case the patient had received treatment and was not leukaemic at the time of testing

Δ T lymphocytes as measured by spontaneous sheep erythrocyte rosettes

o 36% cytoplasmic Ig^+ lymphoid/plasmacytoid cells present

* Increase in E rosettes after overnight culture in medium containing 10% foetal-calf serum

Table 3.XII Mk rosette formation by cultured human cell lines

Cell line	Nature of cells	Mk rosettes (%)	Cell phenotype++
Raji	B lymphoblasts	92-100	$EBNA^+$ CR^+ γFcR^+ SIg^-
150	B lymphoblasts	70	$EBNA^+$ CR^+ γFcR^- SIg^+
P2	B lymphoblasts	80	$EBNA^+$
241	B lymphoblast	79-83	$EBNA^-$ SIg^+
281	Primary hairy cell culture	92	$EBNA^-$ CR^- γFcR^+ μFcR^+ SIg^+ Ia^+
230	monoblasts	80-98	$EBNA^-$ SIg^- Ia^+
45	T lymphblasts	32-42	E^+ $EBNA^-$ CR^- SIg^- Ia^-

++ Constant features of the cell lines. EBNA, Epstein-Barr nuclear antigen; CR, receptors for the third components of fixed complement; γFcR, receptors for the Fc of IgG; μFcR, receptors for the Fc of IgM; SIg, surface membrane immunoglobulin; E, spontaneous sheep erythrocyte receptors; Ia, the antigen staining with an antiserum to the human Ia-like antigen complex, p29,34.

Table 3.XIII Mk rosette formation by PB cells in HCL

Patient	Date	% of mononuclear cells		
		Mk rosettes	T cells	HCs
KD	18. 1.78	95	5	91
PS	6. 2.78	36	52	47
MH	8.10.77	76	56	30
RH	29. 4.77	46	87	13
	30.10.77	52	76	24
DC	29. 4.77	40	94	6
	18. 5.77	43	85	15
	5. 8.77	2	88	10
	13.10.77	34	75	25
FB	17. 6.77	80	50	50
AD	23. 1.78	40	30	40
LH	18. 5.77	76	50	50
	30. 6.77	84	40	60
	6. 2.78	37	70	30
FW	23. 6.77	61	34	68
	19. 1.78	39	8	70
BN	16. 6.77	39	44	56
NW	17. 6.77	13	87	13
RR	4.10.77	90	44	56
HR	5.10.77	94	19	81
	26.10.77	85	22	78
AB	29. 9.77	77	54	46
	22.11.77	64	32	68
	23.11.77	91	0	98
GH	19.12.77	70	43	47
	24. 1.78	55	46	40
TN	15. 1.78	74	30	70

Table 3.XIII continued

Patient	Date	% of mononuclear cells		
		Mk rosettes	T cells	HCs
LK	18. 1.78	56	20	78
DA	2. 3.78	21	26	70
BC	14.12.77	40	54	40
CJ	5. 8.77	66	7	89

rosette formation between Mk and HCs which was generally observed is illustrated in Figure 3.5

3.6 Factors affecting Mk rosette formation and a possible complement

Chemical reagents When mononuclear cells freshly isolated from patients with CLL or HCL were treated with sodium azide (10^{-1}M) or cytochalasin B (20 µg/ml), which affects microfilaments, no effect on Mk rosette formation was observed. The chelating agent EDTA, on the other hand, markedly inhibited Mk rosette formation, even at 10^{-3}M. Neither pH or the suspending medium appeared to influence Mk rosette formation, but the EDTA tests were carried out in phosphate-buffered saline at pH 7.0.

C3 on the leucocyte surface membrane Deposits of C3 on the leucocyte membrane in CLL and HCL were sought using direct immunofluorescence with a rabbit anti-C3 serum. One case of CLL and both cases of HCL were shown to have membrane-associated C3 by this relatively insensitive method. Since $F(ab)_2$ fragments of antiserum were not used, γFc-binding of the reagent may be invoked as a possible reason for the positive results; however, this seems unlikely since the 2 cases of CLL which were negative with this reagent also had strong γFc receptors. As is shown in Table 3.XIV, the % cells staining with anti-C3 bore some relationship to the % cells forming Mk rosettes. Although these two tests give different %-positive cells, the two CLL cases negative for C3 were also negative for Mk rosettes.

Pretreatment of Mk with cobra venom factor (CVF) Erythrocytes were pretreated with CVF with and without serum, and in the presence or absence of EDTA as shown in Table 3.XV. Subsequent testing of the treated Mk revealed that blocking of the Mk immune adherence receptor (C3b receptor) with alternative pathway-activated complement considerably

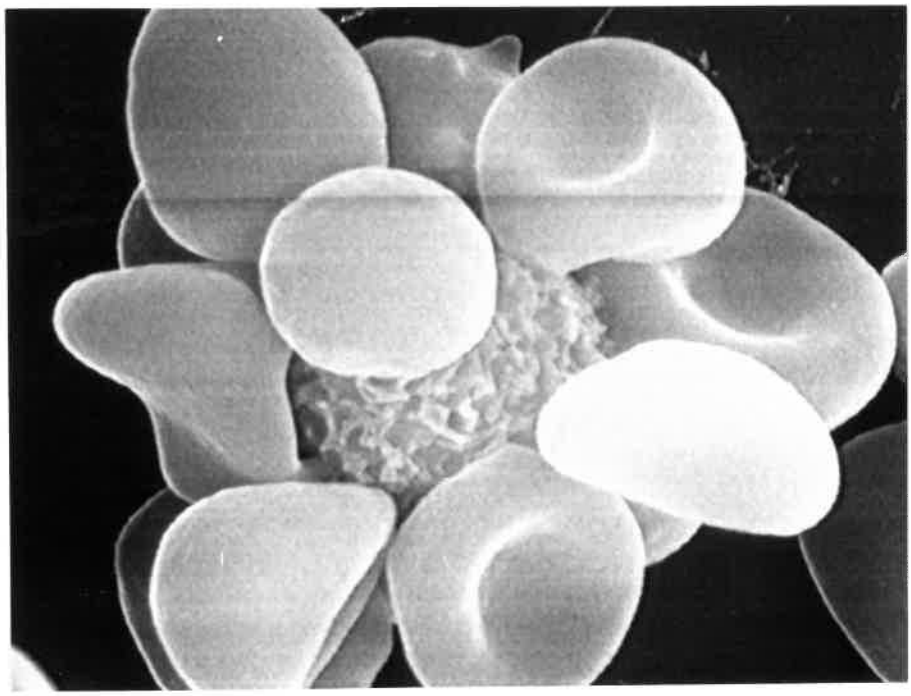

Figure 3.5 Rosette formation between a HC and Mk erythrocytes

Table 3.XIV The relationship between C3 staining and Mk rosette formation in CLL and HCL

Patient	T cells	B[++] cells		Anti-C3[*]	Mk
	E	Ia^+	SIg^+		
CLL	0	100	-[Δ]	0	0
CLL	8	95	89	94	42
CLL	<1	90	80	4	6
HCL(LH)	49	50	50	57	76
HCL(DC)	86	16	16	10	43

[++] Ia-positive and SIg-positive cells are given as an estimate of pathological cell involvement.

Ia^+ from cells staining with the anti-Ia-like antiserum to p29,34.

SIg^+ from cells forming rosettes with a single light chain type.

[*] Cells stained with a direct FITC-labelled technique using rabbit anti-C3 antiserum diluted 1/10.

[Δ] Not done

Table 3.XV The effect of treating Mk erythrocytes with cobra venom factor (CVF)$^{\Delta}$ on subsequent Mk rosette formation

Patient	Reagent*			Mk rosettes (%)
	CVF	EDTA	NHS	
HCL (LH)	-	-	-	76
	-	-	+	77
	+	-	+	35
	+	+	+	76
HCL (LH)	-	-	-	66
	-	-	+	66
	+	-	+	37
	+	+	+	68
	+	-	-	38
	+	+	-	65
HCL (DC)	-	-	-	43
	-	-	+	31
	+	-	+	15
	+	+	+	35
CLL	-	-	-	6
	-	-	+	5
	+	-	+	2
	+	+	+	4
CLL	-	-	-	42
	-	-	+	42
	+	-	+	11
	+	+	+	40
CLL	-	-	-	41
	-	-	+	40
	+	-	+	13
	+	+	+	37
	+	-	-	14
	+	+	-	42

$^{\Delta}$ 20 µl packed erythrocytes incubated with 50 µl CVF in CFT buffer with or without serum (0.2 ml) for 20 min at 37°C. In some cases 10 mM EDTA was added and PBS replaced the CFT buffer. The Mk were then washed and rosette tested in the usual way.

* NHS = normal human serum

reduced Mk rosette formation by HCL and CLL cells. Blocking by CVF alone, in the absence of serum, indicated that the Mk immune adherence receptor binds CVF (cobra C3b). Also, since EDTA blocked this binding reaction, as well as C3b binding by Mk in the presence of serum, it would appear that Mg^{++} and/or Ca^{++} ions are required for immune adherence binding by Mk.

Pretreatment of cultured cells with anti-complement components Cells from various cultured lines were treated with antisera to C3 or C4, or both, in attempts to block Mk rosette formation (Table 3.XVI). Pretreatment of Mk with these reagents did not affect rosette formation. The results show that high concentrations of either anti-C3 or anti-C4 do block Mk rosette formation to some extent, but not completely. A combination of anti-C3 + anti-C4 was more effective at blocking than either of the antisera alone, although at the dilutions used complete inhibition of rosette formation was not obtained. Higher concentrations of the antisera tended to agglutinate the leucocytes. The results also show that the T-cell line 45 (Table 3.XVI) produces some C3 and, or, C4 although less than the other lines. Some lines appear to produce proportionally more C4 to C3 (line P2) than others (Raji, 241). In a control experiment, an antiserum to hairy cells, rendered specific by absorption (Section IV) did not block Mk rosette formation by hairy cells when used at a concentration which strongly stained the cells when tested by indirect immunofluorescence.

Detection of complement deposited by immune complex formation A different approach was employed both to show that Mk rosette formation was detecting deposited complement and to demonstrate the flexibility and potential use of this technique. In preliminary experiments (Table 3.XVII) complement was activated by immune complex formation

Table 3.XVI The effect of pretreatment of cultured leucocytes with anti-complement antibodies on subsequent Mk rosette formation

Cell line	Treatment*	Mk rosettes (%)
Raji	-	96
	Anti-C3 (1/2)	46
	Anti-C3 (1/5)	68
	Anti-C3 (1/10)	82
	Anti-C4 (1/2)	74
	Anti-C4 (1/10)	82
	Anti C3 + C4 (1/10)	74
P2	-	80
	Anti-C3 (1/5)	43
	Anti-C4 (1/5)	55
	Anti C3 + C4 (1/10)	34
241	-	76
	Anti-C3 (1/5)	54
	Anti-C4 (1/5)	79
	Anti C3 + C4 (1/10)	69
45	-	32
	Anti C3 + C4 (1/10)	13

* Washed cells were incubated with antiserum at the dilution indicated for 30 min at room temperature with occasional mixing. The cells were then washed and tested for Mk rosette formation.

Table 3.XVII The use of Mk rosettes for the detection of immune complex formation between Ia-like antigens and monospecific anti-p29,34 antiserum

Treatment*				Mk rosettes (%)
anti-Ia	+ complement	anti-C3	anti-C4	
-	-	-	-	13
+	-	-	-	15
-	+	-	-	12
+	+ (heat treated)	-	-	15
+	+	-	-	27
+	+	+	-	5
+	+	-	+	19

* Normal human lymphocytes were incubated (30 min at room temperature) with (a) anti-p29,34 serum (at 1/100), and washed. They were then incubated (b) with zymosan-absorbed normal human serum (1/10) (5 min at 37°C) as a source of complement. (c) Some tests were then incubated with either anti-human C3 or C4 serum and washed. Control experiments were incubated with steps (a) or (b) alone.

between normal blood lymphocytes and an antiserum to the Ia-like antigen, p29,34. The dilution of antiserum used was outwith the plateau region of this serum when used in indirect fluorescent studies. As can be seen from the table, cells bearing the Ia-like antigen were readily detected by Mk rosette formation and this reaction could be inhibited by antiserum to C3.

4. DISCUSSION

Spontaneous rosette formation with mouse erythrocytes is a marker for a subpopulation of B lymphocytes (Siegal and Good, 1977) and the capacity of HCs to form Mo rosettes has been used as evidence for the B-cell origin of HCL (Catovsky et al, 1975). In this report it is shown that Mo rosette formation is, by no means, a consistent feature of HCs. The rosette test used here is no less sensitive than that of Catovsky et al since a generally higher percentage rosette formation was obtained in CLL than was obtained by these workers. Also, of the only two other studies of Mo rosette formation by HCs which has been reported, one group found them to be negative (Matre et al, 1977), and the other study obtained one case which was negative and two which were positive (Rozenszajn et al, 1976).

These apparently conflicting results are resolved by the demonstration here that Mo rosette formation may depend upon the SIg phenotype. Siegal and Good (1977) have reported that all normal mouse rosetting cells have SIgM and SIgD, and the findings here suggest that this may also be substantially true in leukaemias. Thus, most CLL cells have both SIgM and SIgD and are Mo positive, whereas, with few exceptions, SIgG cases of HCL are Mo negative. Cases of HCL in which the cells bear

SIgD in addition to SIgG (multiple isotype group, Chapter 4) are, in general, Mo positive. The reason why splenic HCs are much more Mo positive than their PB counterparts is not at all clear, but the results suggest that a factor provided by the spleen enhances Mo rosette formation; whether this is also true of CLL cells, and whether it suggests that Mo-positive cells have recently circulated through the spleen, awaits further study.

The results presented in this section show, for the first time, that HCs have a 'receptor' for monkey erythrocytes. They also suggest that previous reports (Lohrmann and Novikovs, 1974; Pellegrino et al, 1975a) suggesting that Mk rosettes are markers for particular cell types are wrong, and that, contrary to one opinion (Siegal and Good, 1977), Mk rosette formation does not depend upon the species of monkey used.

Thus, in this study it is shown that Mk rosette formation with human leucocytes is probably a function of the Mk IA receptor (Cooper, 1969) detecting complement components on the leucocyte membrane. By blocking the Mk IA receptor with CVF, rosette formation with leukaemic cells from HCL and CLL was considerably reduced, and treating cultured lymphoblastoid cells with antisera to complement components also reduced Mk rosette formation. Mk rosette formation by HCs, on the other hand, was not reduced by treatment with an anti-hairy cell serum (Section IV, Chapter 3) indicating that blocking by antisera was not simply steric hindrance. Morever, *in vitro* complement deposition by immune complex formation with normal human lymphocytes enhanced Mk rosette formation and this could be inhibited with antiserum to C3.

This being so, it is of interest that the incidence of membrane associated complement on human leukaemic cells is much higher than has

been previously suspected. Several mechanisms can be envisaged for the presence of complement on leukaemic leucocytes. The anti-lymphocytes antibodies known to be present in some malignancies including Hodgkin's disease (Siegal, 1976), CLL, and chronic myeloid leukaemia (Metzgar et al, 1975) might lead to complement activation leaving C3 deposited on the cell membrane. Also, the high incidence of Mk rosettes with the macroglobulinaemia cells and in myeloma suggest a role for immune complex formation since, in many cases, the paraproteins of these diseases have autoantibody activity (Seligmann and Brouet, 1973). The process of complement deposition would not necessarily lyse the leukaemic cells since human lymphocytes are resistant to cytolysis and CLL cells, at least, are particularly resistant (Logue and Huang, 1978). Passive uptake of serum C3 seems unlikely since many of the leukaemic cells investigated, including myeloblasts (Ross et al, 1978), HCs (Burns et al, 1977c), most acute lymphoblastic leukaemia cells (Tsubata et al, 1977) and myeloma plasma cells (McConnell, 1975) all lack complement receptors, although it is possible that these cell types have picked up serum C4 either via the C4 receptor (Bokisch and Sobel, 1974) or even attached to the C4-binding protein recently described in man (Scharfstein et al, 1978).

Human lymphoblastoid cell lines can activate the alternative pathway of complement when incubated in autologous serum (Okada and Baba, 1974; Budzko et al, 1976), and complement deposition on the lymphoblasts does not depend on the presence of a C3 receptor. Although this method of complement activation has only been demonstrated with cultured cells, it is possible that in some types of leukaemia alterations to the cell membrane during transformation could cause *in vivo* alternative pathway activation. However, CLL cells do not activate

complement in this way (Budzko et al, 1976) and also Epstein-Barr virus infection is probably associated with this type of transformation (McConnell et al, 1978). With this type of complement activation, C3 deposition can be detected by the IA receptor of human group O erythrocytes suggesting that rather more complement is deposited (see below). It is of interest that in infectious mononucleosis, which is associated with Epstein-Barr virus-infected B cells, an increase in spontaneous autorosette (so-called H-rosette formation) has been recorded (Sheldon and Holborow, 1975).

The above mechanisms cannot account for Mk rosette formation by cultured cells, and in these cases it is possible that Mk rosettes are detecting C3 and C4 synthesised by the rosette-forming cells. It has been suggested that C4 is synthesised by human lymphoblastoid cell lines, including Raji (Ferrone et al, 1976), and human monocytes, both normal and leukaemic, can synthesise C3 (Lai A Fat and Furth, 1975; Einstein et al, 1977). The present section suggests that a variety of cell lines can produce both C3 and C4, and that the cultured cells from HCL can manufacture complement components. The detection of complement synthesised by cells also accounts for the reported linkage between Mk rosette formation and the expression of HLA antigens in man-mouse hybrid cells (Pellegrino et al, 1975b; Fellous et al, 1977) in view of the association between HLA and C4 production (Jersild et al, 1976) and of the close spacial association of C4 with HLA determinants on the cell membrane (Ferrone et al, 1976). Also, the apparent synteny between Mk rosette formation and the P blood group system (Fellous et al, 1977) might be tied in with the association between C3 gene expression and certain other human red cell blood groups (Jersild et al, 1976).

The detection of minute quantities of complement demands that the

IA receptor on Mk be very sensitive, more sensitive than the receptor on human group O erythrocytes which are IA negative with untreated Raji cells (Theofilopoulos et al, 1974; Budzko et al, 1976) and with most human leukaemic cells (Sheldon and Holborow, 1975). The measurement of C4 on Raji cells by sensitive absorptive methods (Ferrone et al, 1976) gives an estimate of 487 molecules of C4 per cell, and the IA receptor on human erythrocytes (Hu) requires about 2,500 molecules per cell for adherence (Cooper, 1967). The Mk IA receptor appears, therefore, to be about five times more sensitive than its human counterpart. This report also shows other differences between the IA receptor on Mk and Hu. EDTA abolishes IA rosette formation, and the binding of fluid-phase C3b or CVF, by Mk but not by Hu (Cooper, 1967). A similar difference in cation requirement between the IA receptors of monocytes and polymorphonuclear leucocytes and those of lymphocytes has been observed by Lay and Nussenzweig (1968). Again, the Mk IA receptor clearly binds CVF whereas the Hu receptor does not (McConnell and Lachmann, 1976). CVF is the cobra C3b (Alper and Balavitch, 1976) and it is therefore not surprising that this reagent can bind to Mk but not Hu since such variations in the binding of xenogeneic C3b by IA are frequent. Thus, mouse C3b binds human IA receptors (Ross and Polley, 1976) but fixed rabbit C3b binds poorly (Pepys, 1976). Human C3b, on the other hand, does not bind to mouse IA receptors, also, human CLL cells possess weak IA binding with CVF (McConnell and Lachmann, 1976).

Finally, mention should be made of the possible value of these observations on Mk rosette formation. Rosette formation with Mk provides an exquisitely sensitive and very simple tool for the detection of immune complex formation and deposition of complement in, for example, the leukaemias. The method does not depend upon metabolic

activity of the leucocyte, or even apparently leucocyte viability, and might be used to detect complement deposition in tissue sections. Since γFc-binding of antiserum by leucocytes does not effect complement activation, the method might also be used as an alternative to fluorescent staining in some situations.

IV T lymphocytes and B lymphocytes in HCL

1. INTRODUCTION

Except for two reported cases (Burns et al, 1977d; Saxon et al, 1978a) HCs do not form spontaneous sheep erythrocyte (E) rosettes. E-rosette formation is therefore a reliable indicator of T-cell proportions in HCL, as in normal blood (Jondal et al, 1972), and the use of AET-treated E (Kaplan and Clark, 1974) avoids the problems of weakly-avid T cells occasionally found in chronic leukaemias (Nath et al, 1975; Wenzel and Ichiki, 1977).

Until recently, T cells had not been subclassified because of the paucity of markers, but Moretta et al, (1977b) divided these cells into T_M and T_G depending on whether the cultured T cells had receptors for IgM or IgG respectively. T_M cells are probably helper T cells and T_G cells suppressor cells, and there is a small population of T cells with neither receptor, T null, of unknown function. Imbalances in the distribution of circulating T_M and T_G cells occur in patients with both congenital and acquired immunodeficiencies (Cooper and Seligmann, 1977), and because of the high incidence of infectious disease in HCL (Katayama and Finkel, 1974; Bouza et al, 1978) it was of interest to study T-cell subpopulations in this disorder.

Increasingly, it is being recognised the B-cell neoplasms are characterised by an almost complete replacement of the normal B-cell series by the monoclonal proliferation. Frequently, this replacement is only detectable immunologically since cells involved in the monoclonal proliferation are morphologically normal in such diseases as myeloma (Mellstedt et al, 1974), Waldenstrom's macroglobulinaemia (Preud'homme

and Seligmann, 1972) and non-Hodgkin's lymphoma (Garrett et al, 1977). In this section evidence is presented that the same process occurs in HCL.

2. MATERIALS AND METHODS

2.1 *Patients*

Details of the patients have been given in Chapter 2. In HCL, the proportions of HCs in the PB varies widely and on some occasions virtually no HCs can be morphologically identified. This phenomenon has been described as spontaneous remission (Bouroncle, 1978), but immunological evidence (Chapter 4) reveals that there are always some HCs persisting in the PB. Also, bone marrow biopsy carried out at the time of apparent remission clearly reveals abundant HC involvement (J.C. Cawley, personal communication). However, for the purpose of T cell evaluation in HCL, patients were selected who had undergone apparent remission and in those cases the percentage T cells was very high.

2.2 *T_M and T_G cells*

Isolated mononuclear cells were tested for the proportions of T_M and T_G by the method of Moretta et al, (1975). Briefly, the cells were cultured overnight in tissue culture medium (TC 199) with 10% foetal calf serum. After this time the cells forming spontaneous E, EA_M and EA_G rosettes were counted. The reagents for EA_M and EA_G were the same as described previously. On some occasions SIg^+ B cells (and HCs) were first removed by rosette formation with anti-light chain-coupled red cells (Ling et al, 1977) followed by Ficoll-Isopaque centrifugation.

2.3 Anti-hairy-cell serum (AHS)

Details of the preparation of this serum have been given in Chapter 2. HCs staining with this AHS were scored by indirect fluorescence with FITC-coupled goat anti-rabbit IgG serum (Nordic pharmaceuticals). Positive cells were also examined at the ultrastructural level with either peroxidase- or ferritin-conjugated goat anti-rabbit IgG antiserum (Nordic pharmaceuticals). In all cases the control was preimmunisation serum from the same rabbits.

2.4 Surface markers and electron microscopy

The surface marker tests and the processing for electron microscopy were carried out as described previously.

2.5 Esterase cytochemistry

For cytochemical studies either air-dried smears of PB buffy coats or cytocentrifuge preparations of mononuclear cells were used. The method of Higgy et al, (1977, 1978) with α-napthyl butyrate was employed. Fresh air-dried preparations were fixed in formalin vapour for 4 min, washed, and exposed to α-napthyl butyrate (pH 8.0) in low concentration and Fast blue BBN as a diazonium salt capture agent for 15-30 min. The slides were then washed briefly in distilled water and counterstained in haematoxylin for 5 min. This method stains T cells with a dense localised positivity made up of 1 to 4 course granules (Higgy et al, 1977) while B cells contain no activity. In contrast, HCs often show a highly distinctive pattern of reaction positivity which is not inhibited by fluoride; this positivity is readily distinguished from that of monocytes where the strong granular positivity is scattered throughout the cytoplasm and is inhibited by sodium fluoride (Higgy et al, 1978). The distinctive HC esterase pattern is not observed in a wide variety of other pathological states (Higgy et al, 1978).

3. RESULTS

While examining the surface marker characteristics of HCs, it was often observed that the sum of the % cells forming γFc rosettes and those forming E rosettes exceeded 100%. Some examples are shown in Table 3.XVIII. In these cases, the morphological HCs were not forming E rosettes and this suggested that possibly some T_G cells were expressing the γFc receptor even without culture.

3.1 T_M and T_G cells in HCL

The cells from 3 patients with HCL and from 4 normal volunteers were cultured overnight in TC 199 and then tested for EA_M, EA_G and E rosette formation. The findings are given in Table 3.XIX. The results are difficult to interpret since HCs, almost invariably, have receptors for both EA_M and EA_G and therefore the contribution made by T cells cannot be readily assessed. The results from patient NW (who had a very high proportion of T cells at this time) however, suggest that most of his T cells had receptors for neither IgG nor IgM but belonged to the T null subgroup.

To resolve this question of T cell contribution to EA_M and EA_G rosette formation, the mononuclear cells from the PB of 2 of the cases were enriched for T cells. HCs possess monoclonal SIg (Chapter 4), and thus it was possible to remove HCs by allowing them to form SIg rosettes and then removing the rosetted cells by centrifugation over Ficoll-Isopaque. The T cells remaining on the interface were then cultured overnight, as previously, and tested for rosette formation. This test was carried out on 2 of the same patients as previously, but on different dates. The results obtained (Table 3.XX) show that patient DC - who appeared to possess T_M and T_G cells on the previous occasion

Table 3.XVIII The sum of % rosette formation with EA_G and E by mononuclear cells from cases of HCL, without culture

Patient	% rosette formation		sum
	E	EA_G	
FB	50	82	132
AD	31	82	113
LP (spleen)	18*	94	112
RR	60	63	123
HR	10	95	105
AB	54	64	118
TN	30	92	122

* The % mononuclear cells staining with a specific anti-T cell serum (Chapter 5) was 21.

Table 3.XIX The effect of culture on E, EA_M and EA_G rosette formation by the PB mononuclear cells by HCL

	Patient	% rosette formation		
		E	EA_M	EA_G
Before culture	DC	85	24	23
After culture		89	58	35
Before culture	LH	49	24	79
After culture		54	45	65
Before culture	NW	87	12	15
After culture		NT+	12	16
Before culture	Normal$^{\Delta}$	63	3	28
After culture		58	32	27

+ NT, not tested

Δ Mean value of 4 individuals

Table 3.XIX The effect of culture on E, EA_M and EA_G rosette formation by the PB mononuclear cells by HCL

	Patient	% rosette formation		
		E	EA_M	EA_G
Before culture	DC	85	24	23
After culture		89	58	35
Before culture	LH	49	24	79
After culture		54	45	65
Before culture	NW	87	12	15
After culture		NT+	12	16
Before culture	Normal$^{\Delta}$	63	3	28
After culture		58	32	27

+ NT, not tested

Δ Mean value of 4 individuals

of testing (Table 3.XIX) - now had no T_M cells and only a proportion of T_G cells. Like patient NW (Table XIX), most of DC's T cells appeared to belong to the T null subgroup. Patient LH, on the other had, showed about normal T_M cell proportions, but greatly elevated T_G cells.

A different method of T-cell evaluation is by esterase staining. However, this method probably only detects T_M cells and the results obtained from 5 patients with HCL (Table 3.XXI) make it clear that the proportions of T_M cells are often reduced in this disease.

3.2 *Normal B lymphocytes*

Romanowsky morphology Typical HCs were identified by the presence of prominent cytoplasmic hairs and an eccentrically-placed nucleus which contained largely non-condensed chromatin and frequent single nucleoli. Cells were also identified as HCs when they contained the typical nucleus and possessed the characteristic slate-blue cytoplasm, even when the surface hairs were inconspicuous. The percentage of mononuclear cells scored as HCs using these criteria are listed in Table 3.XXII.

Electron microscopy In addition to typical HCs (Figure 3.8), a variable percentage of cells was observed which in many respects resembled small nature lymphoblasts (Figure 3.6), but were seen to contain the ribosome lamellar complex which has come to be regarded as largely diagnostic of HCs (Katayama et al, 1972a; Catovsky, 1977). Cells of this type were particularly prominent in patient WH (Table 3.XXII). Since the ribosome lamellar complex has not been found in normal lymphocytes, cells containing it are likely to be involved in the leukaemic process.

Esterase cytochemistry The percentage of cells from a number of

Table 3.XX E, EA_M and EA_G rosette formation by negatively enriched T cells from 2 cases of HCL before and after culture

	Patient	% rosette formation		
		E	EA_M	EA_G
Before culture	DC	92	0*	16
After culture$^{\Delta}$		75	0	22
Before culture	LH	96	0	28
After culture$^{\Delta}$		96	43	47

* The same EA_M reagent formed rosettes with HCs before T-cell enrichment.

$^{\Delta}$ Results for EA_M and EA_G were comparable after 12 h, 18 h and 24 h of culture.

Table 3.XXI Comparison of % T cells by E rosette formation and esterase pattern

Patient	T cells	
	E rosette	Esterase
PS	42	10
MH	36	20
HD	24	10
FB	44	25
FW	45	20
Normal	63[+]	67[++]

[+] Mean value of 4 normal individuals

[++] Mean value of 11 normal individuals

Table 3.XXII Studies of PB leucocytes in HCL

Patient	Total WBC count ($\times 10^3/mm^3$)	% of total mononuclear cells								
		HC numbers by different methods				Normal B lymphocytes			T cells	Monocytes
		Romanowsky morphology	EA_M	esterase	SIg	Y-C3b	esterase	SIg	E	Esterase
DC	2.5-3.4	40	40	41	ND	<1	16	ND	ND	0
HD	3.8-5.5	25	20	89	ND	2	5	ND	24	0
WH	3.0-5.2	7	23	65	55(K)	ND	15	0(λ)	36	0
PS	2.8-5.9	35	30	88	62(λ)	0	2	4(K)	42	0
FW	3.3-7.2	24	52	79	57(λ)	ND	1	0(K)	45	<1
FB	3.0-4.0	17	37	67	57(K)	ND	7	2(λ)	44	0
LP	10.0-21.0	60	78	88	70(λ)	ND	2	3(K)	17	0
KD	10.1-14.6	49	51	55	85(λ)	ND	9	0(K)	15	0
AD	5.8-10.0	60	71	ND	66(λ)	<1	ND	<1(K)	18	ND

ND = not done

cases of HCL which showed the distinctive HC esterase pattern (Figure 3.7) is given in Table 3.XXII. It can be seen that this percentage generally exceeded considerably the percentage of cells with typical HCs Romanowsky morphology. This finding suggests that many cells with apparently normal lymphocyte morphology, in fact, belong to the leukaemic clone.

On the other hand, whereas in most cases virtually all the morphologically recognisable HCs, as well as some morphological lymphocytes, showed the HC esterase pattern, in some cases, notably patients DC and WH, morphologically obvious HCs could be found with negative esterase reaction (Figure 3.7). Such cells are recorded under 'Normal B lymphocytes' in Table 3.XXII since B lymphocytes are characteristically negative with this esterase stain.

AHS The absorptions used during the preparation of the AHS have been described in Chapter 2. Before absorption, the serum was exceedingly potent, and stained the HCs from a variety of patients with HCL, in an indirect fluorescent technique, to a dilution of 1/1600. However, after extensive adsorption with a variety of tissues, specific immunofluorescent staining was something of a compromise. Normal PB and splenic (also 1 spleen from a patient with Hodgkin's disease and 1 from a patient with Gaucher's disease) lymphocytes, erythrocytes and most polymorphs completely failed to stain, even at a dilution of 1/5. The end of the plateau region of staining in cases of HCL varied, but in some cases it was about 1/15, in other cases the cells stained at a dilution of 1/100. At a dilution of 1/10, however, the cells from some cases of CLL stained weakly, and the monocytes from a case of acute monocytic leukaemia and from a case of acute myelomonocytic leukaemia also stained weakly at this dilution. At the time of testing the AHS,

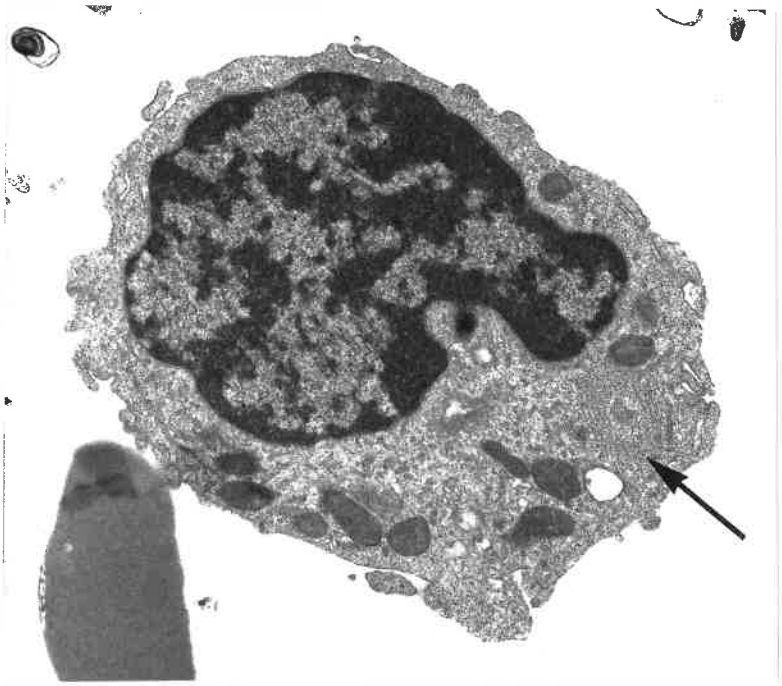

Figure 3.6 (x 11,400) This cell is not readily distinguishable from a normal lymphocyte - there are no typical surface projections and the nucleus contains moderately heavy peripheral chromatin condensation. However, the presence of a ribosome lamellar complex (sectioned largely transversely) indicates that the cell is involved in the leukaemic proliferation.

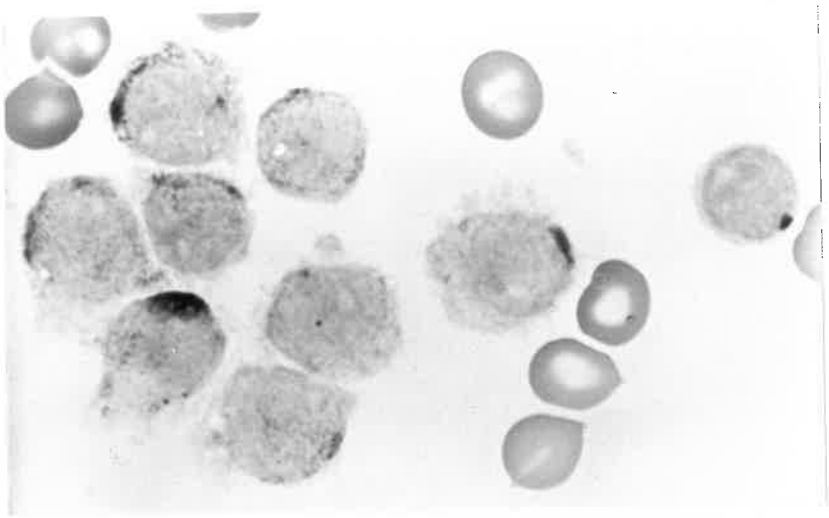

Figure 3.7 HCL, PB buffy coat preparation; α-napthyl butyrate esterase. A group of 8 HCs are seen together with a single mature small lymphocyte. Seven of the HCs show the distinctive reaction pattern with a mixture of fine scattered granule together, in some instances, with coarser granules located in a crescentic configuration. A single HC is virtually negative. The small lymphocyte shows a single block of coarse positivity which is typical of normal T lymphocytes.

$F(ab)_2$ fragments had not been made, and it was considered that γFc binding might be causing non-specific staining. Acetone-fixed cells from a case of CLL and from a case of chronic myelomonocytic leukaemia CMML were then tested, in parallel with cells from cells from a case of HCL which had been stored as a cytocentrifuge preparation at -20°C for 6 months. Some 10% of the CMML cells stained faintly at a 1/10 dilution of serum, and 5% of CLL cells stained quite strongly at this dilution; 73% of the mononuclear cells from the case of HCL stained with a strong membrane and cytoplasmic fluorescence.

Some of the blocking studies with this serum are of passing interest. The exquisite sensitivity of the μFc receptor (and of the apparent C3 receptor) of HCs to blocking by AHS has been described in a previous section. However, EA_M rosette formation by T cells was not blocked by a 1/100 dilution of serum (HCs were regularly blocked to a 1/400 dilution), and in a case of CLL tested with AHS at 1/10, EA_M rosette formation was reduced from 55% to 22% by this treatment. A 1/25 dilution of serum did not block E rosette formation by normal T cells, nor did it block SIg rosette formation (Chapter 4) or EA_G rosette formation by HCs. Mouse rosette formation by splenic HCs, from one case of HCL tested, was only partly blocked by AHS at a dilution of 1/25.

Nevertheless, because normal leucocytes do not stain with the absorbed AHS, it was considered a valuable tool for the estimation of HC numbers within a given case of HCL. This is particularly true because of the virtual absence of monocytes in HCL (confirmed by the esterase staining in Table 3.XXII). The results obtained when percentage scores were made in 3 patients are given in Table 3.XXIII, and the results in this table extend the general theme that normal B cells

Table 3.XXIII Staining of HCs by the AHS

and the correlation with other surface markers

Patient	AHS dilution	% stain	T cells			
			Anti-T*	E	γFc	SIg*
DC$^{\Delta}$	1/100	23	ND	83	12	8
AD	1/15	80	24	31	82	ND
PS	1/10^{++}	73	ND	42	73	73

+ The anti-T cell serum (Chapter 5) was used in an indirect fluorescent method.

* The % cells rosetting with a single light chain type of anti Ig.

Δ This patient had 10% plasmacytoid cells in his mononuclear cell preparation.

++ Acetone-fixed slide used.

ND Not done on this date

are virtually absent from the PB of patients with HCL. Also, of special interest are the results from patient DC. At the time of study, this patient had an infection and some 10% of his PB cells had a transformed, plasmacytoid, appearance. These cells did not possess any of the surface markers examined, but they did stain with the AHS.

Figure 3.8 illustrates a hairy cell stained with the AHS at EM level by the 2 methods used. Non-specific staining was more of a problem at the EM level than it was by light microscopy.

Other marker studies The extra data added to Table 3.XXII: SIg; Yeast C3b (Y-C3b); EA_M; and E rosettes are taken from the results given in other chapters.

The SIg results tabulated refer only to the light chain findings with a rosette method (Chapter 4), but the percentage of heavy chain rosettes (not tabulated) closely paralleled the percentage rosettes obtained for the predominant light chain type, although the class varied from case to case.

In the 5 cases tested for SIg by immunofluorescence the percentage of mononuclear cells staining either for the predominant light chain type or for the particular heavy chain class present, in each case closely approximated to that found by the rosette method. In contrast, by immunofluorescence, no cells bearing the non-predominant light chain type, which presumably reflect normal B cell numbers, were detected.

In the 4 cases tested for complement receptors by the Yeast-C3b method (Table 3.XXII), virtually no cells bearing this receptor were observed, although this method detects normal C3b-receptor-bearing lymphocytes (Section II).

As can be seen from Table 3.XXII, the sum of cells forming E and

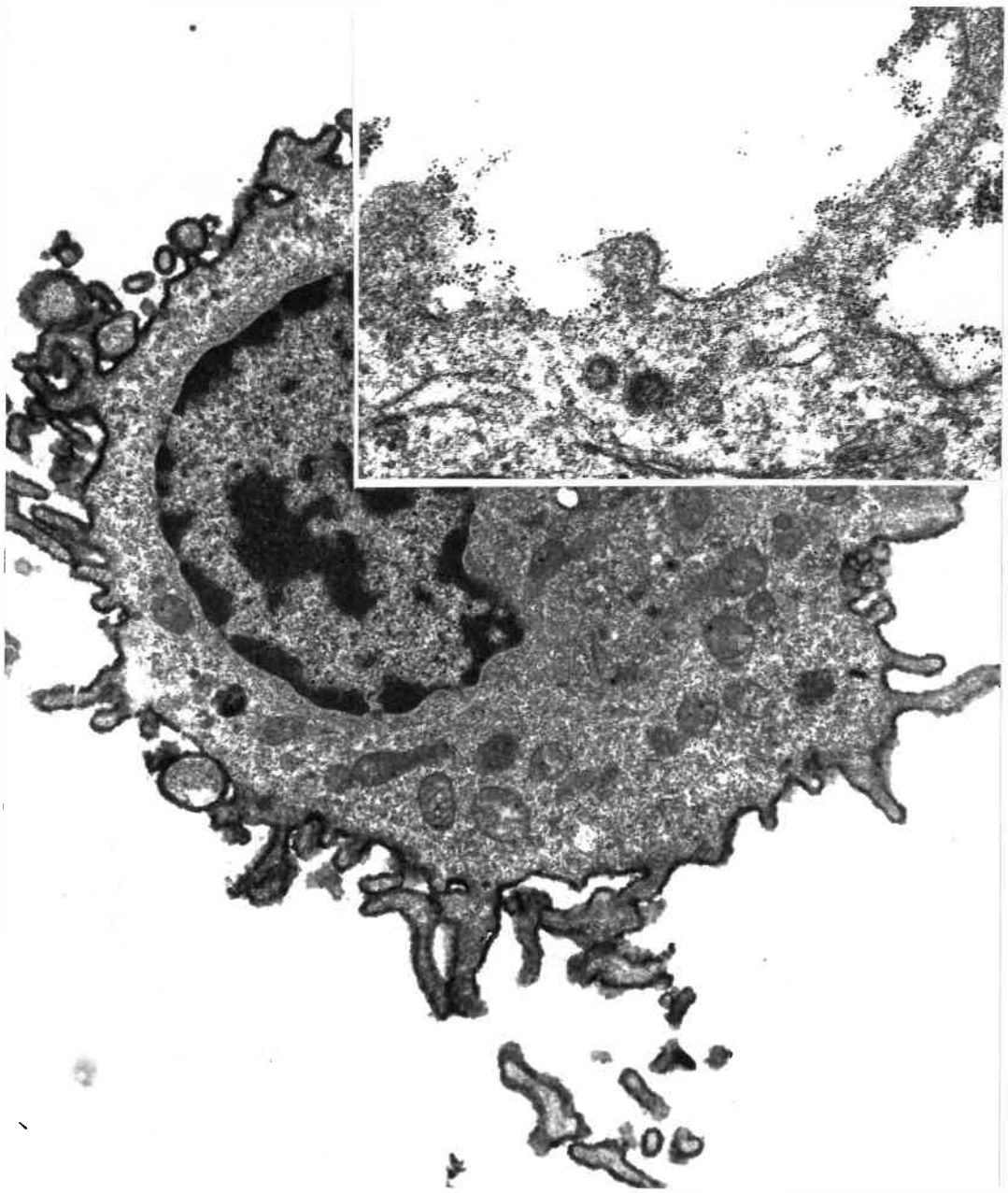

Figure 3.8 Staining with AHS; immunoelectronmicroscopy with an indirect method using ferritin- (inset) and peroxidase-labelled antibody (x 10,000; inset x 22,000). This typical HC shows strong peroxidase activity at its surface indicating the presence there of antibody. In the inset, the presence of AHS is shown by the alternative ferritin method.

monoclonal SIg rosettes approximated to 100%.

4. DISCUSSION

The limited studies of T cell subpopulation in HCL reported in this section suggest that there are imbalances in the T_M, T_G and T null populations. The techniques used to identify T_M and T_G cells cannot account for this imbalance (particularly the reduced proportion of T_M cells) since the values obtained with normal mononuclear cells with the same reagents closely agree with the findings of others (Moretta et al, 1975; McConnell and Hurd, 1976). Moreover, comparison of the percentage of E-rosette forming T cells with the value obtained for T cells by esterase staining, shows that the latter method identifies far fewer T cells in HCL. The identification of T cells by the single block pattern obtained with non-specific esterase stains was independently reported from several laboratories (Ranki et al, 1976; Sher et al, 1976; Higgy et al, 1977; Kulenkampff et al, 1977). In normal blood the difference in percentage scores between this method and E rosette formation for the identification of T cells is small (Ranki et al, 1976; Higgy et al, 1977), but recently Grossi et al, (1978) have shown that this small difference is significant since only T_M, and not T_G, cells have the characteristic T-cell esterase pattern. Thus the esterase results obtained here in HCL probably support the surface marker data in showing a reduced T_M cell subpopulation in this disease. The significance of this finding, however, awaits further study.

Evidence will be presented in the next chapter that HCs bear monoclonal SIg, and HCL can therefore be classified as a B-cell neoplasm. In common with other B-cell proliferations (Mellstedt et al,

1974; Preud 'homme and Seligmann, 1972), it is shown here that the proliferative monoclonal cells of HCL almost completely replace the normal circulating B cell component. Thus, although the majority of cases of HCL had less than 50% morphologically identifiable HCs, several other pieces of evidence indicated that many more circulating lymphoid cells were involved in the leukaemic process. For example, electron microscopy revealed that ribosome-lamellar complexes, in addition to being present in HCs, were also observed in cells that would otherwise be regarded as normal lymphocytes. EA_M rosette formation, without culture, frequently suggested the presence of a higher number of abnormal cells than was apparent by simple morphology. Also, both the esterase pattern defined as specific for HCs (Higgy et al, 1978), and the limited serological studies using the anti-hairy-cell serum, again revealed many more pathological cells than were immediately apparent. Finally, the SIg results further substantiated this theme, since the percentage of cells being the same light chain type as the morphologically unequivocal HCs was in close agreement with the number of leukaemic cells by other methods.

As a corollary to this concept, the results presented here demonstrated a profound depression of circulating normal B lymphocytes in HCL. The evidence supporting this conclusion includes: the very low numbers of cells bearing SIg of the light chain not involved in the monoclonal proliferation; the exceedingly small percentage of cells forming rosettes with Yeast-C3b - since normal B lymphocytes have a receptor for C3b (Bianco et al, 1970); and the generally small numbers of cells showing the lack of esterase activity typical of normal B cells (Higgy et al, 1977).

The number of cells bearing SIg light chains of the type not

involved in the leukaemic process was strikingly low, even when a highly sensitive rosette method was employed (Ling et al, 1977; Haegert et al, 1978). With the traditional direct immunofluorescent method, no cells bearing the non-predominant light chain were demonstrable: this observation is in accord with previous reports where this finding was recorded but not commented upon (Fu et al, 1974; Haegert et al, 1974; Deegan et al, 1976).

The esterase evidence, however, was weakened in a number of cases by a variable number of morphological HCs which failed to stain and this therefore resulted in an artificially high 'normal' B lymphocyte count being recorded in these cases. The esterase cytochemistry also, incidentally, confirmed the virtually complete absence of monocytes recently reported as a feature of HCL (Seshadri et al, 1976). This monocytopenia is an interesting, and curious, finding, but it cannot now be used as evidence favouring a monocytic evidence for HCs (Sehadri et al, 1976; Branda, 1976) in view of the similar B-lymphocytopenia in the disease reported here.

The difficulties which were observed with the extensively absorbed heteroantiserum to hairy cells deserve comment. The immunogenicity of HCs, and the method of immunisation, appeared to be perfectly adequate since the pre-adsorption serum even agglutinated HCs to very high titre. It is probable that entirely monospecific heteroantisera can never be fully achieved by adsorption, and for such sera to be useful this must be recognised. Thus, in immunofluorescent tests, such antisera must be employed at the very end of the plateau region of staining, and more sensitive techniques such as immunoelectron microscopy should probably be avoided. This technique is so sensitive that the slightest cross-reactivity in the serum becomes immediately

apparent, and one published report of an anti-monocyte serum illustrates 'specific' staining with a cell of the wrong type (Stuart et al, 1976).

To conclude the evidence presented here indicates that HCL is a B-cell neoplasm in which the leukaemic cells are invariably present in the PB in much larger numbers than has been previously recognised and in which circulating normal B lymphocytes are profoundly depressed. Within the T cell population, the T_M subpopulation is also considerably reduced, at least in some cases. This depression of both normal B lymphocytes and of T_M lymphocytes is all the more striking when considered in absolute numbers rather than as a percentage, since the majority of patients studied (Table 3.XXII) showed the leucopenia characteristic of HCL.

CHAPTER 4

THE NATURE OF HAIRY CELLS

I Monocytic properties

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2.1 *Sephadex G-10 filtration*

2.2 *Phagocytosis of latex beads*

2.3 *Phagocytosis of opsonised erythrocytes*

3. RESULTS

3.1 *Sephadex G-10 filtration*

3.2 *Phagocytosis of latex beads*

3.3 *Phagocytosis of sensitized erythrocytes*

4. DISCUSSION

II Surface immunoglobulin on HCs

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2.2 *Mononuclear cell isolation*

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2.4 *Cytoplasmic Ig*

2.5 *Culture*

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2.2 *Immunological marker studies*

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3.1 *Immunological markers*

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3.3 *Ig secretion*

4. DISCUSSION

THE NATURE OF HAIRY CELLS

I Monocytic properties

1. INTRODUCTION

The hairy cell was originally thought to arise from a primitive reticuloendothelial cell whose normal progeny included both lymphoid cells and cells of the monocyte-macrophage system (Bouroncle et al, 1958; Mitus et al, 1961). However, more recent concepts of cell kinetics suggest that lymphoid and macrophage cell lines develop independently. Nevertheless, various studies of HCL document that HCs share characteristics with normal B lymphocytes, normal monocytes or combinations of the two cell lines.

Several of the monocytic features of HCs have been described in previous chapters, and these, together with some of the morphological, cytochemical, and immunological, aspects of HCs have led several recent groups to declare that HCs derive from the monocyte-macrophage series (Jaffe et al, 1974; Polliack et al, 1974; King et al, 1975; Seshadri et al, 1976; Rozenszajn et al, 1976; Kass, 1977; Banerjee et al, 1978; Reyes et al, 1978).

In this section, some of the monocytic characteristics of HCs are examined and then the evidence favouring a monocytic lineage for HCs is discussed in relation to the literature.

2. MATERIALS AND METHODS

2.1 Sephadex G-10 filtration

The mononuclear cells isolated from a case of HCL by Ficoll-Isopaque

centrifugation were passed through a column of Sephadex G-10 following the technique of Ly and Mishell (1974). A small column of Sephadex G-10 (4 ml, 6 x 1 cm) was thoroughly washed with HBSSA and then HBSS + 10% foetal calf serum (FCS). To this was added 1 ml of the mononuclear cell preparation at 4 x 10^6 cells in HBSS + 5% FCS. The column was incubated for 60 min at 37°C and non-adherent cells were then eluted with several bed volumes of HBSS + 10% FCS. Weakly adherent cells were eluted with PBS + 10^{-3}M EDTA. Fractions eluted were cytocentrifuged and examined after staining with Giemsa.

2.2 Phagocytosis of latex beads

To investigate the phagocytic potential of HCs, 1 ml of cell suspensions at 4 x 10^6/ml in HBSSA, with and without 10% autologous plasma, was incubated for 1 h at 37°C in a shaking water bath with 1 drop of a suspension of latex particles (1.1 μ). After this time the cells were washed in HBSSA and phagocytosis was assessed by phase contrast microscopy and by the examination of Giemsa-stained cytocentrifuge preparations. Cells were also examined for phagocytosis by transmission electron microscopy.

2.3 Phagocytosis of opsonised erythrocytes

Mononuclear cell preparations from cases of HCL were incubated with ox erythrocytes, or ox erythrocytes sensitized with IgM (EA_M), IgG (EA_G) or IgM + complement (EAC) prepared as described previously. The leucocytes at 4 x 10^6 cells/ml were mixed with the erythrocytes (1%) and incubated on a shaking water bath at 37°C for up to 3 h. After this time, non-phagocytosed erythrocytes were lysed with distilled water and cytocentrifuge preparations were stained with Giemsa. In one case (MB) erythrophagocytosis was also examined by transmission electron microscopy.

3. RESULTS

3.1 *Sephadex G-10 filtration*

The test was carried out on cells from patient LS at a time when this patient had a very high proportion of morphological HCs in his PB mononuclear cell preparation. Very few cells passed through the column when it was eluted with HBSS + 10% FCS, and these had the morphology of small lymphocytes. HCs did not pass through the column, even when it was eluted with 10^{-3}M EDTA.

3.2 *Phagocytosis of latex beads*

Most of the patients available for study were tested for phagocytosis of latex at some time. In all cases, phagocytosis was observed by a proportion of the HCs. At the level of the electron microscope (Figures 4.1 and 4.2), two or more latex particles were frequently observed to be enclosed within phagocytic vacuoles in any one cell.

3.3 *Phagocytosis of sensitized erythrocytes*

Ten patients were tested for erythrophagocytosis, and in only 1 patient (MB, Figures 4.3 and 4.4) was a high degree of phagocytosis observed. Only erythrocytes opsonised with IgG were phagocytosed; unsensitized ox erythrocytes were not ingested and neither IgM, nor IgM and complement, were active as opsonins. In the other 9 patients, an occasional HC could be observed which had internalised a single IgG-coated erythrocyte (Figure 4.5), and such cells were only observed after at least 2 h of culture. Control preparations from patients with acute monocytic leukaemia rapidly phagocytosed EA_G within 30 min but did not ingest unsensitized erythrocytes or EA_M.

3. RESULTS

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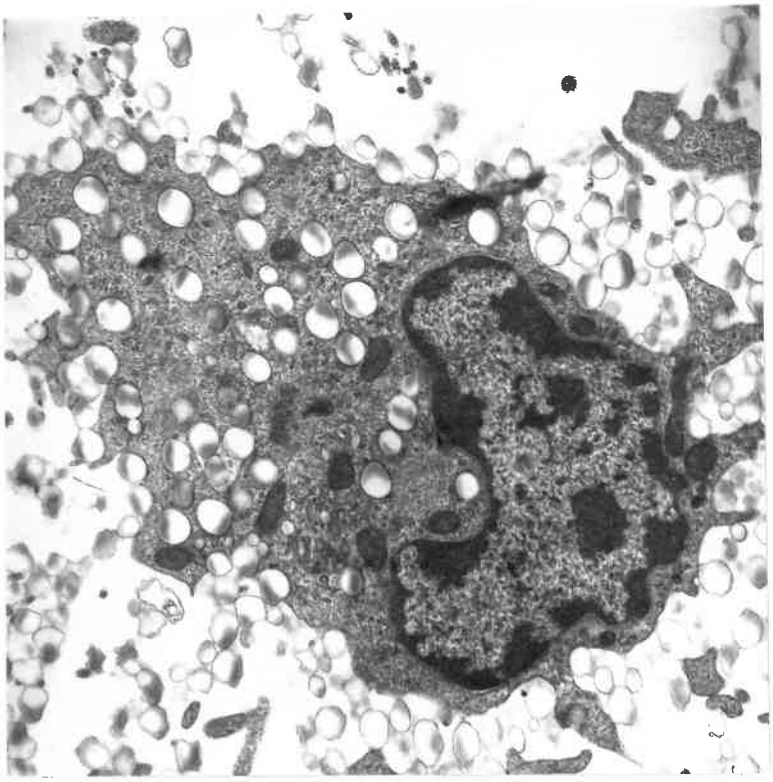

Figure 4.1 HC phagocytosing latex TEM

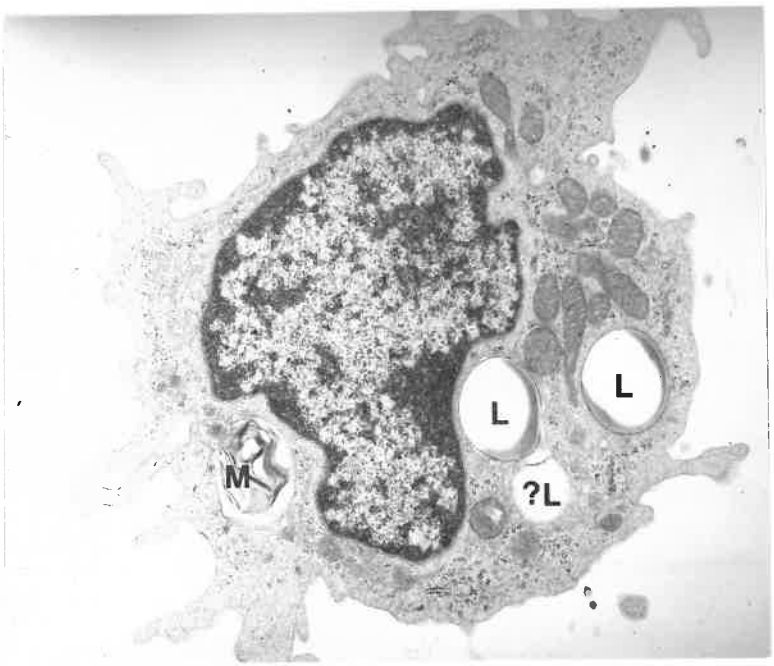

Figure 4.2 HC phagocytosing latex TEM. Two (L) and possibly 3 (?L) latex particles are seen enclosed within phagocytic vacuoles. M = membrane whorl.

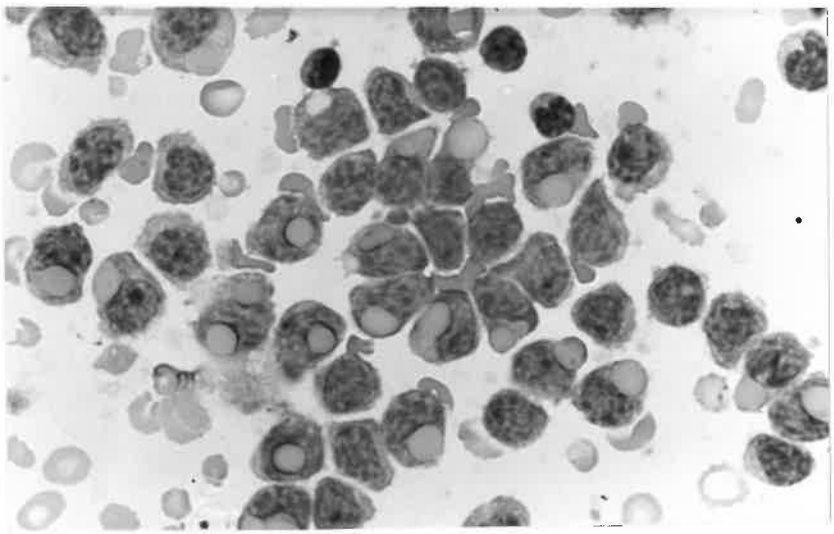

Figure 4.3 Phagocytosis of IgG opsonised ox erythrocytes by the HCs of patient MB.

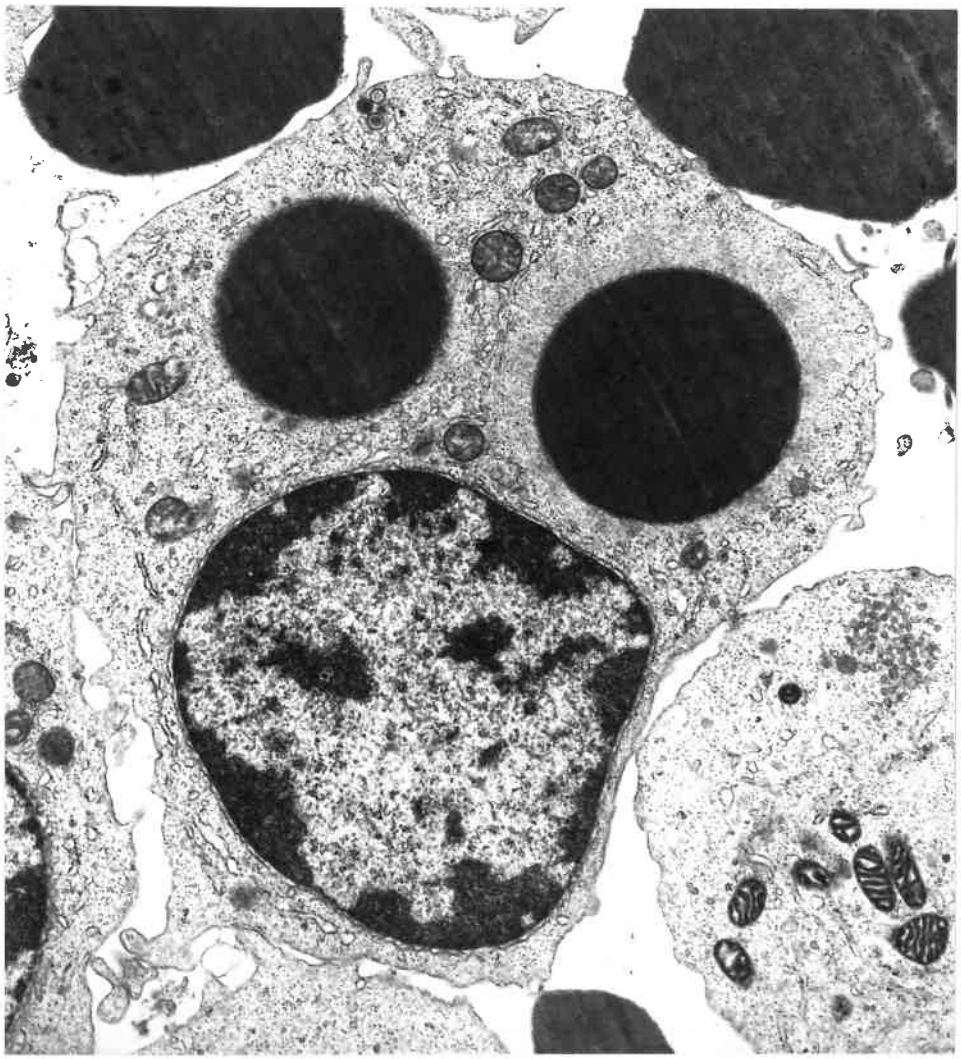

Figure 4.4 Electron micrograph of the phagocytosis of two IgG opsonised erythrocytes by a HC from patient MB.

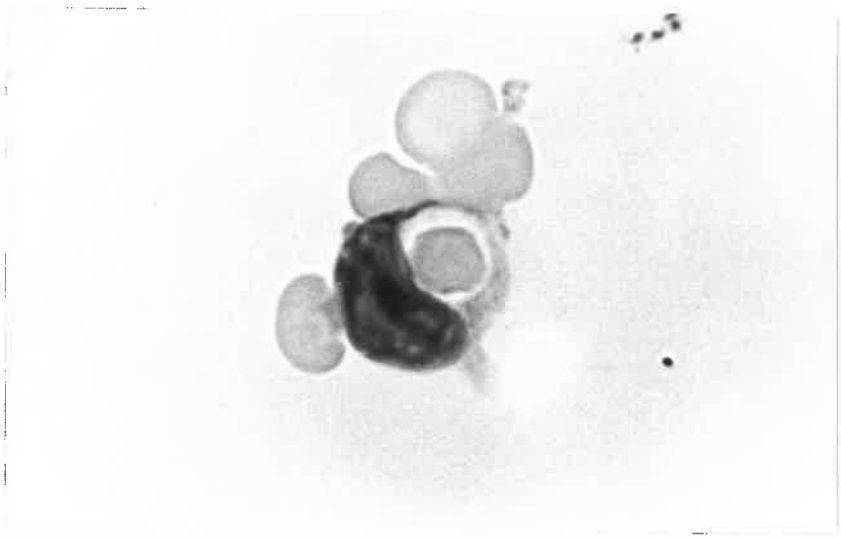

Figure 4.5 HC phagocytosing a single IgG-opsonised erythrocyte. This cell is from patient LS.

4. DISCUSSION

The monocytic properties of HCs are still the subject of some controversy (Catovsky, 1977): this probably results both from variations in the techniques used and from variations from case to case. Adherence to glass slides by HCs was shown in the last chapter, and glass and plastic adherence by HCs is the experience of most workers (Flandrin et al, 1973; Catovsky et al, 1974b; Debusscher et al, 1975; King et al, 1975; Boldt et al, 1977; Rieber et al, 1977; Palutke et al, 1978; Jansen et al, 1978). Rieber et al, (1977) assessed the glass adherence of HCs from three patients quantitively and found the adherent HCs to be more resistant to EDTA-trypsin treatment than normal monocytes. There are, however, reports claiming that HCs do not adhere to glass or plastic (Trubowitz et al, 1971; Rozenszajn et al, 1976; Matre et al, 1977). Another report of a single case (Ruben et al, 1969) showed that HCs passed through nylon fibre columns, but two other groups (Yam et al, 1968; Boldt et al, 1977) both tested a case of HCL by this method and found that the HCs were retained. In this section it was shown that HCs from a single case were also retained by Sephadex G-10 beads which is generally considered to remove monocytes (Ly and Mishell, 1974; Alonso et al, 1978). However, these techniques probably also remove some B lymphocytes, particularly those cells which bear SIg of the IgG or IgA type (Alonso et al, 1978).

Again, with few exceptions (Schrek and Donnelly, 1966; Yam et al, 1968; Matre et al, 1977), there is almost complete agreement that HCs can phagocytose latex (Flandrin et al, 1973; Fu et al, 1974; Catovsky et al, 1974b, 1977; Haegert et al, 1974; King et al, 1975; Rozenzajn et al, 1976; Boldt et al, 1977; Utsinger et al, 1977; Zidar et al, 1977;

4. DISCUSSION

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Rieber et al, 1977; Jansen et al, 1978; Palutke et al, 1978) and this is confirmed in the present section. There is less agreement about phagocytosis of anything other than latex, and, as shown in the present report where only one case effectively phagocytosed opsonised erythrocytes, this ability probably varies from case to case. Ferritin was phagocytosed weakly (less than monocytes) in a single case (Schmalzl et al, 1975), but in most cases carbon black, yeast and bacteria, whether opsonised or not, are not phagocytosed by HCs (Yam et al, 1968; Ruben et al, 1969; Berg and Brandt, 1970; Haak et al, 1974; Fu et al, 1974; Debusscher et al, 1975; King et al, 1975; Kitani, 1977). One report claims weak engulfment of yeast followed by mycocidal activity (Catovsky et al, 1974b), but this was countered by a major study of eight cases (Jansen et al, 1978) which demonstrated that apparently phagocytosed staphylococci were cell associated, rather than internalised, and could be destroyed by lysostaphin which cannot permeate the leucocyte membrane. The single case in this section which phagocytosed opsonised erythrocytes is, however, supported by occasional such cases in the literature and Zidar et al, (1977) (1 of 7 cases) and Boldt et al, (1977) have each reported a similar case. Palutke et al, (1978) reports that most cases will phagocytose erythrocytes, and Rieber et al, (1977) demonstrate occasional erythrophagocytosis of opsonised cells in most cases.

Phagocytosis is not a property exclusive to the monocyte-macrophage series. When using CLL cells as a control, Zidar et al, (1977) observed that two cases adhered to glass and one of these phagocytosed latex, but other groups have failed to demonstrate phagocytosis by CLL cells (Fu et al, 1974; Rieber et al, 1977). Catovsky et al, (1977) demonstrated that all of nine cases of CLL, and three cases of prolymphocytic leukaemia, phagocytosed latex, and claimed that serum was

required in the incubation mixture. In the present report, the presence of serum in the mixture of latex and HCs was found to be unnecessary and Fu et al, (1974) considered that the presence of high concentrations of serum in the initial mixture inhibited phagocytosis by HCs. In some cases of acute lymphoblastic leukaemia, the lymphoid blast cells can occasionally phagocytose erythrocytes (Foadi et al, 1978), and normal T lymphocytes bearing γFc receptors have been demonstrated to phagocytose opsonised erythrocytes (Grossi et al, 1978).

The other evidence for a monocytic origin for the HC mainly rests on the nature of the γFc receptor - and thus of the intrinsic nature of SIg - and on cytochemical staining. The monocytopenia in HCl used as evidence by Seshadri et al, (1976) was shown in the last section to be no more marked than the lymphopenia.

It will be shown conclusively in the next section that the SIg on HCs is intrinsic and not acquired either by the γFc receptor (Jaffe et al, 1974; King et al, 1975) or as autoantibody (Banerjee et al, 1978) as has been suggested. Nevertheless, the γFc receptor on HCs bears many resemblances to that of monocytes (Chapter 3), and it is fair to describe this receptor as a monocytic feature of HCs. However, the UCG (unclassified cell group - null cells, K cells, L cells) also have an exceedingly avid γFc receptor, and some of these cells are B cells as evidenced by immunoglobulin synthesis and secretion (Chess and Schlossman, 1977).

Hairy cells possess non-specific esterase (NSE) enzymes (Rozenszajn et al, 1976; Kass, 1977; Higgy et al, 1978) which are more characteristic of monocytes than of lymphocytes (Li et al, 1973). One group has consistently used this feature as evidence for a monocytic origin for the HC (Kass and Schnitzer, 1973; Kass, 1977) and claims

that the esterase staining is markedly inhibited by sodium fluoride. This last point is crucial since it has recently been demonstrated that some T lymphocytes and null cells also have NSE activity (Higgy et al, 1977), but complete fluoride inhibition is a feature of the monocyte enzyme. However, in a study of 14 cases of HCL, Higgy et al, (1978), whilst observing a distinctive pattern of NSE staining (quite different from that of monocytes), did not find that the enzyme was inhibited by fluoride. Moreover, NSE activity which was inhibited by fluoride has been recorded in megaloblasts and normoblasts in patients with anaemia (Kass and Peters, 1977), and therefore this reaction is probably not entirely specific for monocytes.

Several other features of HCL are very unusual, and by being almost invariably present in HCL, but rarely in other conditions, they are useful as diagnostic aids. However, none of these distinctive features is diagnostic of HCL since one or more may be absent in any particular case and since any may occur in other pathological states. Thus, the tartrate-resistant isoenzyme 5 of acid phosphatase in HCL (Yam et al, 1971) is also found in some cases of Hodgkin's disease and in CLL (Katayama et al, 1972b; Katayama and Yang, 1977). Both the receptor for IgM on HCs (Burns et al, 1977a), and the distinctive ribosome-lamellar complex (Katayama et al, 1972a), can be found in CLL (Cawley et al, 1975; Burns et al, 1977b; Pichler and Knapp, 1977) as can a high leucocyte alkaline phosphatase score (Hayhoe et al, 1977).

There remain other findings which appear to be restricted to the HC and thus add to the interest of HCL. The cytoplasmic projections both at light and electron microscopy level which give the cell its characteristic hairy appearance are not commonly found in other conditions, nor is the hybrid-type appearance of both microvilli and ruffles on the

cell surface as viewed under scanning electron microscopy (Polliack et al, 1974). Unlike the cells from most chronic leukaemias (Revesz et al, 1976), HCs do not bind cholera toxin (Revesz et al, 1976), and very recently, Reyes et al, (1978) have described a newly discovered peroxidase activity in HCs which is not associated with granules.

In conclusion, while the HC remains an unusual and interesting cell type, the weight of evidence does not appear to necessarily suggest a monocytic origin for HCL.

II Surface immunoglobulin on HCs

1. INTRODUCTION

The strong γFc receptor on HCs has often been invoked to account for the varied, and frequently multiple, heavy chain isotypes of surface immunoglobulin (SIg) on these cells (Jaffe et al, 1974; King et al, 1975; Seligmann, 1977; Catovsky, 1977), and these continues to be uncertainty about the heavy chain phenotype of HCs.

A detailed study of the complete SIg phenotype of the cells from a large series of cases of HCL was therefore undertaken, both to clarify this previous uncertainty and to place HCL firmly within the B-cell disorders.

2. MATERIALS AND METHODS

2.1 Patients

Twenty four patients with clinically and morphologically typical HCL were studied. The diagnostic criteria used for most of these patients has been given in Chapter 2. In brief, in all the patients, the bone marrow aspirate or trephine biopsy was extensively infiltrated with HCs, and in all the 15 patients splenectomised, the spleen was diffusely replaced by HCs. In all instances, the diagnosis was confirmed by the demonstration of tartrate-resistant acid phosphatase (Li et al, 1970), of a typical ultrastructure (Daniel and Flandrin, 1974) and of a surface receptor for IgM (Burns et al, 1977a). Only 1 of the patients (FB) had a paraprotein, and this patient is discussed in more detail in the next section.

In most cases the PB was examined sequentially for periods of up to 15 months. Splenic (8 patients), bone marrow (4 patients), and lymph node (1 patient) cells were also examined. In order to determine the possible effect of splenectomy in PB populations in HCL, in two patients blood was studied daily in the immediate post-splenectomy period.

For comparison, the PB of 25 patients with typical chronic lymphocytic leukaemia (CLL) was also examined.

2.2 *Mononuclear cell isolation*

Mononuclear cell suspensions were prepared from the various tissues as described previously. In all instances, the viability by trypan-blue dye exclusion was greater than 95%, and the virtual absence of monocytes characteristic of HCL was always confirmed by non-specific esterase staining of cytocentrifuge preparations.

2.3 *Surface membrane immunoglobulin*

In all experiments, before testing for SIg, the isolated and washed mononuclear cells were incubated for 2 h at 37°C, and in some cases overnight, in serum-free medium in order to remove cytophilic IgG (Winchester and Fu, 1976). A modified rosette method (Molinaro and Dray, 1974; Ling et al, 1977) was used: this employs chromic chloride coupling to indicator ox erythrocytes of purified sheep IgG antibody, monospecific for human heavy and light chains. The antisera used were a gift from Dr. N. Ling, Department of Experimental Pathology, University of Birmingham, and details of their specificity have been given by him (Dhaliwal et al, 1978). In addition, the purity of the antisera was confirmed by the specific staining in indirect fluorescent preparations from cases of IgG, IgA, IgM, and IgD myelomas (Burns et al, 1978d). In particular, the anti-IgG and anti-IgA antibodies did not stain the

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In all experiments, before testing for SIg, the isolated and washed mononuclear cells were incubated for 2 h at 37°C, and in some cases overnight, in serum-free medium in order to remove cytophilic IgG (Winchester and Fu, 1976). A modified rosette method (Molinaro and Dray, 1974; Ling et al, 1977) was used: this employs chromic chloride coupling to indicator ox erythrocytes of purified sheep IgG antibody, monospecific for human heavy and light chains. The antisera used were a gift from Dr. N. Ling, Department of Experimental Pathology, University of Birmingham, and details of their specificity have been given by him (Dhaliwal et al, 1978). In addition, the purity of the antisera was confirmed by the specific staining in indirect fluorescent preparations from cases of IgG, IgA, IgM, and IgD myelomas (Burns et al, 1978d). In particular, the anti-IgG and anti-IgA antibodies did not stain the

cytoplasm of either of two cases of IgM myeloma or of the two cases of IgD myeloma tested; the anti-light chain antibodies were monospecific for the appropriate light chain type of each myeloma studied. Sheep IgG does not readily bind to the γFc receptor of human cells (Alexander and Sanders, 1977), but this possibility was controlled to non-immune sheep IgG; in all instances rosette formation with this reagent was less than 1%. It was also observed that the percentage of cells rosetting with the non-predominant light chain type was negligible. Furthermore, the possibility of soluble complex formation with residual serum Ig causing apparent SIg by γFc-binding of the anti-Ig reagent (Winchester and Fu, 1976) is not a problem with the rosette method (see Discussion).

In some patients, a direct fluorescent staining technique employing FITC-coupled, affinity purified, monospecific rabbit or sheep anti-human Ig was used to detect SIg. The antisera were purchased from Dr. J.L. Smith, Immunology Unit, Tenorus Research Laboratory, Southampton, and details of their preparation have been published (Gordon and Smith, 1978). The antisera were ultracentrifuged immediately before use, and an FITC-coupled normal rabbit serum control was negative in each test.

2.4 Cytoplasmic Ig

Cytocentrifuge preparations of washed mononuclear cells, which had been pre-incubated for 2 h at 37°C to remove extrinsic Ig, were fixed in acetone at -20°C overnight and stained with FITC-coupled, affinity purified, monospecific rabbit anti-human Ig immunoglobulin. These antisera were all monospecific for the appropriate myeloma cell types. A normal rabbit serum used as a control was in each test completely negative. For the staining procedure, a method employing very small quantities of antisera and a cytoplasmic counterstain was used (Worman, 1978). In brief, this method involved placing a 3λ drop

of antiserum on a coverslip which was then inverted onto the cytocentrifuged cells. After incubation and thorough washing, the cells were counterstained by incubation for 5 min at room temperature with 0.2% Evan's blue dye in physiological saline. The counterstain was washed off and the slides read under combined phase contrast and u.v. epifluorescence. The cytoplasm of negative cells fluoresced a bright red thus greatly facilitating percentage cell counts. Positive (green) plasma cells were only red around the Golgi region and the Evan's blue counterstain in no way interfered with specific immunofluorescence.

2.5 *Culture*

Splenic cells from one case of HCL were established in culture for more than 60 days by methods described elsewhere. During this time, the cells consistently maintained a hairy cell morphology and continued to display tartrate-resistant acid phosphatase activity. They retained the same surface membrane marker phenotype as before culture and, in particular, remained negative for the Epstein-Barr nuclear antigen.

2.6 *Other surface markers*

E rosette formation and γFc receptor expression were determined as described previously.

3. RESULTS

3.1 *Distribution of SIg*

The results are shown in Table 4.I. The pathological cells in all 24 cases expressed only a single light chain type (16K, 8λ), thus showing the probable monoclonality of HCL and, incidentally, demonstrating a 2:1, K:λ ratio. As regards heavy chain isotype expression,

Table 4.I Distribution of SIg on isolated mononuclear cells in HCL

Patient	Date	Tissue	SIg method 1. Rosette 2. Fluorescence	% SIg^- positive cells						% E rosettes	% γFc rosettes	Comments	Type
				κ	λ	μ	δ	γ	α				
KD	2. 2.77	PB_*	1	0	73	41	70	85	-	10	93	*After overnight culture	II
		PB	1	0	69	37	70	73	-	-	-		
	18. 1.78	PB	1	2	86	55	75	79	2	5	91		
	13. 4.78	PB	1	0	83	9	78	70	0	10	93		
MB	24. 9.76	Spleen	2	0	90	5	0	90	0	3	78	*After overnight culture	I
	4. 2.77	PB	1	5	41	0	0	60	-	-	-		
	4. 3.77	PB_*	1	0	24	0	0	48	-	6	95		
		PB	1	0	66	0	0	70	-	-	-		
PS	30. 9.76	PB	2	0	60	41	-	64	0	44	64		II
	19.10.76	Spleen	2	0	86	51	-	85	0	13	91		
	19. 2.77	PB	1	4	62	45	48	73	-	42	73		
	6. 2.78	PB	1	0	35	10	43	25	0	52	47		
MH	6. 7.77	PB_*	2	55	0	0	0	48	0	36	60	*Many plasmacytoid cells present. SIg persisted after overnight culture	I
	8.10.77	PB	1	11	0	0	0	18	0	56	33		
HD	13. 4.78	PB	1	36	0	10	43	5	0	40	70		II
RH*	29. 4.77	PB	1	6	0	0	0	6	-	87	14	*This patient had previously demonstrated 87% polyvalent anti-Ig staining when E = 16; γFc = 88 (18.9.75)	I
	28. 4.78	PB	1	3	0	0	0	4	0	58	46		
	28. 4.78	BM	1	9	0	3	0	12	0	46	50		

Table 4.I continued

Patient	Date	Tissue	SIg method 1. Rosette 2. Fluorescence	% SIg- positive cells K	λ	μ	δ	γ	α	% E rosettes	% γFc rosettes	Comments	Type
DC	14. 1.77	PB	1	68	1	8	0	65	-	30	72	* SIg persisted after 4 days in culture	I
	14. 1.77	Spleen	2	90	0	0	0	90	9	22	90		
FB*	21. 1.77	PB	2	47	0	2	3	53	0	50	62	* This patient has a paraprotein (see Section III)	I
	20. 8.78	PB	1	40	0	0	0	40	0	43	63		
AD	25. 1.77	PB*	1	0	66	1	2	63	-	18	78	* SIg persisted after overnight culture	I
	23. 1.78	PB	1	0	38	3	0	40	0	50	40		
LP	28. 1.77	PB*	1	3	64	18	70	24	-	17	83	* G and D capping + synthesis experiments performed	II
	28. 1.77	PB	2	-	-	-	70	4	0				
	3. 2.77	Spleen	1	0	74	14	85	71	-	18	94		
LH*	12. 1.77	Spleen	1	-	-	3	78	-	-	73	96	* The HCs in this case had combined B and T cell features (Chapter 5)	II
	12. 1.77	Spleen	2	78	0	5	82	-	0				
	12. 2.77	PB	1	52	4	11	44	10	-	79	65		
	24. 2.78	BM	1	50	1	15	55	10	0	33	65		
FW	5. 2.77	PB	1	0	50	10	57	32	-	43	60	* Many normoblasts present in mononuclear cell preparations	II
	23. 6.77	PB*	1	1	67	11	66	28	-	34	74		
	19. 1.78	PB	1	<1	40	59	57	27	1	8	73		
	27. 1.78	PB	1	0	29	7	30	13	0	8	65		
	2. 2.78	PB	1	0	17	4	23	15	0	56	46		
	9. 2.78	PB	1	0	0	2	5	2	0	63	48		

Table 4.I continued

Patient	Date	Tissue	SIg method 1. Rosette 2. Fluorescence	% SIg- positive cells κ	λ	μ	δ	γ	α	% E rosettes	% γFc rosettes	Comments	Type
BN	16. 6.77	PB	1	37	5	0	5	52	-	42	56		I
RR	3. 9.77	PB*	1	42	0	0	0	41	0	60	63	* SIg persisted after overnight culture	I
	4.10.77	PB	1	25	0	0	1	37	-	44	68		
HR	5.10.77	PB	1	58	2	1	67	5	8	19	88	* See SIg kinetic experiments	II
	5.10.77	PB*	1	44	3	4	55	6	-	15	85		
	11.10.77	PB*	1	70	2	39	80	29	65	19	89	‡ All SIg classes persisted after overnight culture	
	11.10.77	Spleen‡	1	83	5	53	83	36	84	6	91		
	15.10.77	PB	1	31	3	17	47	10	31	20	83		
	22.10.77	PB	1	49	1	2	69	40	66	18	85		
	22.10.77	PB	2	44	2	15	45	17	-				
	26.10.77	BM	1	66	0	0	64	9	80	13	85		
AB	29. 9.77	PB*	1	52	0	0	0	55	-	54	64	* SIg persisted after 2 days in culture	I
	16.11.77	PB	1	68	0	1	2	67	1	26	80		
	22.11.77	PB	1	31	0	<1	1	47	1	32	75		
	22.11.77	Spleen	1	74	0	0	1	81	0	11	90		
	22.11.77	BM	1	27	0	0	1	45	0	18	73		
	22.11.77	LN	1	4	0	0	0	8	0	31	76		
GH	19.12.77	PB*	1	31	0	4	3	36	-	43	58	* Many plasmacytoid cells present following a systemic candida infection	I
	24. 1.78	PB*	1	8	1	1	1	22	5	46	53		

Table 4.I continued

Patient	Date	Tissue	SIg method 1. Rosette 2. Fluorescence	% SIg- positive cells κ	λ	μ	δ	γ	α	% E rosettes	% γFc rosettes	Comments	Type
TN	15. 1.78	PB	1	72	<1	80	73	4	1	30	92		III
LK	18. 1.78	PB	1	65	0	56	67	43	70	20	78		II
DA	2. 3.78	PB	1	48	0	7	8	55	7	26	70		I
	6. 4.78	PB	1	21	2	<1	1	31	0	51	43		
JC	27. 4.78	PB	1	0	72	0	0	80	0	9	91		I
	9. 5.78	PB	1	0	98	0	0	99	<1	<1	96		
BC	14.12.77	PB	1	<1	23	16	28	28	2	54	47		II
CJ	12. 5.78	PB	1	65	0	0	0	67	0	12	92		I
AF	15. 6.78	PB	1	65	<1	0	63	32	0	26	80	* SIg persisted in culture for 14 days	II
	26. 7.78	PB *	1	52	<1	0	40	2	5	52	50		
	27. 7.78	Spleen	1	94	0	0	94	0	3	5	95		
	28. 7.78	PB	1	90	0	0	73	0	2	15	95		
	2. 7.78	PB	1	90	0	0	67	35	22	17	84		
	8. 8.78	PB	1	57	0	<1	43	10	6	36	74		
	11. 8.78	PB	1	65	0	2	44	5	4				

the cases fell into two, or possibly, three groups. In 13 cases the cells expressed only IgG (10K, 3λ) - type I. The cells in all the other cases displayed multiple heavy chain isotypes, but invariably showed restricted light chain expression. Ten cases (5K, 5λ) had 3, or 4, heavy chain isotypes (type II) which always included IgD, and usually, IgM. The low level of IgM expression in nearly all of the type II cases of HCL was a constant feature, and parallel tests with the cells from cases of CLL (Table 4.II) demonstrated that this was not due to the anti-IgM antibody.

The combination of M and D alone (type III) was observed in only one case (K). In all these mixed cases, the sum of two or more of the heavy chain isotypes greatly exceeded both the percentage of cells bearing the exclusive light chain type and the percentage of $Fc^{+}E^{-}$ (non-T) cells. This indicates that two or more heavy chain isotypes were simultaneously expressed on the same cell. The light chain restriction observed in the multiple isotype groups (types II and III), together with the equal number of λ light chain isotypes in type II, make it unlikely that the heavy chain findings are attributable to extrinsic autologous serum Ig binding.

Table 4.II which shows the results of similar studies in CLL, using the rosette method, demonstrates a clear difference in SIg expression in the two diseases. The distribution of types in CLL shows a preponderance of type III, which was rare in HCL. Type I which was the most common in HCL, has only one representative in 25 cases of CLL. Again, within group II, which is common to both diseases, IgM expression in CLL is much more marked and IgA was not detected in the 21 cases tested; IgA was observed in 2, or possibly 3, of the group II HCL cases. Non-T cells which did not express SIg were not

seen in HCL although this group (type IV) represented some 16% of the CLL cases. However, 2 of the type I group from the HCL cases (RH and FW) appeared to be progressively losing their SIgG, and may eventually become type IV cells.

3.2 *Fluctuations in SIg expression*

Table 4.I shows that, at different times of study of a single case, whereas wide variations were observed in the percentage of SIg expression and in relative proportions of the different heavy chain isotypes (type II), the cells maintained the same isotypes. Moreover, the major heavy chain type was consistently expressed on approximately the same percentage of cells as bore the exclusive light chain type.

In 2 patients (HR and AB), SIg expression was studied sequentially over short time intervals after splenectomy; these results are shown graphically in Figure 4.6. The marked variations observed cannot be attributed to technical artifact since the specimens were sometimes collected on the same day and tested with identical reagents. Also, the anti-Ig-coated indicator cells have been shown to give reproducible results, with no loss of activity, for up to two weeks (Ling et al, 1977). In the present study, the same batch of indicator cells was used over any 8-day period, with frequent checks of reproducibility.

As is seen in Figure 4.6, immediately post-splenectomy there is a marked rise in the total white cell (wbc) count which is attributable mainly to a rise in the absolute count of HCs. The estimate of total HC numbers (% $E^{-}\gamma Fc^{+}$ cells x wbc count corrected for polymorphonuclear leucocytes: this method of calculation is justified because of the virtual absence of both monocytes and non-neoplastic B lymphocytes in HCL) very closely parallels the absolute number of cells bearing the predominant heavy chain isotype (% SIg^{+} cells x wbc count corrected for

Table 4.II SIg phenotype of isolated PB mononuclear cells in CLL

Patient	% SIg positive cells by rosette method						% E rosettes	% γFc rosettes	Type ‡
	κ	λ	μ	δ	γ	α			
1	71	5	0	0	80	0	0	100	I
(repeat 6 mo)	57	0	0	0	67	0	4	97	
2	91	0	95	81	89	0*	10	95	
3	<1	97	80	91	82	ND	3	98	
(repeat 8 mo)	0	85	75	90	30	0	5	91	
4	46	5	58	62	26	ND	29	85	II
(repeat 10 mo)	78	0	ND	68	85	0	25	80	
5	89	0	31	86	22	0	8	94	
6	80	0	80	63	65	<1	10	70	
7	0	82	0	63	31	0	4	65	
8	0	50	62	57	6	ND	<1	97	
(repeat 4 mo)	0	45	15	76	0	0	3	98	
(repeat 8 mo)	0	64	50	76	0	0	<1	95	
9	93	5	92	87	2	0	1	93	
10	0	45	14	32	4	0	2	92	
11	50	0	30	63	0	ND	31	85	
12	<1	90	71	74	6	ND	5	94	
(repeat 4 mo)	0	94	70	27	0	0	4	96	III
13	71	0	47	51	0	0	1	95	
14	91	1	32	41	8	ND	0	94	
15	0	47	43	23	0	0	20	91	
16	40	0	20	26	0	ND	10	87	
17	97	0	12	91	0	0	4	97	
18	0	52	20	50	5	0	<1	95	
19	0	38	36	56	0	0	1	91	
20	0	0	0	0	0	0	12	80	
21	0	0	0	0	0	0	5	93	
22	2	0	0	0	0	0	<1	98	IV
23	0	3	4	4	3	0	0	95	
24	0	15	2	13	0	0	4	93	IV/V
25	0	90	0	90	1	0	<1	96	V

‡ Types I-III as for Table 4.I: Type IV No SIg detected: Type V SIgD only.

* Not done

polymorphs). The predominant heavy chain type was IgG in one case (Figure 4.6, lower), but the similar pattern of heavy chain expression in the other (mixed) case, in which IgD was the dominant isotype, is further evidence against γFc-binding of autologous, but extrinsic, Ig. The second peak observed in both patients around days 5-8 post-splenectomy (Figure 4.6) was attributable mainly to a rise in absolute polymorph numbers.

3.3 *Removal and re-expression of SIg*

Attempts to remove surface immunoglobulin using trypsinization or papainization under standard conditions (Preud 'homme and Seligmann, 1972) were uniformly unsuccessful and complete removal could not be achieved without killing the cells.

SIg was then removed by a capping process using the anti-Ig-coated erythrocytes as a stripping agent. Mononuclear cells were mixed with the indicator erythrocytes as for the SIg rosette test, and the mixture incubated at 37°C. Aliquots were removed at time 0 and at various time intervals thereafter, and read for percentage SIg rosette formation. Figure 4.7a illustrates the rate of removal of SIgD in one case of multiple heavy chain isotype HCL (LP, Table 1), and Figure 4.7b demonstrates the removal of SIgG in a case of IgG only (Type 1), HCL (FB, Table 1). As will be discussed later, the failure to re-express SIg in this case after 10 h of culture may be attributable to the continued presence of the indicator red cells constantly stripping off re-synthesised SIg. However, when anti-IgG, rather than anti-IgD, indicator cells were used to strip the same multiple heavy chain case of HCL, an increase in SIgG rosette formation was seen to occur, and this is shown in Figure 4.7c. Since this unexpected result suggested IgG secretion, an experiment was set up to establish whether Ig was

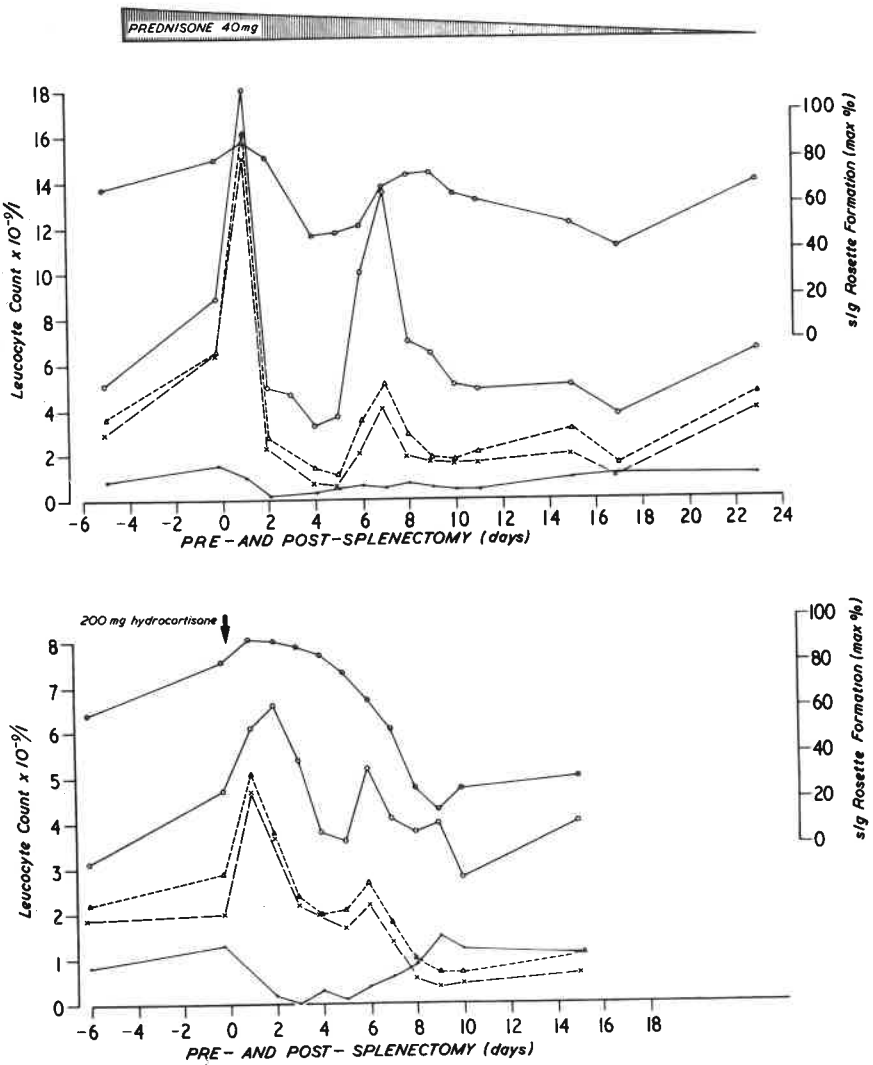
PREDNISONE 40mg
Leucocyte Count x 10⁻⁹/l
s/g Rosette Formation (max %)
PRE- AND POST-SPLENECTOMY (days)
200 mg hydrocortisone
Leucocyte Count x 10⁻⁹/l
s/g Rosette Formation (max %)
PRE- AND POST- SPLENECTOMY (days)

Figure 4.6

Legend to Figure 4.6 Peripheral blood leucocyte changes following splenectomy. Patient HR (upper) and AB (lower). (O—O) indicates the total peripheral white cell count, including polymorphonuclear leucocytes; (Δ···Δ) charts the estimated total HC count assessed by the non-T, γFc-rosetting mononuclears; (●—●) records the total mononuclear cell count forming E-rosettes (×---×) charts the absolute number of cells bearing the major heavy chain isotype (IgD upper, IgG lower). All these counts are expressed as 10^{-9}/l. Also charted ( ) are the maximal percentage of SIg rosette-forming cells (scale indicated on the right ordinate). For most of the period of study, patient HR (upper) was on continuous prednisone as indicated on the graph. In patient AB (lower), up to 10% of plasmacytoid cells were observed in the PB between days 7 and 9; these cells were SIg^-, E^-. Patient AB (lower) received only a single dose of steroid (200 mg hydrocortisone intravenously) at the time of splenectomy (↑ on graph).

secreted into the culture supernatant. The mononuclear cells from the peripheral blood of four HCL patients, none of whom had recognisable plasma cells in their differential count, were thoroughly washed and cultured for between 3 h and 12 h (1×10^7 cells/ml) in serum-free medium at 37°C. Control cultures were incubated for the same time at 0°C, or at 37°C in the presence of puromycin (2.5 µg/ml). The supernatants from these cultures were then titrated for agglutination of indicator erythrocytes coupled to sheep IgG against the various Ig classes and the results are set out in Table 4.III. Control supernatants were completely negative. The results suggest that all four HC cultures were secreting IgG (3λ, 1K) and that the cells from the SIgG-only group (type I) were secreting an excess of light chain. The same experiment, modified and quantitated, was also carried out on patient FB (Table 4.I) who has a paraprotein (IgG K), and cells from this patient also secreted IgG. These results will be documented fully in the next section.

Additional stripping experiments were performed with free, fluoresceinated, rabbit anti-Ig and mononuclear cells from a multiple isotype (type II) case (HR, Table 1). Washed mononuclear cells were incubated with antiserum for 40 min at room temperature, washed thoroughly, and then incubated at 37°C for 30 min to allow capping to take place. An aliquot of cells was then tested by immunofluorescence to confirm that the SIg had been removed. In all the experiments described below, the SIg was seen to be either absent or completely capped when assessed by fluorescence. Culture was continued and aliquots were removed for SIg rosette testing at various time intervals up to 36 h. The results of these experiments are graphed in Figure 4.8. In the initial experiment, polyvalent anti-Ig was used and the re-expression

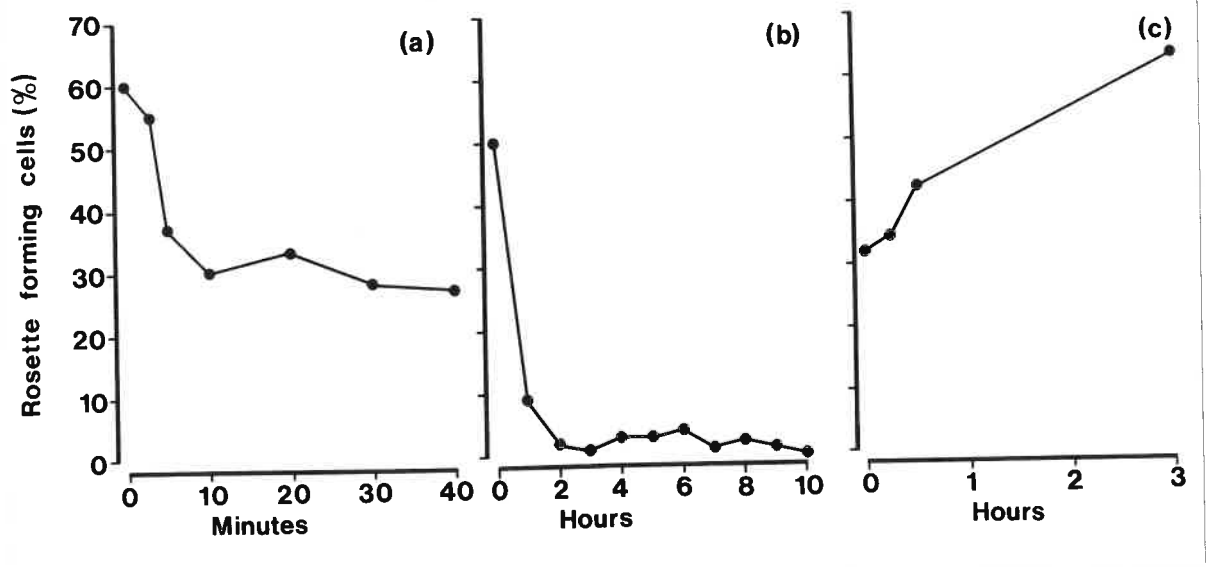

Figure 4.7 (a) The rate of removal of SIgD over a short period in the continued presence of anti-IgD coupled to indicator erythrocytes is shown (patient LP). (b) The complete removal of SIgG (patient FB) by anti-IgG-coupled erythrocytes is shown. (c) The increasing percentage of SIgG rosette formation observed in the continued presence of anti-IgG-coupled erythrocytes is shown (same patient as (a) LP). These results are the mean of two separate experiments.

Table 4.III Agglutination titre of indicator erythrocytes by PB HC cultures

Patient	Type	Immunoglobulin class					
		K	λ	μ	δ	γ	α
KD	II	0	neat	0	0	1/2	-
DC	I	1/32	0	0	0	neat	0
AD	I	0	1/8	-	-	neat	-
LP	II	0	1/16	-	0	1/16	-

of SIgD examined by the rosette method; having shown an initial marked reduction in SIgD (the cells were completely capped or negative by fluorescence) by 5-6 h the SIgD had returned to levels approaching those observed before stripping. Repeat stripping experiments were performed with monospecific anti-Ig antisera (including three different anti-δ reagents) and rosette tested for re-expression of the heavy chain type stripped off. Incidentally, testing for other heavy chain types showed that the anti-α and anti-δ antisera stripped only α or δ respectively, no reduction being observed in the other heavy chain classes. This independent capping provides further evidence for the specificity of the antisera and for the non-association of the heavy chain isotypes; it also eliminates the possibility of SIgM having anti-serum Ig activity (Preud 'homme and Seligmann, 1972). As is also seen in Figure 4.8, stripping of IgD was almost complete even when tested by the sensitive rosette method, and re-expression was taking place, although incomplete, by 18 h in one test and by 36 h in another test. Complete stripping of SIgA could not be achieved even with monospecific anti-IgA (but again stripping appeared complete by fluorescence) and the percentage SIgA positive cells remained similar at 18 h: this probably reflects a low level of IgA synthesis and secretion, especially since SIgA was expressed after more prolonged periods of culture (Table 4.V).

Stripping and re-expression of SIgG was performed, in the same way on the cells of patient FB. SIgG was demonstrated to re-express on stripped cells in a time of between 24 h and 72 h (Section III).

3.4 Cytoplasmic Ig

The results of cytoplasmic staining are set out in Table 4.IV. All the cases studied exhibited cytoplasmic staining of restricted light

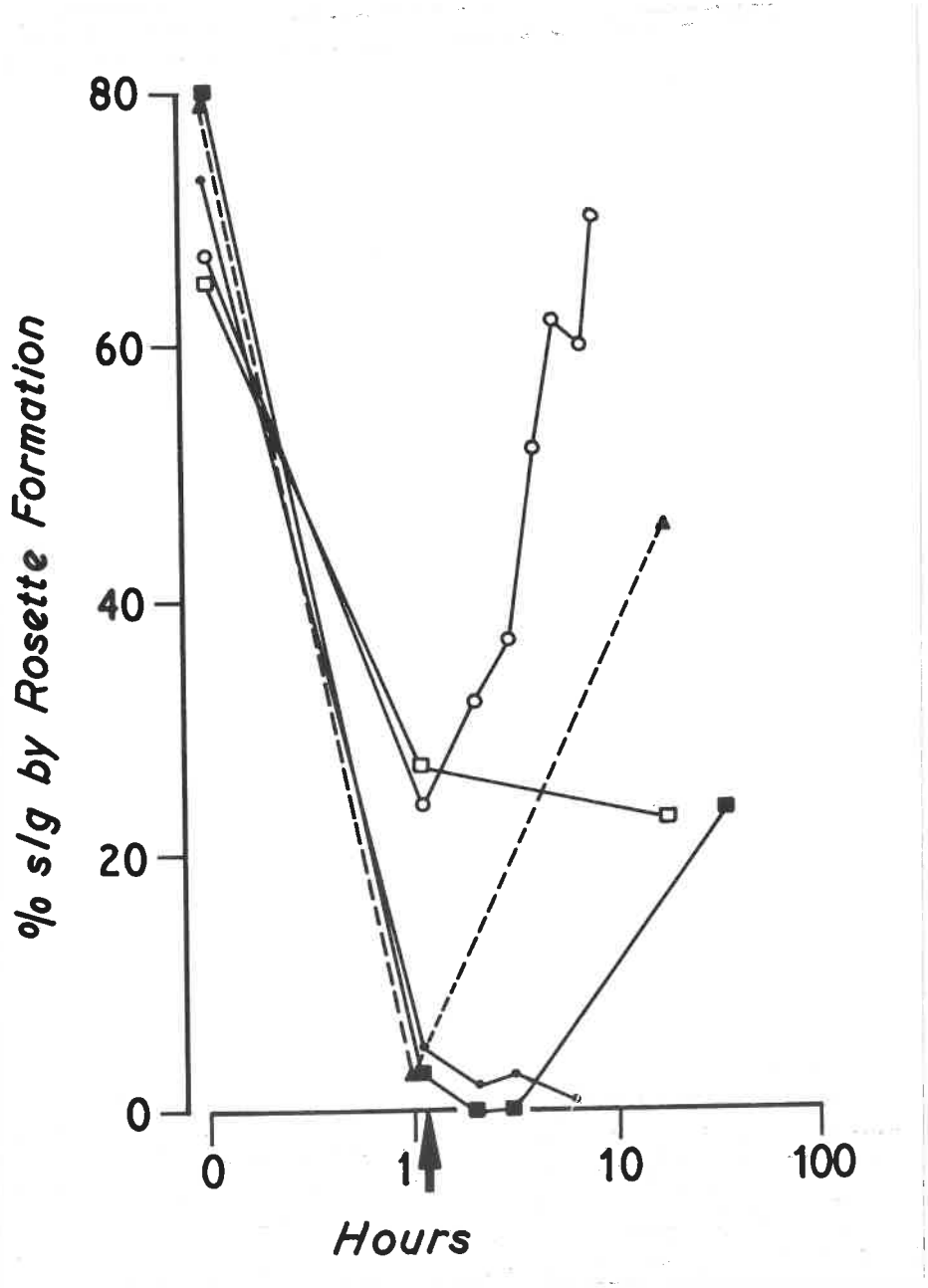

Figure 4.8 The stripping and subsequent re-expression of SIg, as monitored by SIg rosette formation, is shown. The experiments all involved leucocytes from patient HR. Stripping was performed with polyvalent (○—○), with anti-α (□—□) and with three different anti-δ (●—●, ■—■, ▲···▲) antisera. At the time indicated by the arrow, all cell preparations (including ○ and □) were completely capped or negative for SIg when tested by fluorescence.

Table 4.IV Cytoplasmic immunoglobulin staining of HCL cells

Patient	Tissue	Type	Percent mononuclear cells staining					
			κ	λ	μ	δ	γ	α
KD	PB	II	0	76^{7}	$-^{\Delta}$	0	70^{3}	0
DC	Spleen	I	90^{3}	0	-	-	-	-
FB	PB	I	67^{3}	0	0	0	69^{3}	0
	BM		49^{3}	0	0	0	48^{3}	0
AD	PB	I	0	43^{5}	0	0	0	0
LP	Spleen	II	0	79^{3}	0	0	-	-
LH	Spleen	II	96^{3}	2	$24^{2}/100^{6}$	0	$20^{2}/90^{6}$	-
FW	PB	II	0	53^{7}	0	0	0	0
GH	PB	I	26^{1}	0	12^{3}	0	26^{1}	17^{1*}
TN	PB	III	70^{6}	0	0	74^{3}	12^{4}	0
LK	PB	II	60^{3}	0	0	0	0	0

Superscript numbers indicate the pattern of fluorescence:

1, strong diffuse; 2, strong granular; 3, moderate diffuse;
4, moderate granular; 5, weak/moderate diffuse; 6, weak, diffuse;
7, very weak, diffuse.

Δ = not done

* = some cells showing strong IgA fluorescence resembled plasma cells

chain type. Although the staining was much weaker than that of control plasma cell preparations, it seemed unlikely that the fluorescence was attributable solely to SIg since in the multiple isotype cases, all of which had IgD at the surface, no intracytoplasmic IgD was detected, except in one case (TN, Table 4.IV). The possibility that γFc-bound extrinsic Ig was causing apparent cytoplasmic staining was minimized by the routine use of preincubation and washing. The possibility of interalization by either the phagocytosis or pinocytosis of extrinsic Ig during this preincubation cannot be excluded, but the light chain restriction and the diffuse nature of staining (except in one case - LH) make this an unlikely explanation for the intracytoplasmic staining pattern. The cytoplasmic Ig findings are therefore further evidence of immunoglobulin synthesis by HCs.

3.5 *Culture*

During the period of culture, the great majority of cells resembled HCs morphologically although, after 60 days, increasing numbers (up to 10%) of larger cells with a transformed appearance began to appear. Virtually all the leucocytes, including the larger cells, were positive for tartrate-resistant acid phosphatase and all the HCs, but not the larger cells, were positive for the μFc receptor. Other surface marker data are presented in Table 4.V. The cells consistently expressed SIg of a single light chain type (K). After short-term culture (18 days), only 36% of cells expressed SIg (mainly δ), while after more prolonged culture a greatly increased percentage of cells expressed SIg of multiple heavy chain isotypes, including IgA. After 66 days culture, the cells were tested for cytoplasmic Ig and only faint diffuse K reactivity was detected, λ and all the heavy chain isotypes being absent in both

Table 4.V Marker data of culture established from splenic HCs (patient HR)

Time in culture (days)	E	γFc	μFc	Mouse	SIg					
					K	λ	μ	δ	γ	α
18	4	99	92	0	36	<1	6	35	5	5
29	-	-	95	-	-	-	-	75	-	50
46	-	-	93	-	78	0	4	80	<1	82
66	0	93	86		77	<1	36	79	41	71

the morphological HCs and in the larger transformed cells. It should be stressed that no human serum was present in the culture medium, that the culture fluid was changed repeatedly, and that almost all the cells contained tartrate-resistant acid phosphatase; also it is noteworthy that, of the heavy chain isotypes, only δ was detected on an appreciable number of the cells in the first three weeks of culture. All these points make it highly likely that the results reflect the increasing expression on the same cell of genuine monoclonal SIg of multiple heavy chain isotypes.

4. DISCUSSION

All 24 cases of HCL in this study expressed SIg of restricted light chain type (16K, 8λ). In 13 cases, the only heavy chain isotype expressed at the surface was IgG, while in the other 11 cases multiple surface heavy chain isotypes (including IgG in 10 and IgA in 3), were detected.

The existence and incidence of genuine intrinsic SIgG on B cells has been the subject of much controversy, although very recently it has become widely agreed that SIgG is present on a small population of normal human lymphocytes (Cooper and Seligmann, 1977; Gathings et al, 1977) and on the cells of some cases of chronic lymphocytic leukaemia (Cooper and Seligmann, 1977; Brouet and Seligmann, 1977; Dhaliwal et al, 1978). In mice, most memory B cells carry IgG (Coffmann and Cohn, 1977; Black et al, 1978) and the less mature memory cells also carry IgD (Black et al, 1978). It is also of interest that a very successful method of removing SIg-positive B cells in man is the use of glass beads coated with $F(ab)_2$ fragments of human IgG and $F(ab)_2$ fragments of

anti-human IgG (Perlmann et al, 1976) indicating that IgG-bearing cells are probably more common than has been suspected.

The controversy over SIgG arose because of a number of important technical points. These include: the necessity of eliminating monocytes (Winchester and Fu, 1976; Cooper and Seligmann, 1977); the need to remove labile extrinsic autologous IgG (whether autoantibody or immunoglobulin bound by the γFc receptor) (Winchester and Fu, 1976; Horwitz and Lobo, 1975); and the avoidance of problems arising from the attachment of heterologous reagent antibody (Cooper and Seligmann, 1977; Siegal and Good, 1977). Therefore, in the present study particular care was taken with these technical aspects. First, HCL is characterised by an almost complete absence of monocytes (Seshadri et al, 1976), and this was shown to be the case in the present series (Section I, Chapter 4). Secondly, a routine preincubation step at 37°C removes the problem of autoantibody attachment since such antibody, although potentially monoclonal, is rapidly removed by short periods of incubation *in vitro* (Horwitz and Lobo, 1975; Winchester and Fu, 1976). Detection of extrinsic autologous IgG attached by the γFc receptor is unlikely to show the restricted light chain pattern consistently observed in the present study, more especially since only one of the patients (Section III, Chapter 4) had a paraprotein and half of the type II group expressed only λ light chains. Furthermore, SIg persisted unaltered in the cases tested after short-term culture for from 12 h to 4 days. In the case cultured for a prolonged period, although the percentage of cells expressing SIg varied, they continued to express multiple heavy chain isotypes, all of a single light chain type.

The problem of binding of heterologous reagent Ig via the Fc receptor, either because of soluble complex formation between reagent

Ig and residual serum Ig or as a result of aggregates formed in the reagent, is usually overcome by employing $F(ab)_2$ reagents (Winchester and Fu, 1976). The former problem does not arise with the rosette method used in the present study since the anti-Ig reagent is irreversibly bound to a solid matrix and therefore not free to form soluble complexes. Sheep IgG does not readily bind to the human γFc receptor (Alexander and Sanders, 1977), and Haegert has also demonstrated that the rosette method yields exactly comparable results for SIg, whether whole serum IgG or $F(ab)_2$ preparations are coupled to the indicator erythrocytes (Haegert et al, 1978; Haegert, 1978). Moreover, in the present study, the control employing non-immune sheep IgG was always negative, thus refuting Fc binding and also controlling for the possibility of attachment of reagent via the receptor for the $F(ab)_2$ fraction of IgG recently described on some human lymphocytes (Hofman et al, 1977).

Another important technical consideration is the specificity of reagents. In addition to the details given in the Methods, it should be noted that in the IgG-only cases as demonstrated with the rosette method, the affinity purified fluorescent reagents also detected only IgG. Also, the existence of these G-only cases, together with the case of SIgM and D, without G, substantiates the lack of cross-reactivity of the reagents used in the rosette method. This was further substantiated by the data obtained in CLL, where M and D alone was common. Similarly, the demonstration of SIgA in only three cases of HCL, and none in CLL, provides evidence of the lack of cross-reactivity of the anti-α reagent.

Because of the close relationship between HCL and CLL, the distribution of SIg phenotypes in these two chronic leukaemias was compared.

In this work, like others using the rosette method (Dhaliwal et al, 1978), it is shown that there is a group of CLL patients with multiple heavy chain isotype expression (type II) but that G-only cases (type I) are rare. The present study found a lower incidence of type II CLL patients than Dhaliwal et al, (1978), and indeed M- and D-only cases (type III) constituted the major group. Similar results in CLL have been obtained by the fluorescent method of SIg detection (Preud 'homme and Seligmann, 1972). The SIg results obtained in CLL with the more sensitive rosette method (Haegert et al, 1978) confirm the presence of a SIg negative group (Cooper and Seligmann, 1977; Jayaswal et al, 1977; Dhaliwal et al, 1978) (type IV) and the virtual absence of SIgA previously observed in both fluorescent and rosette studies (Cooper and Seligmann, 1977; Jayaswal et al, 1977; Dhaliwal et al, 1978). This is one difference between CLL and HCL, but more striking is the very low level of SIgM expression in HCL. In a very recent study of 15 cases of HCL by Jansen et al, (1978), who employed fluorescent staining of formaldehyde-fixed cells, 7 cases expressed SIgA (all type II) and only 6 cases expressed a low level of SIgM.

Attempts to remove SIg enzymically using trypsin or papain under standard conditions were unsuccessful since this treatment killed the HCs before removing SIg. This has also been the experience of others in HCL (Rieber et al, 1977; Braylan et al, 1977), although claims of complete removal and re-expression of SIg following trypsinization of HCs have been made (Leech et al, 1975; Kitani, 1976; Utsinger et al, 1977). Anti-Ig reagent, however, produced rapid and virtually complete removal of SIg as assessed by fluorescence and by the rosette method, and re-expression was seen to be taking place after 18 h of culture. In the one case where stripping, although complete by the fluorescent method,

was only partial as monitored by the rosette method (thus demonstrating the greater sensitivity of the latter method), re-expression was seen to have occurred in less than 12 h. This is interpreted as redistribution of persisting SIg, rather than genuine resynthesis. Since others have shown re-expression times of greater than 48 h in HCL following stripping with anti-Ig reagent (Rieber et al, 1977, 1978), reports of SIg re-expression after overnight culture following trypsinization (Leech et al, 1975; Kitani, 1976; Utsinger et al, 1977) must be interpreted with caution. However, HCs stripped by exposure to erythrocytes coupled to anti-Ig failed to re-express SIg in their continued presence, probably as a result of continuous stripping (Loor et al, 1972). It is conceivable, therefore, that the anti-Ig method of stripping, which is known to result in the persistence of some anti-Ig on the cell membrane for up to 3 days (Nash and Ling, 1976), results in a falsely long re-expression time (Ault and Unanne, 1977).

One of the stripping and resynthesis experiments suggested that the cells from this case were secreting IgG. This evidence of IgG secretion was substantiated both by the demonstration of IgG (of a single light chain type) in the medium from short-term cultures of HCs, and by the detection of cytoplasmic Ig in a number of cases of HCL.

It is therefore concluded that the majority of cases of HCL express at the surface IgG which is a product of the cell. Whether this SIg is inserted into the membrane as 'true SIg', or whether it is attached via the Fc receptor immediately after secretion, requires further investigation. As an extension of this point, IgG secreting cells may, at least partially, account for the higher percentage SIg positive cells detected by fluorescence when whole Ig rather than $F(ab)_2$ reagents are used, especially since a population of normal human PB cells actively

synthesising and secreting Ig has recently been identified (Strelkauskas et al, 1977). Thus it is likely that intrinsic secreted IgG in the immediate micro-environment of the secreting cell is bound to anti-Ig and the soluble complex formed with whole anti-Ig is able to attach via its Fc region, whereas the $F(ab)_2$ complex is not. This seems at least as plausible an explanation of the difference in SIgG positive cells detected by whole and $F(ab)_2$ anti-Ig reagents as that of Winchester and Fu (1976) who proposed Fc-binding of complexes formed with extrinsic serum IgG. This is substantiated by the fact that, when the precautions of preincubation and thorough washing are carried out, leukaemic monocytes, which have a strong γFc receptor (Koziner et al, 1977), are completely negative for SIg even by the rosette method (Cawley et al, 1978b).

The results in this section clarify the previously confused situation in the literature concerning SIg expression and Ig synthesis in HCL. Work showing polyclonal SIg patterns (Preud 'homme and Seligmann, 1972; Stein and Kaiserling, 1974; King et al, 1975; Reyes et al, 1976; Zidar et al, 1977; Banerjee et al, 1978) is probably explicable in terms of Fc binding. The literature showing a monoclonal pattern of SIg expression is fully compatible with the present findings since examples of SIgG alone (Aisenberg et al, 1973; Leech et al, 1975; Kitani, 1976; Rieber et al, 1977, 1978; Catovsky, 1977; Jansen et al, 1978) and mixed heavy chain expression (including IgG and IgA) have been described (Haak et al, 1974; Haegert et al, 1974; Stein and Kaiserling, 1974; Deegan et al, 1976; Kitani, 1976; Boldt et al, 1977; Rieber et al, 1977, 1978; Jansen et al, 1978). These mixed heavy chain types are in accord with recent evidence that the onset of expression of different Ig classes is completely random and that any class can appear singly or in combination with others and that this need not be a sequential step-wise phenomenon (Gearhart, 1977).

Similarly, the demonstration of IgG (and IgM) secretion in HCL has precedents in the literature (Rubin et al, 1969; Stein and Kaiserling, 1974; Debusscher et al, 1975; Gordon and Smith, 1978), and the presence in a paraprotein in one case (Section III, Chapter 4) suggests *in vivo* synthesis.

In several of the mixed phenotype cases, and in several mixed cases reported by others (Haak et al, 1974; Haegert et al, 1974; Deegan et al, 1976), the percent cells bearing SIgG is lower than that bearing SIgM or (more especially) SIgD. It is likely, therefore, that these mixed cases bear relatively low levels of SIgG which require sensitive methods of detection such as the rosette method employed in the present study, or autoradiography (Rieber et al, 1977, 1977). Prefixation of the cells with formaldehyde before immunofluorescent staining appears to enhance the sensitivity of this method and SIgG is readily detected on most cases of HCL (Jansen et al, 1978). The two mixed cases tested on the same occasion by both the rosette method and fluorescence in the present section showed a much lower percentage of SIgG-positive cells by fluorescence.

III Typical HCL with IgG K paraproteinaemia

1. INTRODUCTION

In the last section it was shown that IgG secretion by HCs *in vitro* was probably a general phenomenon. In this section a patient with typical HCL who also had an IgG (K) paraprotein is described, and it is suggested that the HCs are secreting the immunoglobulin.

2. MATERIALS AND METHODS

2.1 The patient, significant case history

The patient (FB) was included in the previous chapters as a typical case of HCL. However, although he exactly fulfilled the immunological criteria of HCL, further details of the diagnosis are given here.

Briefly, the relevant clinical findings were: gross splenomegaly without lymphadenopathy or hepatomegaly; pancytopenia (with a differential showing 37% HCs, and no monocytes, in a wbc count of 2.3 x 10^9/l at presentation); marrow aspiration and trephine biopsy revealed diffuse infiltration with HCs and increased reticulum fibrosis. Shortly after presentation the patient's thrombocytopenia gave rise to increased bruising and this responded to prednisone therapy.

Routine serum electrophoresis showed a paraprotein band in the $\alpha 2/\beta$ region of 6 g/l (8% of total protein) and immunoelectrophoresis showed this was IgG K. There was no immuneparesis (IgG 15 g/l (NR 5-16), IgA 2.4 g/l (NR 1.0-4.5), IgM 0.6 g/l (NR 0.4-1.7)). No Bence-Jones protein was detected in concentrated urine. Skeletal survey was normal,

showing no evidence of lytic bone lesions.

Morphologically, the HCs were typical both at light and electron microscopy. By TEM, some cells contained typical ribosome lamellar complexes (Katayama et al, 1972a), and by SEM the cells showed the characteristic combination of ruffles and villi (Golomb et al, 1975). Cytochemically, the cells possessed the tartrate-resistant isoenzyme of acid phosphatase (Yam et al, 1971) and gave the characteristic HC esterase pattern (Higgy et al, 1978) in a high number of cells and, incidentally, esterase staining confirmed the absence of monocytes typifying HCL (Burns et al, 1976). The leucocyte alkaline phosphatase score was repeatedly high (>150), a finding again characteristic of HCL (Hayhoe et al, 1977). Most HCs were capable of ingesting latex. The immunological marker profile of the patient's cells will be given below, but in addition, on the 2 occasions studied, the membrane turnover of the μFc receptor showed the 6 h peak-to-peak re-expression time typical of HCL (Burns et al, 1977b).

2.2 Immunological marker studies

The reagents and the methods used for all of the marker studies in Results have been described in previous sections. The microtitre agglutination method described in Section II, Chapter 4, was however, modified slightly as follows.

Isolated mononuclear cells from PB were suspended in HBSSA and incubated for 24 h at 37°C. Control cultures were incubated for the same time at 0°C, or at 37°C in the presence of puromycin (2.5 μg/ml). The Ig content of the supernatants was determined by microtitre (Cooke Engineering Co.) agglutination of sheep erythrocytes coupled by chromic chloride to monospecific, affinity purified, sheep IgG against human IgD, IgM, IgG, IgA, K and λ. These antisera were purchased from

Dr. J.L. Smith, Southampton. The concentration of secreted IgG and K was calculated by comparing the agglutination titre with that produced by normal human IgG of known concentration; for K a 2:1 ratio of K:λ in normal IgG was assumed. Supernatants from the control cultures were invariably negative for coupled erythrocyte agglutination.

3. RESULTS

3.1 *Immunological markers*

The immunological marker studies carried out on patient FB are shown in Table 4.VI. A high proportion of the cells possessed the μFc receptor, and he consistently belongs to SIg type I. Cytoplasmic staining was performed on three occasions, and both PB and BM HCs stained with a moderate diffuse pattern for IgG and K, but for no other Ig class.

3.2 *SIg re-expression*

On 20.8.78, PB mononuclear cells were tested for SIg by the rosette method. Only IgG and K were detected (Table 4.VII). Both the IgG and K were independently stripped off, with monospecific sheep antisera for 30 min at 37°C, and the cells were thoroughly washed with sterile HBSS. Upon retesting for SIg, it was observed that the anti-IgG had also stripped off K, and the anti-K had removed IgG-indicating that no other Ig classes were present on the cells.

The stripped cells were then cultured in TC 199 + 10% foetal calf serum (tubes gassed with CO_2) and, as a control, untreated cells were cultured in parallel. At various time intervals (Table 4.VII), the cells were tested for SIgG and K re-expression. The small number of cells which were available for study (the patient was still severely

Table 4.VI Immunological marker studies on patient FB

Date	Tissue	% rosette formation															
						SIg						% cells staining‡					
		E	γFc	μFc	EAC	κ	λ	μ	δ	γ	α	κ	λ	μ	δ	γ	α
21.1.77°	PB	50	62	55	33	47	0	2	3	53	0	-Δ	-	-	-	-	-
19.2.77	PB	38	68	37	-	57	2	3	3	46	-	-	-	-	-	-	-
17.6.77	PB	50	82	55	-	40	2	2	2	38	-	42	0	0	0	17	0
12.5.78	PB	37	73	58	15	24	2	0	0	13	0	-	-	-	-	-	-
11.8.78	PB	43	63	49	4	27	0	0	0	30	0	67	0	0	0	69	0
11.8.78	BM	23	72	23	8	44	0	0	0	43	0	49	0	0	0	48	0

‡ Moderate diffuse fluorescence

° SIg determined by immunofluorescence

Δ Not done

Table 4.VII Re-expression of SIg following stripping with anti-Ig reagents

Time	Control		Capped with anti-IgG		Capped with anti-K	
	IgG	K	IgG	K	IgG	K
Pre-cap*	40	40	-Δ	-	-	-
0	46	46	0	0	3	0
12 h	30	30	3	7	2	5
18 h	-	28	-	14	-	13
72 h	31	33	-	-	26	22

* Other SIg classes were negative

Δ Not done or not applicable

leucopenic), and the appreciable decrease in viability, particularly of some cultures, limited the number of tests which could be carried out. However, with the fluorescein diacetate method routinely employed for scoring, only viable cells are counted and the dead cells did not present a major problem, especially since no particular sub-population appeared to be dying off. The results in Table 4.VII indicate that SIgG and K were progressively restored, and that by 72 h of culture this process was virtually complete. At no time were any plasma cells seen in cytocentrifuge preparations of the cultured cells.

3.3 *Ig secretion*

The culture supernatants from PB HCs cultured for 24 h in serum-free medium were tested for agglutination of sheep erythrocytes coupled to sheep IgG against human Ig of different classes on two occasions (12.5.78 and 11.8.78). On both occasions only IgG and K synthesis was detected. On the earlier date, there appeared to be an excess of light-chain secretion: IgG, 1.95×10^{-1} µg/10^7 cells; K, 5.1×10^{-1} µg/10^7 cells. On repeated testing, there was an equal secretion of both IgG and K: IgG $0.77 \times 10^{-1}/10^7$ cells; K $0.8 \times 10^{-1}/10^7$ cells, and the level of secretion appeared to have decreased. However, it should be stated that at such dilutions of the standard IgG solution, the margin of experimental error may be high, and also, the well selected as the end point is somewhat subjective since clear-cut positive to negative was not obtained, (although at the lower dilutions the agglutination by supernatants was very definite and this was confirmed microscopically).

4. DISCUSSION

In this section, a patient with clinically, morphologically and immunologically typical HCL is described with an IgG paraprotein, and the evidence suggests that the HCs are producing the paraprotein.

The HCs are synthesizing IgG as evidenced by the cytoplasmic staining, SIg re-expression and the supernatant agglutination experiments.

It was shown in the previous section, that cytoplasmic staining (CIg) in HCL is a regular feature when powerful antisera are used. Also since SIgD positive cells were negative for cytoplasmic IgD, the technique is probably not detecting SIg, and the diffuse nature of staining, together with light chain restriction, makes pinocytosis an unlikely explanation. Cytoplasmic Ig is readily detected in plasma cells, but in CLL intracytoplasmic staining by immunofluorescence is generally only detectable in cases with a paraprotein (Preud 'homme and Seligmann, 1974): this type of staining, therefore, probably reveals only quite high levels of active Ig synthesis.

In the present case, the demonstration of SIgG re-expression after stripping was particularly important, to eliminate γFc binding of the paraprotein as a possible explanation for HC SIg. The time taken for the SIg to re-express during culture is of some interest. Dhaliwal et al, (1978) have suggested that SIgG may take longer to reappear on the membrane than other Ig classes, and certainly in this case the time taken (72 h) far exceeds the routine culture time used by Seligmann's group (Preud 'homme and Seligmann, 1974; Brouet and Seligmann, 1977). As discussed in the last section, a long time for re-expression may also be a feature of HCs, but the routine culture time of 6-7 h for

SIg re-expression in CLL employed by Seligmann and colleagues might account for their repeated failure to detect SIgG re-expression in this disease.

The detection of IgG and K in the supernatant of cultured HCs suggested IgG K synthesis by these cells. No plasma cells were detectable in cytocentrifuge slides of the mononuclear cell preparations, and Ig was not detected in control cultures which contained puromycin. It is possible that the test was detecting SIgG released from the membrane (Vitetta and Uhr, 1972) but the quantity of Ig suggested active secretion. Also, type II SIg HCs, with both SIgD and SIgM, appear to secrete only IgG by this method (Section II, Chapter IV).

The demonstration of paraproteinaemia in HCL strongly supports the evidence favouring a B lymphoid origin for HCs. No previous cases of IgG paraprotein in this disease have been reported although the evidence of secretion by HCs reported here, and in the previous section, suggests that more might be expected. The present patient only had a small paraprotein peak and more sensitive methods of detection may well reveal more cases as has occurred in CLL. In this disease, the incidence of paraproteins (IgM and IgG) is about 20% (Preud 'homme et al, 1975), but with more sensitive methods, monoclonal serum Ig has been found in 75% of cases (Eipe et al, 1976).

The only previous report of HCL with paraproteinaemia was that of Golde et al, (1977a) in which an IgM paraprotein was present. However, their patient was atypical in a number of respects and the diagnosis has been questioned (Katayama, 1977; Banerjee et al, 1978). Thus, Golde's case had marked leucocytosis, no marked thrombocytopenia, only 20% "hairy cells" in a count of $66 \times 10^9/l$, and there was immuneparesis. The ultrastructure of the cells was described as being lymphoid, rather

than that characteristic of HCs, and no ribosome lamellar complexes were seen. No histopathological information was given and some of the immunological marker characteristics, in particular the presence of a receptor for C3, were atypical for HCs. Their main diagnostic criterion, the tartrate-resistant isoenzyme of acid phosphatase (Yam et al, 1971) typical of HCL, can frequently be found in the cells of CLL (Katayama et al, 1977). Moreover, the patient's paraprotein had been present for some twenty years, and light chain studies were not performed to establish that the pathological cells bore the same isotype.

The evidence of SIg expression and Ig secretion by HCs provided in this chapter, suggest that further cases of paraproteinaemia in HCL can be predicted, but that these are more likely to be IgG than IgM in type.

CHAPTER 5

PROBLEMS IN IMMUNOLOGICAL MARKER STUDIES

I CLL with SIg antibody having activity against ox erythrocytes

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II HCL with hybrid membrane phenotype

1. INTRODUCTION
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PROBLEMS IN IMMUNOLOGICAL MARKER STUDIES

I CLL with SIg having antibody activity against ox erythrocytes

1. INTRODUCTION

The immunoglobulin nature of SIg on lymphocytes has been known for some years (Warner et al, 1970), and antibody activity has been described for the monoclonal SIg on the cells of some lymphoproliferative disorders (Preud'homme and Seligmann, 1974). The range of antibody activities of such monoclonal SIg is remarkably limited and most appears to be directed against self antigens (Brouet and Seligmann, 1977). Only one case of heteroantibody activity by the monoclonal SIg of CLL has been reported (Brouet and Prieur, 1974) and this was against the Forssman antigen of sheep erythrocytes.

In the present section, a case of CLL is described with monoclonal SIg on the cells directed against an antigen on ox erythrocytes. Since many of the surface marker studies employ ox erythrocytes as indicator cells, the initial findings from this patient were something of a problem and this case provides a warning on the importance of controls in rosette testing.

2. MATERIALS AND METHODS

2.1 Patient

The patient was an old man of 97 who died shortly after presentation. He had a high leucocyte count and an IgM (K) paraprotein.

2.2 Immunological marker studies

Mononuclear cells were isolated as previously described. Also, all the reagents for marker studies and the conditions employed were as described in previous chapters.

3. RESULTS

3.1 Surface markers

The initial studies on this patient showed: γFc 93%; μFc 88%; EAC 84%; mouse erythrocytes 49%; sheep erythrocytes 1%. However, the unsensitized ox erythrocyte (ox) test used as a control for γFc and μFc revealed 79% rosette formation. Trypsinization of the ox erythrocytes did not reduce rosette formation, indeed ox rosette formation increased to 84%.

3.2 Investigation of ox rosette formation

'Antigen' capping The first premise was that SIg on the CLL cells had antibody activity against an antigen on the ox. To determine whether SIg, and thus antibody activity, could be capped off with the ox antigen, the formed ox rosettes were incubated at 37°C and aliquots examined periodically. After 30 min at 37°C, no marked polarity of the rosetting ox was seen and rosette formation was only reduced by 10%.

Antibody capping of SIg Lymphocytes from the patient were stained for SIg by direct immunofluorescence, and a monoclonal M, D, K pattern of staining was obtained for approximately 90% of the cells (Table 5.I). The stained cells were allowed to cap at 37°C for 40 min and aliquots removed at this time showed that the fluorescent pattern of staining (for M, D and K) was capped, or extensively patched, on all the positive cells. The capped cells were then tested for ox rosette formation

Table 5.I The effect of removing SIg on ox rosette formation

Test	κ	λ	μ	δ	γ	α
Fluorescent SIg staining (%) ‡	93	2	92	87	2	0
Capping and ox rosette testing *	5	70	14	58	68	72

‡ Lymphocytes were treated with FITC-labelled monospecific rabbit antiserum to the class indicated for 20 min at 4°C. The stained cells were then thoroughly washed at 4°C and read for % SIg staining.

* The stained and washed cells were incubated for 40 min at 37°C and tested for ox rosette formation.

and the results are shown in Table 5.I.

Since virtually no cells possessed SIgG, A or λ, these results provide the necessary control for rosette formation after the capping treatment. From Table 5.I it can be seen that most of the antibody activity was removed with K, and that removing SIgM also reduced ox rosette formation to a major extent. Capping off of SIgD reduced ox rosette formation by only 14%: the reduction in rosetted formation caused by removing SIgM + SIgD was 100%.

4. DISCUSSION

The leukaemic lymphocytes from this patient possessed monoclonal SIg directed against an antigen on ox erythrocytes. The capping experiments described demonstrated that the SIg was restricted to IgM and IgD, and confirms the idiotypic identity of these two classes on the cells of CLL (Fu et al, 1975). The results also suggested that IgM predominated over IgD in this case.

Naturally occurring antibody activity of the SIg on lymphocytes against erythrocyte antigens is not uncommon in the lymphoproliferative diseases (LPD) since the cells from patients with chronic cold agglutinin disease will form rosettes with human O erythrocytes which have I or i antigen (Feizi et al, 1973). Antibody activity against a heteroantigen is, however, very rare in the LPD, and only one case of CLL, which bound the Forssman antigen of sheep and cat erythrocytes, has been described (Brouet and Prieur, 1974). Another case which probably displayed the same phenomenon, since the CLL cells spontaneously bound both ox and sheep erythrocytes, was mentioned by Pichler and Knapp (1977). The specific antigen detected by SIg in the present case was not determined because of the early demise of the patient, however, it

was not the Forssman antigen since sheep erythrocytes did not bind; another type of heteroantigen activity can therefore be added to SIg antibody activity in the LPD - against an ox erythrocyte antigen which is not destroyed by trypsin.

Not surprisingly, the serum from the present patient agglutinated untreated ox erythrocytes, thus confirming previous work in animal models that SIg and secreted Ig have the same idiotype (McConnell, 1971). Ox erythrocytes themselves, however, did not strip the SIg. After 5 min of culture at 37°C, preformed ox rosettes showed marked polarity, but by 30 min complete morulla-like rosettes were again seen and rosette formation was reduced by only 10%. Murine splenic lymphocytes with SIg activity for sheep erythrocytes (induced by immunisation) provided a similar phenomenon (Ashman, 1973). Thus although sheep erythrocytes induced some cap formation when rosettes with the lymphocytes were incubated at 37°C, cap formation was much slower than that caused by anti-Ig antibodies and only caused some 14% cells to cap. It was suggested that the bulky erythrocytes physically resist the efforts of the lymphocyte to pack SIg together into caps (Ashman, 1973).

Ox erythrocytes are widely used as indicator cells in rosette tests, since, in contrast to sheep, mouse or human erythrocytes they do not adhere to any lymphocyte subpopulation (Hallberg et al, 1973). The case described in the present section illustrates that non-adherence of ox cannot be assumed, particularly in the LPD with a clonal expansion of cells bearing monoclonal SIg, and that care in checking the unsensitized erythrocyte control must always be observed.

II HCL with hybrid membrane phenotype

1. INTRODUCTION

Individual surface markers differentiate B and T cells with varying degrees of certainty, and it is generally agreed that a panel of immunological markers is required to assign a particular cell to either category. In general, the ability of human lymphocytes to form E rosettes and to react with anti-T-cell antisera are characteristics of T cells, whereas B lymphocytes are characterized by the presence of SIg, C3 receptors and the presence of readily detectable Ia-like antigen. However, in some instances lymphocytes are detected which exhibit combined B- and T-cell features (Cooper and Seligmann, 1977) and this has been particularly true in human leukaemias where a variety of unusual phenotypic markers has been described (Sandilands et al, 1974; Hsu et al, 1975; Toben and Smith, 1977).

In this section a case of HCL with unusual combined B- and T-cell membrane characteristics is described. In addition, the changes in membrane phenotype observed in the cells from the patient over a 13 mo period amply confirm the observation of Hsu et al, (1975) that sequential studies in such cases are essential.

2.1 Patient

The patient was clinically and haematologically absolutely typical of HCL. He has been included in the series of patients described in Chapter 2. The unusual marker phenotype of his cells (below) did not give rise to any unusual features.

2.2 Immunological marker studies

The reagents and tests used for the study of this patient have

all been described in previous chapters, except for the anti-T-cell serum. This serum (Greaves and Janossy, 1976) was an extensively absorbed rabbit antiserum to monkey thymic cells and was kindly given by Dr. G. Janossy, ICRF Membrane Immunology Laboratory, Lincoln's Inn Fields, London. Before use, the antiserum was ultracentrifuged, and in staining tests an indirect immunofluorescent technique was employed using FITC-labelled goat anti-rabbit serum.

3. RESULTS

3.1 Morphological and cytochemical studies

In all experiments, mononuclear cell preparations were cytocentrifuged and stained to exclude granulocyte contamination (always less than 5%) and the characteristic absence of monocytes was confirmed.

The percentage PB mononuclear cells (11.1.77) showing the HC esterase pattern was 65%, while some 28% possessed the single dot pattern characteristic of normal T lymphocytes.

In the splenic cell suspension (12.1.77), 92% of the cells showed acid phosphatase activity, and 90% gave the HC esterase pattern. Some 3% of splenic cells showed the T-cell dot pattern with esterase staining.

Transmission electron microscopy of the PB showed many morphologically typical HCs, and a proportion of these were demonstrated to contain the ribosome-lamellar complex. The almost total replacement of splenic tissue by HCs was confirmed at the ultrastructural level and some of the HCs were again seen to contain the ribosome-lamellar complex.

3.2 *Immunological marker studies*

The marker studies performed on the unusual cells from patient LH over a 13 mo period are shown in Table 5.2. The expression of the individual B and T markers fluctuated over this time and two main patterns are discernable

In the first week of December, 1977, the patient suffered a severe trauma (unrelated to HCL) and, during his stay in hospital, he contracted a bacterial infection. After this time the patient underwent apparent 'spontaneous remission' and very few HCs could be detected in his PB either morphologically or immunologically. There were, however, many HCs still present in his bone marrow (24.2.78) although the E rosette forming capacity seen in the patient's HCs in earlier studies was no longer evident.

Before this traumatic event, the HCs from this patient, in addition to bearing the conventional B-cell markers normally present on HCs (SIg, Ia), appeared to possess T-cell features (E rosettes and anti-T cell staining). The summation of B-cell and T-cell features, on most occasions, greatly exceeded 100%. Also, morphologically typical HCs were seen to form E rosettes. The HCs did not form rosettes with erythrocytes from a variety of other species including ox, rabbit, guinea pig, rat and donkey. They were also negative for the Epstein-Barr nuclear antigen and for the C3b receptor.

The combination of B and T cell markers on the cells of the patient are particularly well documented for his splenic cells (Table 5.2) which were morphologically and cytochemically almost all HCs. In addition to the data presented in Table 5.2, over 90% of splenic mononuclear cells possessed cytoplasmic IgM, IgG and K as detected by diffuse staining by direct immunofluorescence.

Table 5.2 Immunological marker studies on isolated mononuclear cells from PB and spleen

Material	Date	E	Anti-T cell staining	SIg						Ia-like p29,34 antigen	γFc	μFc	Mouse erythrocytes
				κ	λ	μ	δ	γ	α				
PB	11.1.77	84	-[+]	-	-	-	-	-	-	-	30	34	-
PB	12.1.77	95	75	-	-	-	-	-	-	-	62	-	9
Spleen	12.1.77	73	-	78	0	5	82	-	0	90	96	23	47
PB	26.1.77	76	-	19	3	5	18	5	-	-	-	22	-
PB	12.2.77	79	-	52	4	11	44[Δ]	10	-	-	65	-	1
PB	26.2.77	52[*]	70	70	16	77	37	72	0	79	78	66	-
PB	13.3.77	84	-	34	-	-	33	-	-	-	47	38	-
PB	18.5.77	49	-	50	3	9	46	9	-	50	79	24	-
PB	30.6.77	40	-	44	2	0	44	27	-	-	80	-	-
PB	2.12.77	22	-	64	<1	25	53	18	2	-	77	21	<1
PB	6.2.78	70	-	11	2	3	3	8	1	-	50	10	-
PB	20.2.78	85	-	6	0	2	2	2	0	-	38	7	-
BM	24.2.78	33	-	50	1	15	55	10	0	-	65	1	-

[+] Not done: [Δ] SIg persisted for 3 days in culture: [*] E rosette formation increased to 76% after 6 h of culture at 37°C (Table 5.3).

2.3 Investigations of the nature of E rosette formation

Conditions affecting E rosette formation Table 5.3 shows some of the physical and chemical conditions which affected E rosette formation by this patient's HCs. It was considered that some serum factor attached to the cell membrane (perhaps anti-heterophile antibody attached via the Fc receptor) was causing spurious E rosette formation. Culture of the cells for 6-12 h, followed by thorough washing in warm medium, however, increased rosette formation (Table 5.3). On the other hand, a reduction in E rosette formation by HCs, similar to that seen with normal T-cell controls, was observed when rosette formation was carried out at 4°C, without centrifugation, or in the presence of sodium azide or cytochalasin B. Thus, like E rosette formation by normal T lymphocytes, this E rosette formation was energy dependent and required an intact micro-filament system.

Blocking of E rosette formation E rosette formation by HCs was specifically blocked by an anti-T serum (which also stained a percentage of the HCs (Table 5.2)), but antisera to the Ia-like antigen, p29,34, Sig, or anti-hairy-cell serum (all of which also stained most HCs) did not block rosette formation (Table 5.4). The anti-T-cell serum did not block HC SIg rosette formation, although it did reduce γFc rosette formation from 65% to 47% (12.2.77).

The effect of SIg removal The possibility existed that E rosette formation might be caused by SIg antibody activity which was not blocked by antisera directed against the Fc region of SIg. SIg was therefore removed by stripping with antiserum and E rosette testing performed on the SIg-negative cells. E rosette formation was not reduced by this treatment, and in some cases it was actually enhanced (Table 5.4).

Table 5.3 Factors affecting E rosette formation[+]

E Control	Treatment	E after treatment
52	Cells cultured for 6 h and washed	76
84	Cells cultured for 12 h and washed	93
76	Formed rosettes incubated 30 min 37°C	2
52	Rosette formation at 4°C	26
76	Azide (10^{-1}M)	18
84	Cytochalasin B (20 µg/ml)	58

[+] Results from testing on different occasions. Some tests were repeated on different dates with similar results.

Table 5.4 The effect of blocking with antisera

and of removal of SIg on E rosette formation

Type of Experiment	% E rosette formation							
	Control	Anti-T‡	Anti-Ia$^{\Delta}$	AHS*	Anti-K+	Anti-μ+	Anti-δ+	Anti-γ+
Blocking	52	0	59	51	59	53	36	52
	79	4	-	67	72	-	77	-
Capping§	52	-	-	-	65	69	66	82
	77				66	77	65	-

‡ The anti-T cell serum blocked at 1/10 and 1/20 and had no effect on SIg rosette formation

Δ Antiserum to the p29,34 Ia-like antigen used at 1/20

* Anti-HC serum at 1/25

+ Anti-Ig reagents blocked SIg rosette formation

§ SIg shown to have capped with each of the antisera by direct immunofluorescence and by the failure of cells to form SIg rosettes

4. DISCUSSION

In this section, a case of HCL is described in which the morphologically typical HCs possess both B and T cell surface markers. Analysis of the marker studies showed that several B-cell and T-cell features were present on the same cell since the sum of cells possessing features of both usually greatly exceeded 100%.

Thus, the patient's HCs possessed the B-cell features shown to be typical of HCL. They bore SIg and the Ia-like antigen, and, splenic cells at least, synthesized cytoplasmic immunoglobulin. A proportion of splenic HCs also formed rosettes with mouse erythrocytes which is considered to be a B-cell marker (Siegal and Good, 1977).

However, a high percentage of the patient's PB and splenic mononuclear cells both formed E rosettes and stained with an anti-T-cell serum. In addition, formation of these E rosettes required conditions known to be necessary for E rosette formation by normal T lymphocytes (Jondal, 1976) and it was specifically blocked with the anti-T cell serum but not by an antisera to the Ia-like p29,34 antigen or by an anti-hairy cell serum. Removal of SIg did not reduce rosette formation, indeed in some tests this treatment enhanced E rosette formation perhaps by clearing the membrane area surrounding the E receptor. Clearly E rosette formation was not caused by SIg antibody activity for an antigen on sheep erythrocytes as was described for a case of CLL in the last section.

This patient's HCs therefore appeared to be true hybrid cells with the surface marker profile: E^+, anti-T^+, μFc^+, SIg^+, CIg^+, Ia^+, γFc^+, Mo^+, $C3b^-$, $EBNA^-$.

The existence of a human leukaemia with true hybrid cells bearing

both an E receptor and SIg is not without precedent. Hsu et al, (1975) have reported a case of lymphosarcoma cell leukaemia in which sequential studies of leukaemic T cells revealed an increasingly large proportion of cells generating SIg. In addition, Brouet and Prieur (1974) have reported a case of CLL that may have been similar since it expressed SIg of restricted light chain type and rosetted with sheep erythrocytes, but no blocking studies were reported. There is also a recent report of an Ig secreting, human B-lymphoblastoid cell line which both binds an anti-T-cell serum and forms E rosettes (Pellegrino and Ferrone, 1978).

Hsu et al, (1975, 1976) have emphasized the importance of sequential studies in unusual cases of this kind, since the marker profile may change and their case showed increasing SIg expression on E-positive cells. The present case appears to have been studied during a different transition with SIg^+, E^+ cells gradually losing the E receptor. Thus shortly before the patient's infection and 'spontaneous remission', and subsequently, very few, if any, HCs are forming E rosettes. The high percentage of E-rosette forming cells observed in the most recent studies appear to be normal T lymphocytes by cytochemical and morphological criteria. Also, the SIg^+ HCs seen in bone marrow no longer formed E rosettes. Certainly this work bears out Hsu's point about the necessity of sequential studies but the reasons for the changing phenotype remain unclear.

Scattered cases of T-cell derived CLL have been reported (Brouet and Seligmann, 1977), and very occasionally such cases, while lacking SIg, have been shown to possess less definitive B-cell markers such as receptors for γFc and fixed C3 (Toben and Smith, 1977). These T-cell cases of CLL were frequently associated with unusual clinical

features such as skin lesions (Brouet and Seligmann, 1977). Only one other case of HCL with T-cell features has been described (Saxon et al, 1978a), and the cells from this case did not possess any of the common B-cell markers. Like the present case, however, the cells were morphologically typical HCs and the patient was clinically and haematologically typical of HCL.

Finally, the existence of cases of HCL with T-cell features provides additional evidence for the lymphoid, rather than monocytoid, nature of HCs.

Chapter 6

CONCLUSIONS

The classification of HCL and its position in a scheme of B-cell differentiation

CONCLUSIONS

The classification of HCL and its position in a scheme of B-cell differentiation

Hairy-cell leukaemia is a distinct clinicopathological entity in which characteristic mononuclear cells, whose most distinctive feature is the possession of numerous surface projections or hairs, circulate in varying numbers and infiltrate a variety of organs, especially the bone marrow and spleen.

The first unequivocal recognition of HCL was by Bouroncle et al, (1958) who identified 'reticulum cells' with a serrated border in a group of 26 patients presenting a well-defined clinicopathological picture. The authors concluded that this constituted a distinct entity that they chose to term leukaemic reticuloendotheliosis. Subsequent work has amply confirmed this conclusion (Catovsky et al, 1974b; Burke et al, 1974; Katayama and Finkel, 1974) and there is now wide recognition of this distinct disease entity.

In the years following Bouroncle and colleague's classic paper, the terms leukaemic reticuloendotheliosis and the more descriptive hairy-cell leukaemia (Schnitzer and Kass, 1974) came to be regarded by many authors as synonymous, whereas in fact leukaemic reticuloendotheliosis originally had a broader usage covering what are now regarded as several distinct entities including monocytic leukaemia and a variety of non-Hodgkin's lymphomas, as well as HCL. The selection of this unfortunate name by Bouroncle et al may account, at least in part, for the large number of confusing terms which have been regarded as synonyms for the entity, but which have probably not

referred to HCL; these include histiocytic leukaemia, histioleukaemia, reticulosis, malignant leukaemic reticulo-histiocytosis and aleukaemic reticulosis (early literature reviewed by Bouroncle et al, 1958). Hence, confusion about the clinicopathological entity under consideration has been one of the reasons for controversy over the nature of the hairy cell and the term hairy-cell leukaemia is preferred.

The early literature considered the HC to be derived from primitive undifferentiated reticulum cells whose normal progeny would have included cells of the lymphoid, myeloid and monocyte-macrophage series (Bouroncle et al, 1958; Mitus et al, 1961). The hybrid features of HCs are readily understood in terms of this scheme, but since more recent work has shown that the lymphoid and monocytoid series develop independently from a very early stage, the existence of such a hybrid cell type has proved both intriguing and perplexing to immunologists and haematologists alike.

Many of the monocytic features of HCs can possibly be explained by the nature of the cell membrane. In almost all manipulations HCs are 'sticky': they tend to clump together during handling and before any immunological tests they must be very thoroughly washed and dispersed, much more than normal lymphocytes. In lymphocytes, the hairs are microvilli (MV) which can be quite well visualised with immunoperoxidase staining; indeed with this technique a proportion of normal circulating lymphocytes (many of which are SIgG positive) appear quite hairy (Mason et al, 1977). It is likely that the expression of MV on circulating lymphocytes corresponds to an 'activated state' making the cells capable of recognising the endothelial cell wall and to adhere to it (reviewed by Loor, 1977). Thus the quiescent state of lymphocytes is characterized by a smooth surface. To enter the specific

homing area in the lymphoid organs they have to pass between the high endothelial cells forming the wall of the post-capillary venules, which are the main site of entry of lymphocytes in lymph nodes. In these venules, the lymphocytes become quite villous and it has been proposed that they use MV to adhere to the endothelial wall and that they progressively lose the MV as they traverse the wall to arrive smooth in the specific homing area (Loor, 1977).

It is proposed here that HCs are lymphocytes 'frozen' at the villous 'activated state'. This theory accounts for some of the monocytic features of HCs. In Chapter 4 it was demonstrated that while HCs adhered to glass and plastic, they were only weakly phagocytic for latex (with occasional exceptions) and were not professional phagocytes. Cell adherence to glass and plastic is a function of microvilli (Loor, 1977), and it can be argued that weak phagocytosis of latex is a function of most cells but made much more conspicuous in HCs because of the strong attachment phase caused by the 'sticky' microvilli. Such a hypothesis might also explain the frequent appearance of nucleoli in cells which are otherwise morphologically mature, and possibly accounts for the failure of HCs to populate lymph nodes since they cannot complete the smooth phase required for homing; however this last point may reflect the marker phenotype and ontogenic maturity of the cell (see below).

The only convincing evidence left favouring a monocytic origin for HCs is the presence of a strong γFc receptor which deforms attached indicator erythrocytes and is not blocked by anti-Ia-like antigen. However this type of receptor is also a feature of the unidentified cell group (UCG : K cells, L cells, null cells Chapter 3). The UCG is heterogeneous both in markers and function (Chess and

Schlossman, 1977) but it generally agreed that this group contains cells with a strong γFc receptor and with the ability to mediate antibody dependent cell-mediated cytotoxicity (ADCC). HCs have not been properly tested for the ability to induce ADCC but it is of some interest that this can, on occasion, be brought about with IgM antibody (Wahlin et al, 1976; Fuson and Lamon, 1977) which suggests a μFc receptor. A population within the UCG can synthesise and secrete immunoglobulin (Chess and Schlossman, 1977), and when sensitive methods of SIg detection are employed only a very small population of non-T SIg-negative cells can be identified (Strelkauskas et al, 1975; Haegert, 1978; Haegert et al, 1978). These results suggest that at least some of the UCG might be B lymphocytes, and while there is controversy about the incidence of γFc receptors on B lymphocytes (Gergely et al, 1977), some B cells, at least, possess this receptor.

The other evidence throughout this thesis favoured a B-lymphocyte origin for the HC. Moreover, several pieces of evidence suggest that HCs derive from a cell which occurs late in B-cell differentiation. A number of partly theoretical schemes of B-cell development in man have been proposed (Cooper et al, 1973; Cooper and Seligmann, 1977; Sweet et al, 1977), and while much of the information has come from the study of B-cell malignancies (and it is therefore rather artificial to reintroduce malignant cells), it is of some interest to establish the relative position of HCL. One scheme of B-cell development is shown in Figure 6.1. The scheme was constructed from the information obtained in a detailed marker study of the plasma cells from 25 patients with a range of immunoproliferative diseases including IgG, IgA, IgD, IgM and IgE myeloma (Burns et al, 1978d). For the purpose of this discussion, the IgG memory cell loop has been added, and it is

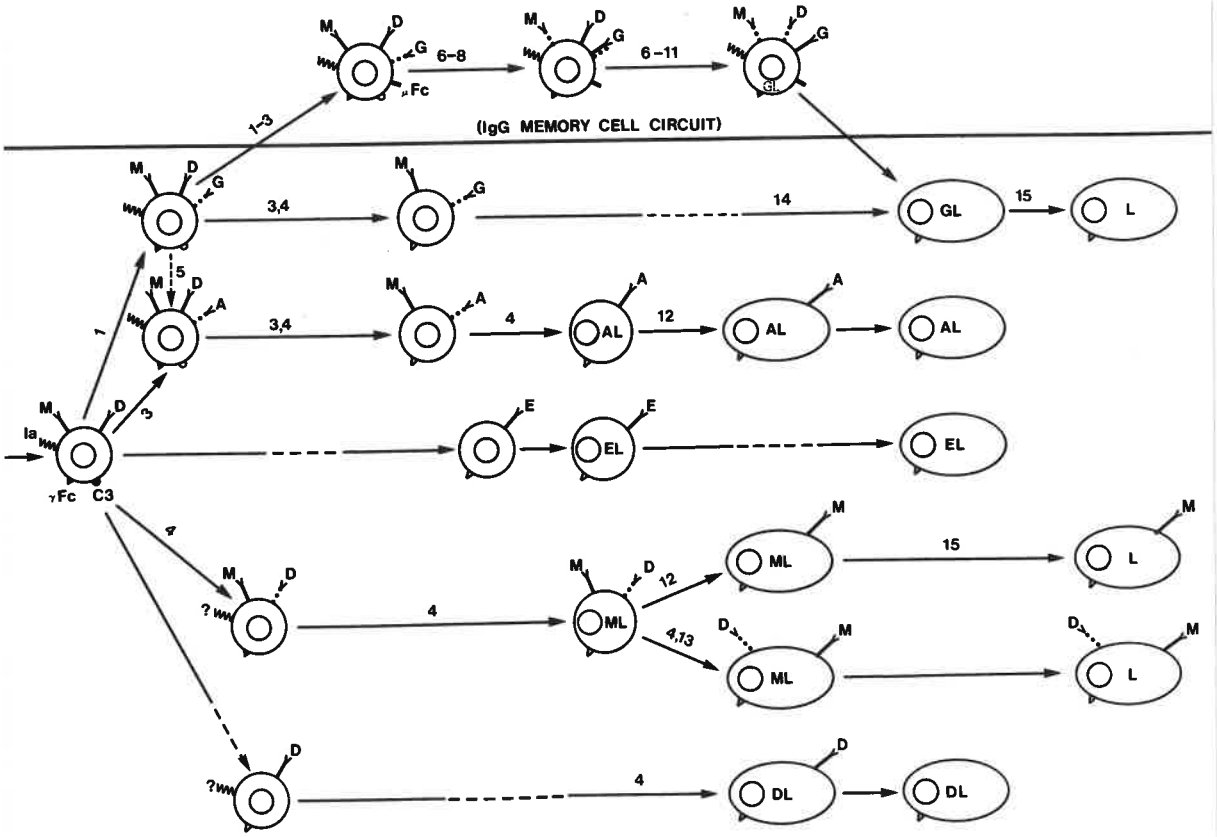
(IgG MEMORY CELL CIRCUIT)
M
D
G
μFc
6-8
6-11
GL
1-3
3,4
14
15
L
5
A
4
AL
12
1
Ia
γFc C3
E
EL
2
?
4,13
ML
DL

Figure 6.1

Figure 6.1 Schematic representation of the terminal stages of B-cell development.

Key

- Predominant SIg of a given class
- SIg of a given class weakly expressed
- Ia-like antigen
- C3 receptor(s)
- C3 receptor(s) weakly or partially expressed
- γFc receptor strongly expressed
- γFc receptor weakly expressed
- μFc receptor

——- - → Intermediate steps unknown, if they exist.

L cytoplasmic light chain (K or λ)

The scheme is based partly on results in the dissertation and in a separate study (Burns et al, 1978d), but some stages are obtained, or confirmed, from previous studies and from the reports of others - these references are numbered and given below.

1. Dhaliwal et al, 1978.
2. Burns et al, 1978a.
3. Parkhouse and Cooper, 1977.
4. Preud 'homme et al, 1977a.
5. Cooper and Seligmann, 1977.
6. Burns et al, 1978c.
7. Coffman and Cohn, 1977.
8. Burns et al, 1977c.
9. Black et al, 1978.
10. Gathings et al, 1977.
11. Cawley et al, 1978.
12. Preud 'homme and Seligmann, 1974.
13. Ferrarini et al, 1976b.
14. McConnell, 1971.
15. Preud 'homme et al, 1976.

conceded that there are possibly similar loops for the other isotypes and that the scheme is oversimplified.

Leukaemic cells may express neoantigens or receptors which are not the result of development along the B-cell pathway. Nevertheless several features suggest that HCs are memory cells. In relation to Figure 6.1, it is suggested that most HCs occupy the final stage of the IgG memory cell circuit whereas CLL cells would mostly derive from the first stage. Thus the features of multiple heavy chain SIg isotype (Gathings et al, 1977) and, in particular, SIgG expression and secretion (Coffman and Cohn, 1977; Parkhouse and Abney, 1977; Zan-Bar et al, 1978; Black et al, 1978) are features of B memory cells in the mouse. Also, it has been suggested that a slow SIg replacement time is a feature of memory cells (Melchers et al, 1975; Lewis et al, 1976).

The loss of the C3 receptor illustrated in the figure is a constant feature of all types of plasma cells and appears to have taken place in the memory circuit as well; this may start with the loss of C3b as for CLL cells (Ross and Polley, 1975) which do not carry a C3b receptor but still bear a C3d receptor, but HCs bear neither receptor. The γFc receptor is weakly expressed by plasma cells which, however, lack the Ia-like antigen. As was discussed above HCs bear a strong γFc receptor which perhaps suggests a more complex memory circuit than illustrated.

One weakness of the scheme, as illustrated, is the emergence of the μFc receptor on the memory circuit (proposed CLL and HCL counterparts). Most of the other receptors are progressively shed with maturation and it is therefore unusual for a new membrane marker to arise. This may represent a neoantigen feature. However, a subpopulation of normal B

cells express a μFc receptor (Ferrarini et al, 1977) and it can be argued that the receptor becomes revealed with the loss of the C3b receptor and only continues on memory cells. The function of the μFc receptor, incidentally, remains obscure, but in their discussion of a similar receptor on mouse peritoneal macrophages Ehlenberger and Nussenzweig (1976) suggest that the IgM-antigen complex is bound but not interiorised by the receptor, and thus remains exposed and available for the stimulation of immunocompetent cells.

The distinctive clinical picture of HCL - bone marrow replacement and splenomegaly without prominant lymphodenopathy - presumably reflects the migratory pattern of HCs. Little is known about the various factors influencing cell migration (reviewed by Sprent, 1977) but it is thought that surface markers play a role, and certainly the SIg isotype has some effect on the migratory properties of cells. One example relevant to HCL is the absence of receptors for complement on HCs. Splenic histiocytes do not bind EAC reagents whereas lymph node histiocytes do (Shevach et al, 1973; Jaffe et al, 1974). Also, low concentration trypsin treatment of lymphocytes before injection completely prevents localization of the cells in lymph nodes and leads to above normal numbers homing to the spleen. After several hours the cells begin to enter lymph nodes, and this capacity can also be restored by *in vitro* culture for 6-12 h before transfer. This reversal of the homing defect is temperature dependent and requires protein synthesis (Sprent, 1977). These data suggest a role for a trypsin-sensitive surface receptor in the control of lymph node, but not splenic, homing capacity: the complement receptor on lymphocytes is trypsin sensitive (Lay and Nussenzweig, 1968) and might therefore be the receptor in question.

To conclude, the work described in this thesis suggests that HCL is a clonal proliferation of a population of memory B lymphocytes 'frozen' at an activated state.

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APPENDIX A

Parts of this thesis have been published in the scientific literature as follows:

Barker, C.R., Burns, G.F., Cawley, J.C. and Hayhoe, F.G.J. (1976) IgM receptors on the surface of hairy cells of leukaemic reticuloendotheliosis. Lancet, i, 1303.

Burns, G.F., Cawley, J.C. and Barker, C.R. (1978) Characteristics of the receptor for IgM on human B lymphocytes. Immunology. In press.

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Burns, G.F., Cawley, J.C., Barker, C.R., Goldstone, A.H. and Hayhoe, F.G.J. (1977) New evidence relating to the nature and origin of leukaemic reticuloendotheliosis. British Journal of Haematology, 36, 71.

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Burns, G.F., Worman, C.P., Roberts, B.E., Raper, C.G.L., Barker, C.R. and Cawley, J.C. (1978) Terminal B-cell development as seen in different human myelomas and related disorders. Clinical and Experimental Immunology. In press.

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APPENDIX B

Additional publications submitted during the period of research for the dissertation but unrelated to the main thesis.

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