MICROENVIRONMENTS OF T AND B LYMPHOCYTES

A LIGHT- AND ELECTRONMICROSCOPIC STUDY

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Aan mijn ouders
Aan Tilly

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Chapter 1

General Introduction

1.1 Pluripotent Haemopoietic Stem Cells

Peripheral blood cells - erythrocytes, granulocytes, monocytes, thrombocytes and lymphocytes-are the end products of a differentiation process which occurs in the bone marrow and, in rodents, also in the spleen. Normal haemopoietic tissue is a cell renewal system with an accurate balance between cell production originating from pluripotent haemopoietic stem cells and continuous cell loss. The important function of haemopoietic stem cells was emphasized by Till and McCulloch (1961) in bone marrow transplantation studies in mice. They noted that intravenous injection of small numbers of bone marrow cells into lethally irradiated syngeneic recipient mice caused the appearance of haemopoietic colonies in the spleen of the recipient mice. These colonies consisted either of erythroid, granuloid, megakaryocytic or mixed cell populations (Curry and Trentin, 1967). The technique used by Till and McCulloch is known as the "spleen colony assay" and has established two major qualities of haemopoietic stem cells: (1) they have the capacity of self replication (Trentin and Fahlberg, 1963; Curry et al., 1967) and (2) they are pluripotent since they give rise to clones of different cell types of which the differentiated "end" cells recirculate in the blood (Till and McCulloch, 1961; Becker et al., 1963; Till, 1976). In contrast to erythroid and myeloid colonies, lymphoid colonies were not detectable with the spleen colony assay; however, Ford et al. (1966), Micklem et al. (1966), and Wu et al. (1968) demonstrated with chromosome marker techniques that lymphoid cells were also derived from pluripotent haemopoietic stem cells.

One of the major questions in cell biological investigations of haemopoiesis concerns the factors which determine the commitment and differentiation of pluripotent haemopoietic stem cells. At present it is generally accepted that two types of factors are involved in the regulation of haemopoiesis: (1) microenvironmental factors (see 1.2), and (2) humoral factors (see 1.4).

1.2 Haemopoietic microenvironments

A haemopoietic microenvironment is defined as a special region in a haemopoietic organ which differs from adjacent areas and in which local conditions influence the proliferation and differentiation of immigrant – haemopoietic stem – cells (Metcalf and Moore, 1971). It is assumed that a microenvironment is created by stromal cells in haemopoietic organs (Curry and Trentin, 1967; Trentin, 1970). Convincing evidence that microenvironments determine the differentiation pathway of haemopoietic stem cells came a.o. from the work of Curry et al. (1967), Wolf and Trentin (1968), Bernstein (1970), Rauchwerger et al. (1973) and Wolf (1974). Curry and coworkers found a constant ratio between erythroid (E) and granuloid (G) colonies in the spleen (E/G ratio $\approx$ 3), whereas this ratio in the bone marrow was about 0.5 (Wolf and Trentin, 1968). Implantation of small fragments of bone with adjacent bone

marrow into the spleen of lethally irradiated recipients restored haemopoiesis in a similar way as intravenously injected bone marrow cells (Wolf and Trentin, 1968). Microscopic inspection of the bone marrow implanted spleens showed that the marrow stroma was clearly demarcated from the splenic stroma a.o. by textural differences in connective tissue and by the presence of bone spicules in the marrow stroma. Haemopoietic colonies inside the implanted marrow stroma were predominantly granuloid. (E/G ratio $\approx$ 0.5). Mixed colonies, occurring at the junction between both types of stroma were mostly granuloid at the bone marrow side and erythroid at the spleen stroma side. In the spleen colony assay, mixed colonies generally occur relatively late during the regeneration period after irradiation and bone marrow transplantation. Their appearance is most probably caused by stem cells at the periphery of the expanding colony encroaching upon a microenvironment for another haemopoietic line (Curry and Trentin, 1967). These findings strongly indicate that the nature of the microenvironment influences the differentiation pathway of haemopoietic stem cells. Two other types of experiments contribute to this notion. Firstly, in rats the spleen is deficient in the production of granuloid cells when compared to mice. However, injection of rat spleen cells into lethally irradiated mice leads to both erythroid and granuloid colonies of rat origin (Rauchwerger et al., 1973). These colonies grow as fast as spleen colonies from mouse cell origin. Secondly, the "Steel" anemic mutant mouse (Sl/Sl^d) has a genetically determined defect in haemopoietic microenvironments. This type of mouse cannot be cured by injection of bone marrow cells from non-anemic littermates. Implantation of spleen stroma from non-anemic littermates, however, restored haemopoiesis (Bernstein, 1970; Wolf, 1974).

The cellular architecture of haemopoietic microenvironments is largely unknown. McCuskey et al. (1972) speculated that haemopoietic microenvironments are morphologically composed of (1) a microvascular component regulating the cell flux, the oxygenation and the pH of the tissue; (2) a connective tissue component, concerned with the transfer of metabolites and (3) neural elements associated with blood vessels and with the stroma. From *in vivo* microscopic inspections of the spleen, and from histochemical analysis of the spleen and bone marrow they concluded that the haemopoietic inductive microenvironment for erythropoiesis was well vascularized, had a high rate of blood flow and contained neutral mucopolysaccharides. Conversely, the microenvironment inductive to granulopoiesis had lower rates of blood flow and contained acid mucopolysaccharides.

In the spleen it has been shown that erythroid, granuloid and lymphoid microenvironments are situated in different compartments. Erythroid microenvironments are more or less randomly distributed throughout the red pulp, whereas granuloid microenvironments are more located under the splenic capsule and along trabeculae (Curry and Trentin, 1967). Lymphoid microenvironments are found in close association with branches of the splenic artery.

Haemopoietic cells in the bone marrow are located within haemopoietic cords, which lie between the sinuses (Weiss, 1970). According to Weiss and Chen (1975), the microenvironments regulating the different lines of haemopoietic differentiation are also spatially separated within these cords. Megaka-

ryocytes occur near the outside wall of vascular sinuses and release strips of thrombocytes into the circulation. Erythroblasts also lie in the peripheral part of the haemopoietic cords, whereas granuloid cells develop in the central part of the cords. Lymphoid cells and monocytes are predominantly found in a peri-arteriolar sheath surrounding radially directed arterioles, which branch from the central longitudinal artery in the bone marrow.

The nature of the stromal cells which induce differentiation and proliferation of haemopoietic stem cells in microenvironments – designated by McCulloch (1973) as "managerial cells" – is not clear. Possibly, the central macrophages found in erythroblastic islets represent the managerial cell in erythropoietic microenvironments. This macrophage extends cytoplasmic processes, enveloping each of the erythroblasts in the islet (Le Charpentier and Prenant, 1975; Ploemacher and van Soest, 1977). However, it may also be that the central macrophage just phagocytizes nuclei extruded from erythroblasts and red cells dying during ineffective erythropoiesis (Weiss and Chen, 1975).

It is evident that the structural constituents of microenvironments determining the commitment of pluripotent stem cells into the various haemopoietic cell lines are far from clear and can only be speculated upon. Further analysis of these microenvironments may be stimulated by the development of specific anti-haemopoietic stem cell antisera. The use of these antisera in combination with specific immuno-histological and immuno-electronmicroscopic techniques may then provide further insight into the architecture of these haemopoietic microenvironments.

1.3 Differentiation of lymphoid cells.

Differentiation of lymphoid cells occurs at two *levels* (see review by Rosse, 1976). The first level deals with antigen. independent differentiation and is located in so called "primary" or "central" lymphoid organs, like – in rodents – the bone marrow and the thymus. The second level concerns the antigen dependent differentiation of lymphoid cells. The latter process takes place in "secondary" or "peripheral" lymphoid organs, such as the spleen, lymph nodes, Peyer's patches, tonsils, adenoids, appendix, omental milky spots and other organized collections of lymphoid tissue. During the antigen driven differentiation (the immune response) lymphoid cells differentiate into "effector" cells which specifically eliminate the antigen which evoked the immune response, as well as into specific "memory" cells. Subsequently, upon renewed contact with the antigen memory cells differentiate into effector cells. This secondary immune response is enhanced and more vigorous when compared with the primary immune reaction.

There are two major *lines* of lymphoid differentiation in rodents. (1) Part of the lymphoid cells in the bone marrow leaves the bone marrow with the blood stream to migrate directly to peripheral lymphoid organs. These "B" cells are characterized a.o. by surface immunoglobulin (Ig) and differentiate upon antigenic stimulation in antibody producing plasma cells. This process is known as the "humoral" immune response. (2) Other lymphoid cells in the bone marrow leave this organ (also with the blood stream) and localize in the thymus. Here, these lymphoid cells further differentiate to "T" lymphocytes.

During this process several types of membrane markers are acquired on the cell surface, a.o. the theta-antigen and receptors which recognize antigen. Upon maturation T cells may leave the thymus and migrate towards peripheral lymphoid organs. Mature T lymphocytes are concerned with "cell-mediated" immunity, such as delayed type hypersensitivity, reactions against fungi, cytotoxic killing of organ transplants and cytotoxic killing of antigenic tumour cells, and have a "helper" function in antibody formation.

1.3.1 B cell differentiation

The antigen independent differentiation of B lymphocytes in adult rodents probably starts in bone marrow microenvironments which induce the early commitment of pluripotent haemompoietic stem cells to precursor B lymphocytes. Precursor B lymphocytes are still immuno-incompetent, but after separation and injection in irradiated recipient mice, generate immunocompetent lymphocytes in the spleen within 7 days (Lafleur et al., 1972). Precursor B lymphocytes have a high mitotic activity since in the continuous presence of tritiated thymidine 90 per cent of the bone marrow lymphocytes is labeled within 4 days (Osmond and Nossal 1974). Upon a mitotic phase these cells enter a maturation phase of about 2 days in which they gradually acquire immunoglobulin in the cell membrane. Each B cell acquires immunoglobulins of one idiotype only, i.e. which recognize one antigenic determinant.

Already during the maturation phase, B cells start to leave the bone marrow and seed to peripheral lymphoid organs (Osmond and Nossal, 1974; Rozing et al., 1977). During migration or upon entry in the peripheral lymphoid organs B cells acquire full immunocompetence and finally have about 10^5 immunoglobulin molecules on the cell surface (Nossal, 1976). Antigen induced triggering of immunocompetent B cells leads further to two types of differentiation processes: (1) the formation of clones of antibody producing plasma cells and (2) the formation of memory B lymphocytes (Strober, 1975). Memory cells probably have an increased amount of surface-Ig with a high affinity for the inducing antigen. Renewed contact with antigen causes a rapid differentiation of memory B cells into antibody producing plasma cells, resulting in a "secondary" immune response.

1.3.2 T cell differentiation

The early commitment of pluripotent haemopoietic stem cells to precursor T cells probably also occurs in the bone marrow. Roelants et al. (1975), and Loor (1976), using a very sensitive indirect double immunofluorescence assay, found that the bone marrow and spleen of normal mice contains a small but distinct population of lymphocytes which lack membrane Ig and possess a low concentration of Θ antigen. Nude mice (congenital athymic mice) were found to have large numbers of these particular cells, especially in the spleen. Similar findings were obtained in adult thymectomized, lethally irradiated and bone marrow reconstituted mice. The authors suggested that these Ig negative, low Θ positive cells were precursor T lymphocytes, which in the absence of a thymus accumulated preferentially in the spleen. This concept was supported

by the fact that these cells disappeared when such athymic mice received a thymus graft.

Upon early commitment of pluripotent haemopoietic stem cells to precursor T cells, these cells seed to the thymus, localize in the superficial thymic cortex and start to proliferate (Weissman, 1973). Convincing evidence that most mitotic cells in the thymus indeed are bone marrow derived came from T_6 chromosome marker studies reported by Ford et al. (1966), Micklem et al. (1966) and Wu (1968). According to Greaves et al. (1974), precursor T cells in the thymus are clearly visible in microscopic sections as large basophilic blast cells. These blast cells are retained within a microenvironment created by epithelial reticular cells, which form a cytoplasmic web by means of long cytoplasmic processes (Hoshino, 1963). The reticular cells are supposed to trigger off mitosis in the cortical lymphocytes (Metcalf and Ishidate, 1962; Clark, 1968). Proliferating blast cells in the superficial thymic cortex give rise to a progeny of small lymphocytes (Weissman, 1973). During differentiation these cells express increasing concentrations of specific antigens on their cell surface (Θ, TL, Ly, H2), but in contrast to B lymphocytes, do not express surface immunoglobulin (Cantor and Weissman, 1976). Lymphocytes in the cortex are small lymphocytes, they show a high mitotic index, are sensitive to hydrocortisone and lack immunocompetence, whereas lymphocytes in the medulla are medium sized, show a low mitotic index, are hydrocortisone resistant and immunocompetent. The latter cells therefore have characteristics similar to peripheral T cells.

By transcapsular labeling of the thymus with tritiated thymidine it has been shown that the medium-sized immunocompetent cells in the thymic medulla are the progeny of cortical lymphocytes (Weissman, 1973). They probably enter the circulation from the medulla. However, Shortman and Jackson (1974) have shown that an unidirectional migration pathway for lymphocytes from the cortex to the medulla is not the only source of medullary thymocytes. Experiments in which the thymus was intravenously labeled with ^{3}H-thymidine followed by isolation of the two subpopulations showed uptake of the label by both thymic subpopulations. This indicated that the medullary cell population was mitotic as well and the authors concluded that the cortisone-resistant medullary thymocytes also contained a self generating population. These experiments point out that there exist more than one differentiation line in the thymus (c.f. Fathman et al., 1975).

1.4 Humoral factors involved in haemopoiesis

Although an extensive review of humoral factors involved in the regulation of haemopoiesis is beyond the scope of this thesis a few major aspects of humoral regulation will be summarized.

1.4.1 Erythropoietin

Already in 1906 Carnot and Deflandre noted that plasma of rabbits, made anemic by bleeding, increased the red cell counts when injected into normal rabbits. Reissman (1950) showed in experiments with parabiotic rats that the introduction of anoxia in one member induced an increase in erythropoiesis in both members of the parabiotic pair. These and other experiments led to the conclusions that erythropoiesis is regulated by a humoral factor. This factor was called "erythropoietin".

A major site of production of erythropoietin seems to be the kidney, since resection of this organ resulted in a fall of the erythropoietin level (Jacobson et al., 1957). More direct evidence that the kidney regulates erythropoiesis came from experiments with isolated perfused kidneys. Kuratowska et al. (1961) and Fisher and Birdwell (1961) perfused kidneys of anoxic animals and noted an erythropoietic activity in the perfusate. Although the kidney is the main source of erythropoietic activity, also extra-renal sites produce erythropoietin (see review by Mirand and Murphy, 1970).

1.4.2 Thrombopoietin

Also thrombopoiesis is regulated by a humoral factor. This factor, thrombopoietin, is found in the serum of patients with acute blood loss and it increases platelet levels when injected into rabbits (Toi, 1956). Thrombopoietin probably acts on megakaryocytes since a single injection induces a peak in the number of platelets 3-4 days after injection. This delay corresponds well with the maturation time of platelets (Ebbe and Stohlman, 1965). The site of production of thrombopoietin is unknown although several organs have been suggested like the adrenals, pituitary, liver and spleen (reviewed by Metcalf and Moore, 1971).

1.4.3 Colony stimulating factors

Factors stimulating the proliferative capacity of granuloid and monocytic cells are known from *in vitro* tissue culture experiments. These factors are called "colony stimulating factors" (CSF's) (Metcalf and Moore, 1971, van den Engh, 1974). They stimulate granulopoiesis *in vitro* and they are produced by a feeder layer of cells supporting the haemopoietic cells. CSF's are produced in tissue cultures by various types of cells, like neonatal mouse kidney cells, lymphoma cells (Bradley and Metcalf, 1966), and fibroblasts (Pluznik and Sachs, 1965). CSF's are detectable in the serum of normal mice (Metcalf and Foster, 1967) and are thought to be secretory products released by many organs in the body (Metcalf and Moore, 1971). Van den Engh (1974) reviewed the physico-chemical properties of CSF's isolated from different sources and tested their biological activity. He stressed the heterogenous nature of CSF's

and suggested that a part of the CSF molecule is common in all CSF molecules and as such responsible for the biological activity.

1.4.4 Humoral factors involved in lymphopoiesis

The activity of humoral factors regulating lymphopoiesis is much more difficult to assess since lymphopoiesis is also influenced by the stimulatory effect of antigens and mitogens. At present, however, there is firm evidence that at least the thymus produces factors which regulate lymphopoiesis independent of other influences (van Bekkum, 1975). Indirect proof of the existence of thymic humoral factor came from experiments reported by Osoba and Miller (1963). These and other investigators implanted thymic tissue in cell tight diffusion chambers in neonatally thymectomized mice. They were able to demonstrate that the immunological reactivity of thymus deprived mice against typically thymus dependent antigens (e.g. reactivity to allogeneic skin grafts, antibody formation to sheep erythrocytes) was restored after implantation of the diffusion chambers.

Direct evidence for the influence of thymic humoral factors in lymphopoiesis came from experiments reported by Goldstein et al. (1971), in which parental bone marrow cells were incubated with thymic extracts (thymosin) and subsequently injected into F1 recipients. The incubated bone marrow cells caused a significantly enhanced graft-versus-host reaction in the recipient. Bach et al. (1971) suggested that the incubation of bone marrow cells with thymic extracts induced the appearance of T cell specific surface antigens on lymphocytes as indication of T cell maturation. This hypothesis was experimentally confirmed by Komura and Boyse (1973). In similar experiments as performed by Goldstein and Bach, they noted that about 2 percent of the nucleated bone marrow cells incubated with the thymic extract became susceptible to lysis by complement and specific anti T cell antisera, indicating the appearance of T cell antigen on thymosin incubated bone marrow cells.

In the normal *in vivo* situation the concentration of thymic hormone is very low and T cell "capacitation" outside the thymus occurs therefore at a much lower level than in the *in vitro* situation (Rosse, 1976).

Humoral factors involved in the stimulation of proliferation and differentiation of B cells are described in birds (Jankovic and Leskowitz, 1965). In mammals, however, such factors have seemingly not been described.

1.5 Purpose of Investigation

So far it has been discussed that haemopoietic microenvironments and humoral factors play an important role in the commitment and differentiation of pluripotent haemopoietic stem cells to erythrocytes, thrombocytes, granulocytes, monocytes as well as T and B lymphocytes. The first three types of differentiated cells are typical "end cells" which exert only one specialized function.

Monocytes and lymphocytes are subject to further differentiation. Upon stimulation, monocytes are able to leave the blood stream and transform into macrophages. These cells non-specifically remove foreign material and cellular debris from the tissues. Lymphocytes, although having a relatively simple morphology, are the most sophisticated progeny of haemopoietic stem cells. They differ in a number of aspects from the other differentiated cells. Firstly, they have the unique property to recognize "foreignness" by means of specific antigen receptors. Interaction between antigen and antigen receptors may induce further differentiation of lymphocytes to effector cells, resulting in a specific elimination of the antigen. During this differentiation process, interaction is required between antigen, lymphocytes and non-lymphoid cell types (e.g. macrophages). Such interactions may only proceed efficiently if microenvironments exist which facilitate: (1) contact between antigen depot sites and lymphocytes and (2) subsequent proliferation and differentiation of antigen-activated lymphocytes. Such microenvironments are present in peripheral lymphoid organs. Hence a second difference to the other types of blood cells is the fact that lymphocytes use specialized microenvironments to exert their physiological function. A third difference is the ability of lymphocytes to recirculate between blood, peripheral lymphoid tissues and lymph (Gowans and Knight, 1964). Recirculation guarantees an optimal chance for lymphocytes to encounter and recognize antigens on invading micro-organisms and developing malignant tumor cells ("immuno-surveillance").

The objective of this thesis is to gain more insight into the ultrastructure of lymphocyte subclasses and the architecture of the lymphoid tissue. In the literature many papers concern the kinetics and functional aspects of lymphoid cells, however the morphological substrate of cellular interactions in immune function is far less understood. The experimental work described in the following papers attempts to indicate interrelationships between lymphoid cells and cells of the lymphoid stroma, both under normal conditions, after irradiation and reconstitution, as well as after antigenic stimulation of the experimental animal.

To introduce the experimental work we shall briefly discuss in the following chapter the complicated architecture of the spleen and lymph nodes.

Chapter 2

Architecture of spleen and lymph nodes

2.1 The Spleen

The spleen is a highly vascularized haemopoietic organ. It can be considered as an encapsulated vascular filter connected to the blood stream. The spleen cleares the blood from damaged blood cells and from foreign substances. Blood-borne antigens evoke an immune response mainly in the spleen. The spleen may sequester blood cells from the circulation for varying periods in time. (Weiss, 1972).

2.1.1 Vascularization of the spleen

Blood enters the spleen at the hilus through the splenic artery which branches off in the trabeculae as "trabecular arteries" (Fig. 1). Upon further ramification trabecular arteries become gradually surrounded by lymphoid cells and are then designated as "central arteries" or "central arterioles". Small "terminal arterioles" branch radially from the central arterioles to terminate in the red pulp. Capillaries extending from central (and terminal) arterioles open freely into the marginal zone. The blood flow towards the red pulp is both of the open type and the closed type (Weiss, 1973; Murakami et al., 1973). In the closed circulation arterioles extend via capillaries into the splenic sinuses; in the open circulation arterioles and capillaries end freely in the splenic cords and in the marginal zone.

The efferent blood in the spleen is collected in large splenic sinuses, which are connected to splenic venes. These venes enter the trabeculae as "trabecular venes". At the hilus trabecular venes are continuous with the splenic vene outside the organ. Lymphatics also occur in the spleen. They originate in the white pulp and run closely along branches of the splenic artery (Janout and Weiss, 1972; paper 1).

2.1.2 Haemopoietic compartments in the spleen

The spleen of rodents contains two major haemopoietic compartments: (1) the red pulp (red: due to the presence of large numbers of erythrocytes), and (2) the white pulp (white: due tot the presence of large number of lymphocytes) (Fig. 1). The red pulp is diffusely distributed throughout the spleen. It consists of splenic sinuses and thin plates of tissue, the splenic cords. In the splenic cords the microenvironments regulating the differentiation of erthrocytes, granulocytes and megakaryocytes can be found. The white pulp represents the lymphoid compartment in the spleen, and is structurally organized around branches of the splenic artery. The white pulp is separated from the red pulp by a loose meshwork of connective tissue, the marginal zone.

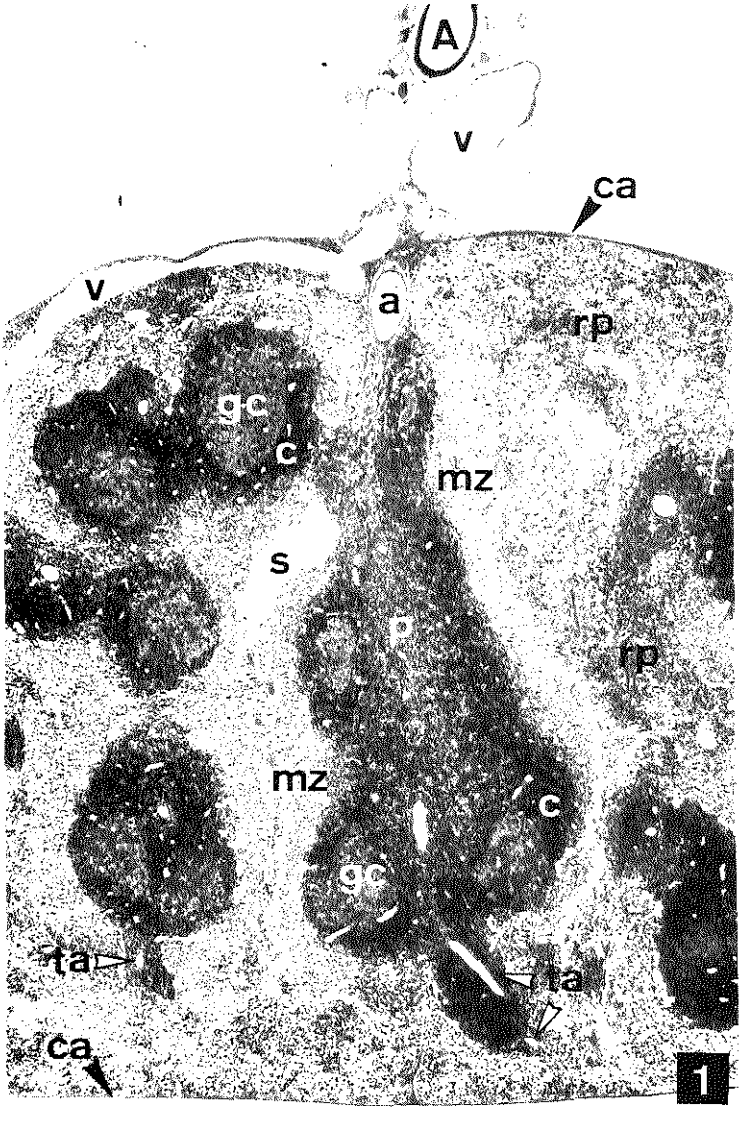

Fig. 1. Histological section of the spleen of a (DBA/2 x C57BL/Rij) F1 mouse, 10 days after i.v. stimulation with sheep erythrocytes. Due to the perfusion fixation, the white pulp is clearly demarcated from the marginal zone (*mz*) and red pulp (*rp*). Follicles at the periphery of the peri-arteriolar lymphoid sheath (*p*) contain distinct germinal centers (*gc*), which are surrounded by a corona (*c*) of small lymphocytes. *A* = splenic artery; *a* = trabecular artery; *ta* = terminal arteriole; *s* = vascular sinus; *v* = vene; *ca* = capsule. Perfusion fixation. 1 µ Epon section, stained with toluidine blue.

2.1.3 *The white pulp, T and B cell compartments*

In histological sections of the white pulp three different areas can be distinguished: (1) the peri-arteriolar-lymphoid-sheath (PALS), (2) lymphoid follicles at the periphery of the PALS and (3) a surrounding marginal zone.

In the PALS reticulum cells form a stroma which consists of layered concentric sheaths around the central artery. Due to the arrangement of the reticulin sheaths the lymphocytes lie in more or less parallel arrays. As will be discussed in paper III lymphocytes migrate actively through the white pulp, and the sheaths seem to conduce the lymphoid cells during migration. The reticulin pattern in these sheaths is – especially in rats – more conspicious in the peripheral part than in the central part of the PALS. Based on this distribution pattern, the PALS can be subdivided into a central PALS and a peripheral PALS (paper I).

Follicles – spherical or ovoid accumulations of lymphoid cells – are inserted in the peripheral PALS. When follicles show a homogenous distribution of small lymphocytes they are called "primary"; follicles containing a center of lymphoid blast cells are called "secondary". Secondary follicles are an expression of immunological activity. The follicle center or germinal center can be divided into two subregions: a central region, adjacent to the PALS, which contains many mitotic cells, blast cells and macrophages, and a more peripheral part which predominantly contains medium-sized lymphocytes and dendritic reticulum cells. Germinal centers are surrounded by a corona of dark staining lymphocytes. The marginal zone which surrounds the follicles and PALS is an important traffic area for lymphocytes entering and leaving the white pulp (Goldschneider and McGregor, 1968; Ford, 1969). The structure of the white pulp architecture is schematically presented in Fig. 2.

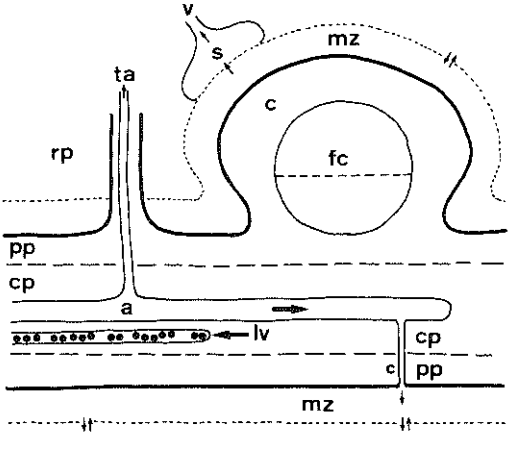

Fig. 2. Schematic presentation of the white pulp. *a* = central arteriole; *ta* = terminal arteriole; *c* = capillary; *lv* = lymph vessel; *mz* = marginal zone; *s* = sinus; *v* = vene; *cp* = central part of the PALS; *pp* = peripheral part of the PALS; *fc* = follicle center; *c* = corona; *rp* = red pulp. The arrow indicates the direction of blood flow. The black dots represent lymphocytes in the efferent lymph vessel.

T and B lymphocytes are not randomly distributed throughout the stroma of lymphoid organs. Several types of experiments have shown that T and B cells localize in distinct spatially separated compartments. Waksman and coworkers (1962) were the first to note that in histological sections of the spleen of neonatally thymectomized rats the PALS but not the follicles were depleted of lymphoid cells. It was concluded that definite regions in peripheral lymphoid organs were "thymus dependent" with regard to their resident lymphoid population. Parrott et al. (1966) confirmed these observations in neonatally thymectomized mice, and de Sousa et al. (1969) described similar findings in congenitally athymic nude mice. Autoradiographic studies on the localization of purified radio-labeled lymphocyte subclasses following i.v. injection provided direct evidence that B cells occur in follicles (de Sousa, 1971; Parrott and de Sousa, 1971; Howard et al., 1972; Sprent, 1973; Gutman and Weissman, 1973; Paper III) and to a minor part also in the peripheral PALS (Mitchell, 1972; Nieuwenhuis and Ford, 1976), whereas T cells localize in the PALS (Parrott et al., 1966; Sprent, 1973 paper II). Detection of lymphocyte subclasses in frozen sections of lymphoid tissue by means of specific fluorescent anti Ig and anti Θ antisera have furthermore confirmed the presence of well defined, spatially distributed compartments for T and B lymphocytes in peripheral lymphoid organs (Gutman and Weissman, 1972; Weissman et al., 1976; Paper IV). For the white pulp in the spleen, the general conclusions of these experiments can be summarized as follows: (1) T cells occur predominantly in the central and peripheral PALS. (2) B cells populate follicles and the peripheral PALS. (3) Germinal centers are thought to contain proliferating B cells but not T cells, but recent immunofluorescent studies have indicated the presence of small numbers of T cells in germinal centers (Weissman et al., 1976). (4) The marginal zone contains both B and T cells, the B cells being in excess to T cells.

2.2 Lymph nodes

Lymph nodes are encapsulated structures connected to the path of lymphatic vessels. Their structure is shown in Figs. 3 and 4. Lymph flows through the lymph node and is filtered by phagocytic cells located in sinuses. In this way systemic distribution of infectious agents is largely prevented.

The afferent lymphatics of lymph nodes drain certain regions in the body and the cells in the lymph node are able to respond immunologically to antigens coming from this region (McMaster and Hudack, 1935). The lymph enters the node by afferent lymphatics which open in the marginal sinus (Fig. 3,4), it passes through trabecular sinuses and leaves the node via the medullary sinuses draining into the efferent lymphatic.

The lymphoid stroma of the node can be divided in three different compartments: (1) an outer cortical region in which lymphoid follicles are situated, (2) a non-follicular paracortical area and (3) medullary cords. Blood vessels enter the node at the hilus. Arterioles run via the medullary cords and the paracortex towards the outer cortex and branch into a capillary plexus. Postcapillary venules collect the blood from these plexuses and run back towards the hilus. Lymphocytes (both B and T cells) enter the node

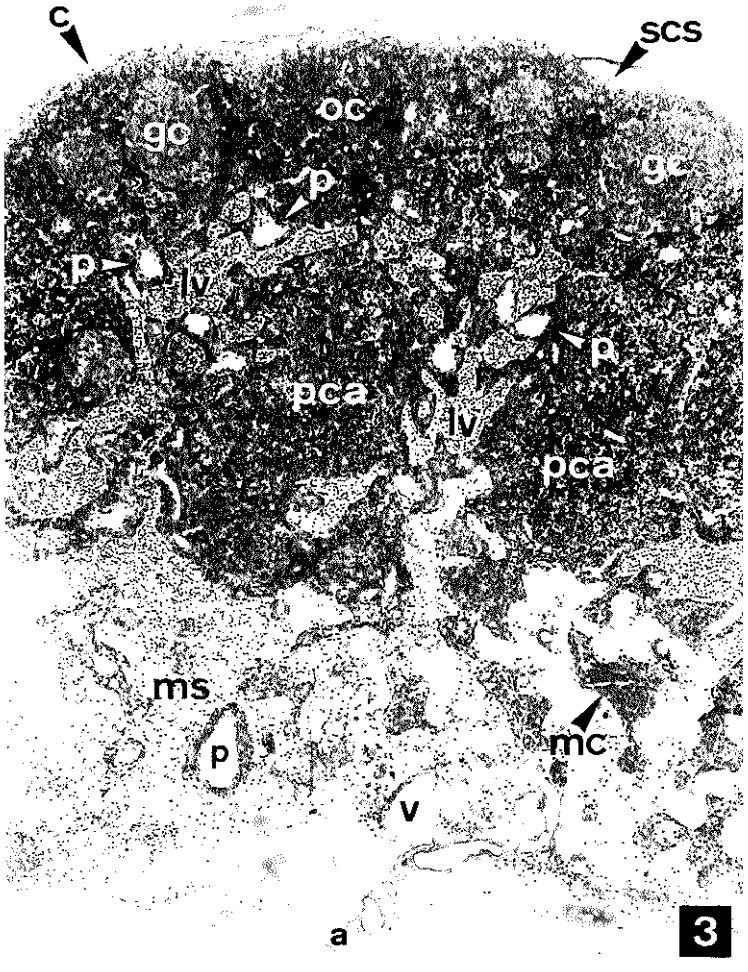

Fig. 3. Histological section of the mesenteric lymph node of a (DBA/2 x C57BL/Rij) F1 mouse. *c* = capsule; *scs* = subcapsular sinus; *oc* = outer cortex; *gc* = germinal center; *pca* = paracortical area; *mc* = medullary cord; *a* = arteriole; *p* = postcapillary venule; *v* = venule; *lv* = lymph vessel; *ms* = medullary sinus. Perfusion fixation. 1 μ Epon section, stained with toluidine blue.

predominantly from postcapillary venules. (Gowans and Knight, 1964; Sedgley and Ford, 1976). Upon entry, they segregate to different compartments (Gutman and Weisman, 1973; Nieuwenhuis and Ford, 1976): B cells localize in the outer cortex and to a lesser extent also in the cortico-medullary junctions. T cells, on the other hand, localize in the paracortical region. Lymphoid cells leave the stroma by short lymphatics which drain the superficial cortex and the paracortical area (Fig. 3). These intranodal lymphatics open in the medullary sinus. Medullary cords predominantly contain plasma cells which migrate during differentiation from the superficial cortex towards this region (Veldman, 1970).

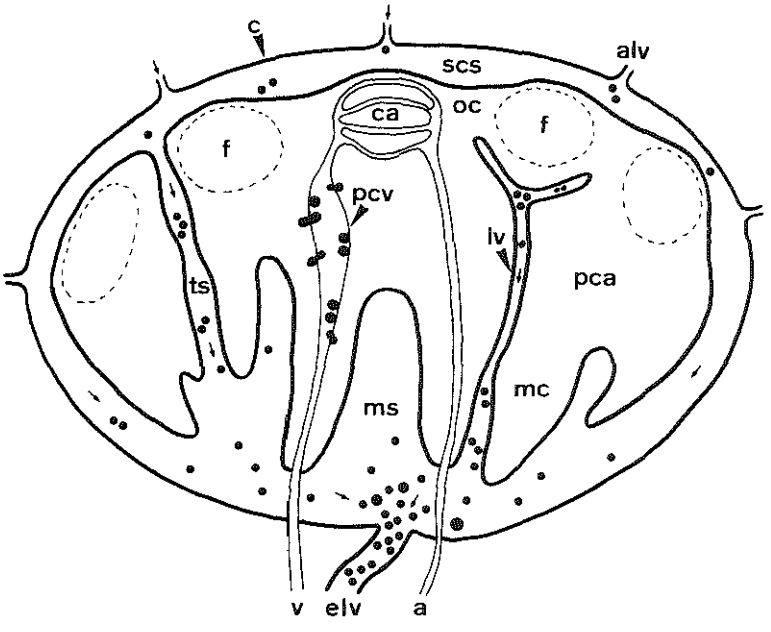

Fig. 4. Schematic presentation of the lymph node. *c* = capsule; *alv* = afferent lymph vessel; *scs* = subcapsular sinus; *oc* = outer cortex; *f* = follicle; *pca* = paracortical area; *mc* = medullary cord; *ts* = trabecular sinus; *ms* = medullary sinus; *lv* = lymph vessel; *a* = arteriole; *ca* = capillary plexus; *pcv* = postcapillary venule; *v* = vene; *elv* = efferent lymph vessel. The arrows indicate the direction of lymph flow. The black dots represent lymphoid cells.

Chapter 3

Discussion of the papers

3.1 Paper I

In paper I the architecture of the splenic white pulp in normal mice and rats is described. Attention is paid to the cellular composition of compartments in which T and B lymphocytes are localized and to the ultrastructure of lymphoid and non-lymphoid cell types residing in these areas. It appears that two areas in the white pulp contain characteristic non-lymphoid cells which possibly determine the nature of lymphoid microenvironments.

(a) In the central PALS (T cell compartment), "interdigitating cells" (IDC) occur. These cells form a cytoplasmic web by means of their long interdigitating, rather low electrondense cytoplasmic processes. Lymphocytes show an intimate contact with these processes and are sometimes totally surrounded by IDC. Fingerlike protrusions emerging from the lymphocytes and tight junctions between lymphocytes and IDC suggest that lymphocytes can be retained at the surface of IDC for prolonged periods. IDC in contrast to macrophages have a low phagocytic potential: they show small numbers of dense bodies which do not show a detectable acid phosphatase activity (Veerman, 1974; Heusermann et al., 1974). IDC do also not show the morphological features characteristic of extensive protein synthesis, yet they synthesize (and probably also secrete) glycoproteins, since the periodic-acid silver-methenamine reaction was positive in the Golgi complex, the tubulovesicular complex, dense bodies and the cell coat. Friesz (1976) recently confirmed the presence of glycoproteins in IDC and provided evidence for the production of two kinds of glycoproteins by IDC. Sialoglycoproteins were found in the surface coat of IDC, whereas asialoglycoproteins occured in the Golgi regions, the tubulovesicular complex and in dense bodies. According to Friesz, the latter of glycoproteins might be destined for secretion and might act as a humoral factor triggering off blast transformation and mitosis in the surrounding lymphocytes. IDC are not only confined to the central PALS of the splenic white pulp, they also occur in thymus-dependent compartments of other lymphoid organs. Veldman (1970) originally described this cell type in the paracortex of rabbit lymph nodes. Subsequently, IDC have been found in thymus-dependent areas of human lymph nodes (Kaiserling and Lennert, 1974), in lymph nodes in rats (Hoefsmit, 1974; Friesz, 1976), and in the spleen of rats (Veerman, 1974). Furthermore IDC have been reported in the human thymic medulla (Kaiserling et al., 1974; Mueller-Hermelink et al., 1976) and in the thymic medulla of rats and guinea pigs (Hoefsmit and Gerver, 1975).

Lymphocytes in the thymic medulla have similar characteristics as peripheral T lymphocytes (Cantor and Weissman, 1976). Their cell membrane is low Θ positive, TL negative and strongly H_2 positive. Both types of cells are cortisone-resistant, show reactivity to mitogens like phytohaemagglutinin and concanavalin A, they show mixed lymphocyte reactivity, graft-versus-host activity, and helper cell activity in the *in vivo* and *in vitro* antibody response. It

is of interest that for the *in vitro* generation of this subclass of thymocytes thymic reticular cells are required, which can be substituted to some degree by splenic surface adherent cells (Mosier and Pierce, 1972). It could well be that IDC serve to induce T cell differentiation in this sytem since IDC occur both in the thymic medulla as in thymus dependent areas in the spleen. The thymic medulla is a compartment which is accessible for antigen (Raviola and Karnovsky, 1972; Benner et al., 1974). It could therefore well be that IDC are involved in the antigen-dependent maturation phase of T lymphocytes, both in the thymic medulla as well as in thymus dependent compartments in peripheral lymphoid organs. Further arguments favouring the hypothesis that IDC (in the spleen) induce the antigen dependent differentiation of virgin thymocites to T helper cells are presented in paper II.

IDC are most probably related to the mononuclear phagocyte system (Langevoort et al., 1970). Arguments for this assumption are the following: (1) Veerman (1974) in an electronmicroscopic study on the ontogeny of the PALS in neonatally thymectomized rats described that before IDC appear promonocytes reside in this area. (2) Cells with a morphology similar to IDC have been observed in *in vitro* cultures of monocytes (Sutton and Weiss, 1966). These cells, described as "epitheloid cells" were observed after prolonged times of culture. According to these authors monocytes give rise to macrophages which subsequently differentiate into epitheloid cells. (3). Macrophages in the macrophage inhibition test show characteristics similar to IDC. Upon stimulation with antigen, the macrophages stop locomotion and phagocytic activity, they form interdigitating processes and come in close contact with surrounding lymphocytes (Smith and Weiss, 1970).

IDC have subcellular characteristics in common with Langerhans cells. Langerhans cells are reticular cells which occur in the epidermis. Like IDC, they show a particular low electron dense cytoplasm which may interdigitate between keratinocytes and melanocytes. They also have an irregular nucleus (Silberberg et al., 1975). In DNCB induced contact allergic reaction in the skin of guinea pigs (a T cell mediated immune response) lymphocytes in the dermis appose closely to Langerhans cells. Similar observations were reported by Levaditi et al. (1973) in tuberculous lesions in the skin. Interestingly, sensitization of the skin of guinea pigs with ferritin (Silberberg et al., 1976) caused the appearance of Langerhans cells containing ferritin in lymph nodes. The authors concluded that Langerhans cells could pick up and transport ferritin from the skin towards draining lymph nodes and in this way acted similarly to macrophages. Obviously, there are ultrastructural differences between Langerhans cells and IDC. Firstly, Langerhans cells contain specific organelles, the "Langerhans cell granules". We did not find these oblong shaped structures in IDC, although the membrane invaginations of IDC have similar dimensions as the Langerhans cell granules. Langerhans granules, however, were found in IDC by Kamperdijk and Hoefsmit (personal communication). These investigators observed that during the secondary immune response to paratyphoid vaccin or to sheep erythrocytes IDC developed Langerhans granules. Furthermore, Hoefsmit noticed that cell suspensions of the thymus, enriched for stromal cells contained IDC with

Langerhans granules, whereas IDC *in situ* did not. Apparently, IDC can develop Langerhans granules under appropriate physiological conditions. A second difference between Langerhans cells and IDC is the extended tubulovesicular complex in IDC, indicating a secretory function of IDC.

The above mentioned data indicate that IDC and Langerhans cells have many characteristics in common. However, definite prove that IDC and Langerhans cells are identical needs further experimentation.

(b) The second lymphoid cell type possibly determining a lymphoid microenvironment was found in the peripheral part of germinal centers. These cells are known as dendritic cells (DC) since they show very fine ramifying dendritic cytoplasmic extensions which penetrate deeply between surrounding mitotic lymphocytes and lymphoblasts. This is a feature in common with IDC; however, the cytoplasmic extensions of DC are much finer, and contain amorphous electron dense material with small electron dense spheres in the extracellular spaces (c.f. Abe and Ito, 1973). Nossal and Ada (1971), investigated the process of antigen trapping by means of autoradiography and found that antigen was retained for prolonged periods on the surface of DC. Experiments published by van Rooyen (1973) indicated that antigen is most probably retained in the form of antigen-antibody complexes which are carried towards DC by Fc receptors on B lymphocytes (Veerman and van Rooyen, 1975). Like IDC, DC show a close contact with surrounding lymphoid cells, however fingerlike protrusions emerging from lymphocytes and tight junctions between lymphocytes and DC were not observed. Since germinal centers are active sites of proliferation of recently immigrated B cells (Nieuwenhuis and Keuning, 1974) we tentatively suggest that DC contribute to the B cell microenvironment involved in antigen dependent B cell proliferation. The origin of DC has not been established although Hoefsmit (1974) suggests that DC have differentiated from reticulum cells (i.e. reticulin producing fibroblasts). Consequently, these cells would not belong to the mononuclear phagocyte system.

3.2 Paper II

Paper II concerns a morphological study of the T cell microenvironment in the spleen. For a precise identification of the T cell microenvironment, mice were lethally X-irradiated and subsequently injected i.v. with 5×10^7 syngeneic thymocytes. These mice are referred to as "T mice". The reconstitution of the thymus dependent area in the spleen was followed for 6 days after injection of the thymocytes. Injection of antigen to these irradiated reconstituted mice is a well known method to generate "educated T cells" or "T-helper cells" reactive to the injected antigen (Mitchell and Miller, 1968; Hartmann, 1970). For example, simultaneous injection of syngeneic thymocytes and sheep erythrocytes within 6 days generates a population of T helper cells in the recipient spleen of the lethally irradiated mouse. This population enhances the anti-SRBC plaque forming activity of B cells *in vivo* and *in vitro* (van Muiswinkel et al., 1975). In the present study some T mice were injected with 4×10^8 SRBC.

The injected thymocyte suspension consists predominently of small dark staining lymphocytes (mean diameter 4.5 μ) which localize around IDC within 24 hrs after injection. They showed peculiar cell contacts with IDC: (1) fingerlike protrusions which indent the surface of IDC, and (2) tight junctions (see also paper I). Both forms of cell contacts indicate that the injected, spleen seeking thymocytes are retained at the surface of IDC. From 2 days onwards, blast cells arose in close conjunction with IDC, and a peak frequency of blast cells in the PALS was found 5 days after injection. By that time, 6 percent of all lymphoid cells in the PALS were lymphoblasts. Addition of sheep erythrocytes to the injected cell suspension doubled the percentage of lymphoblasts on day 5, which is an indication that the differentiation of small lymphocytes into lymphoblasts in this experimental system is antigen driven. At 6 days after reconstitution most of the lymphoblasts had disappeared and large numbers of medium sized (mean diameter 7 μ) moderately electron dense lymphocytes occupied the PALS. In normal rats and mice this type of lymphocyte is the habitual resident of the PALS (see also paper I).

There is now experimental evidence that these medium-sized "end cells" indeed are T helper cells. Zaalberg and Lubbe (unpublished results) separated different fractions of lymphocytes from the spleen of lethally irradiated mice, reconstituted with thymocytes and SRBC, by 1g velocity sedimentation. They tested the helper activity of the spleen cells *in vitro* and *in vivo*, and found a detectable helper activity from day 4. after reconstitution. Preliminary electronmicroscopic analysis of the different fractions by day 6 after reconstitution indicated that most of the active helper T cells were in the fraction which largely consisted of medium-sized T cells (Zaalberg and Van Ewijk, unpublished results).

Together, the morphological changes in the PALS suggest that IDC contribute to the microenvironment of T cells: they are involved in the homing of the injected thymocytes and probably induce the injected small thymocytes to proliferate and differentiate into a population of medium-sized immunologically active T cells. This T cell differentiation is antigen dependent and as such different from T cell differentiation in the thymic cortex which is supposedly antigen independent.

It could well be that under normal physiological circumstances thymocytes seeding from the thymic cortex settle around IDC in peripheral lymphoid organs and differentiate there into immunologically active T cells. In normal mice also small dark staining lymphocytes (recently emigrated cortical thymocytes?) have been observed around IDC, although to a lesser extent than under the present experimental conditions. Whether IDC also play a role in the antigen independent differentiation of T cells is not known. It is clear that autoradiographic studies on the migration of *in situ* labeled thymocytes (c.f. Weissman, 1973) will be necessary to elucidate the role of IDC in T cell differentiation, both in the thymic medulla and in the thymus dependent areas in peripheral lymphoid organs.

3.3 Paper III

The third paper of this thesis is concerned with autoradiographic studies on the migration of ^{3}H-leucine labeled B cells in the spleen and lymph nodes of T mice. Like Goldschneider and McGregor (1968), Ford (1969) and Nieuwenhuis and Ford (1976) we found that the marginal zone is a major site of entry for lymphocytes in the white pulp. In accordance with the observations of Nieuwenhuis and Ford we found that B cells migrated from the marginal zone and also along the reticulin sheaths surrounding terminal arterioles into the white pulp, which in T mice consists only of the PALS (Paper II). Within 6 hrs after injection about $^{2}/_{3}$ of the labeled cells in the white pulp was located in the peripheral part of the PALS, whereas much to our surprise, $^{1}/_{3}$ of the cells was present in the central PALS. The central PALS, as shown in the previous paper, contains microenvironments for T cells. During the course of the reconstitution period, however, the number of labeled cells in the central PALS decreased. This could be explained by assuming that B cells actively migrated from the central PALS towards the stroma which ultimately constitutes B cell compartments. Accordingly, distinct primary follicles were noticed by 18 hrs after injection. Another possibility is that B cells in the central PALS migrated towards efferent lymph vessels which run parallel to branches of central arteries (Snook, 1946; Anderson and Weiss, 1976). This notion was confirmed by the presence of labeled cells in lymphatics near the splenic artery, by 24 hrs after injection.

Migration of B cells through the peripheral and central PALS (see also Fig. 5) may have significance for the immune response. It seems likely that in those immune responses in which cooperation between T and B cells is required, the migration of B cells through T cell compartments serves this purpose, either by cell-cell contact with antigen-activated T cells (Mitchison,

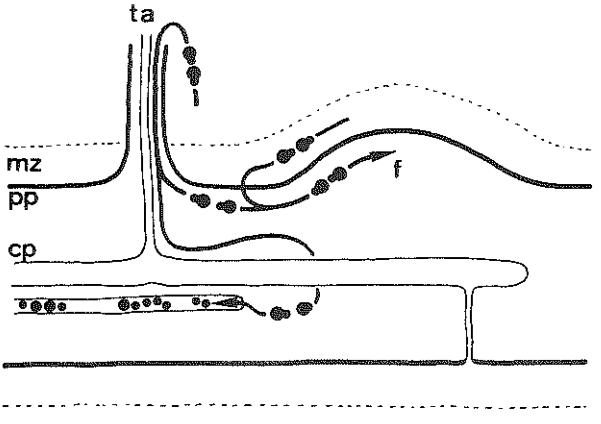

Fig. 5. Schematic presentation of B cell migration in the splenic white pulp of a (T) mouse. B cells enter the white pulp from the marginal zone (*mz*) and along the sheaths which surround terminal arterioles (*ta*). They traverse the peripheral or the central PALS (*pp, cp*), to localize in follicles (*f*) in the peripheral PALS, or to migrate into efferent lymphatics in the central PALS. The black dots represent lymphoid cells.

1971) or by short range activating factors secreted by antigen-activated T cells (Feldman and Basten, 1972; Basten and Feldman, 1972; Katz and Benacerraff, 1972; Taussig, 1974). The retention of antigen-activated T cells in the central PALS by IDC (Paper II) fits well in this concept. As will be shown in the next paper on cellular events during the immune response, B cells indeed transform into Ig-synthesizing plasmablasts in the peripheral PALS.

In lymph nodes, B cells were found to enter the lymphoid stroma through the wall of postcapillary venules (Fig. 6). This observation is in agreement with those of Gutman and Weissman (1973) and Nieuwenhuis and Ford (1976).

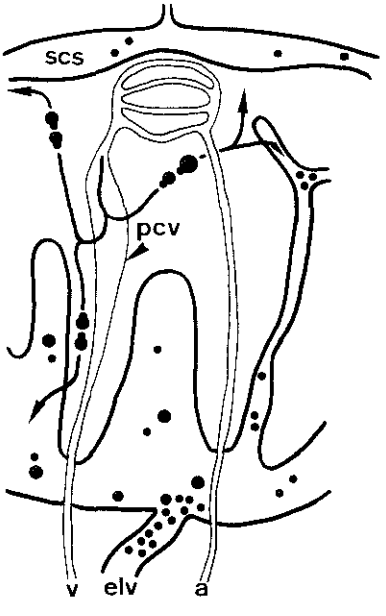

Fig. 6. Schematic presentation of B cell migration in lymph nodes of a T mouse. B cells enter the lymphoid stroma from postcapillary venules (*pcv*) and migrate to the outer cortex or to the medullary cords. They leave the node via the efferent lymph vessel. *a* = arteriole; *v* = vene; *scs* = subcapsular sinus; *elv* = efferent lymph vessel. The black dots indicate lymphoid cells.

Upon entry, B cells migrated towards the superficial cortex and formed follicles from 18 hr onwards. A minority of B cells was also found in the cortico-medullary junction and in medullary cords.

As in the spleen, B cells in lymph nodes were found to migrate across T cell areas. Again this particular migration pathway of B cells might be the basis for T-B interaction during the T cell dependent immune response.

Cell types involved in the ultimate homing of B cells in primary follicles are as yet not identified. In contrast to germinal centers, primary follicles do not show DC with extended cytoplasmic processes. With electronmicroscopy we noted in some follicles the presence of cells with a similar appearance to DC, but with relatively short cytoplasmic extensions. It could well be that antigen-antibody complexes recognized by Fc receptors which possibly occur on both dendritic cells, reticulum cells and lymphocytes form the basis of B cell homing in primary follicles. Further (immuno) electronmicroscopic studies are required to reveal the nature of factors determining B cell homing in primary and secondary follicles.

3.4 Paper IV

This paper deals with the morphology of T and B cell compartments in the white pulp of the spleen during the humoral immune response. To study the cellular changes during immune reactivity we immunized germfree mice with sheep erythrocytes. Normal germfree mice show a very low background stimulation and their lymphoid tissues are therefore histologically in a "quiescent" state. This is witnessed a.o. by the lack of germinal centers in lymph nodes and spleen. Immunization of germfree mice with SRBC therefore causes a distinct primary immune response.

In this study we i.v. injected germfree mice with 4 x 10^8 SRBC and studied the white pulp of the spleen with light-, fluorescence- and electronmicroscopy at intervals of 12 hrs after immunization. The first transformation of lymphoid cells into blast cells was noted in the central PALS by 48 hr after stimulation. These blast cells were located preferentially in the neighbourhood of interdigitating cells and particles (antigen?) containing macrophages. Apparently, differentiation of T lymphocytes into lymphoblasts is a first step in lymphoid differentiation during the immune response to SRBC. Our observations are in agreement with morphological studies on the course of the immune response in lymph nodes of mice subcutanously immunized with SRBC (Davies et al., 1969). These authors studied the histology of lymph nodes of thymectomized, lethally irradiated, bone marrow protected CBA/H mice, grafted with a single lobe of a CBA/H-T6T6 neonatal thymus and subsequently immunized with SRBC. They found that in these mice: (1) initial lymphoblasts and dividing cells were located in the thymus dependent paracortex and (2) these cells were of donor origin, i.e. thymus derived.

In our studies the differentiation of B cells into Ig synthesizing plasmablasts was first noted by 72 hrs after immunization. To detect these cells we incubated cryostate sections of the spleen with rabbit-anti-mouse Ig as a first step, and with rhodamine conjugated to goat-anti-rabbit Ig as a second step. In the fluorescence microscope, plasmablasts were noted in the peripheral PALS, nearly always in the neighbourhood of particle containing macrophages. They accumulated from day 4 onwards around the central arterioles and terminal arterioles. By day 8 the PALS was virtually depleted of Ig synthesizing cells; they were now mostly found around the largest branches of the splenic artery and in the red pulp. Apparently the plasmablasts moved out of the PALS during the course of the immune response by a migration pathway along the reticulin sheaths surrounding central and terminal arterioles towards the red pulp. This migration pattern agrees well with other histological studies on the immune response to thymus dependent antigens in conventional mice and other types of rodents (reviewed by Fitch and Wissler, 1971).

From our experiments we concluded that antigen driven B cell differentiation starts in the peripheral PALS. This process seems to be induced by antigen containing macrophages and conduced by antigen activated T helper cells. Several observations support this hypothesis. (1) The peripheral PALS has been shown to be an important traffic pathway for recently immigrated B cells (Paper III, Nieuwenhuis and Ford, 1976). (2) In perfusion experiments with

isolated spleens, Ford and Gowans (1967) have shown that in the rat spleen the height of the immune response to infused SRBC was determined by the number of nucleated cells in the perfusate rather than by the total number of nucleated cells in the spleen. This indicates that the antibody forming cell precursors are part of a population of B cells which recently had immigrated into the white pulp. (3) Macrophages with phagosomes containing remnants of erythrocytes were initially found in the marginal zone and marginal sinuses and appeared later on in the peripheral and central PALS. This indicates that macrophages pick up and transport antigen from the marginal zone towards the PALS and expose antigen to T and B cells. (4) Plasmablasts were found initially in close relation with macrophages in the peripheral PALS, both in our fluorescence and electronmicroscopic studies. This observation is in accordance with studies on the hemolysin response *in vitro* by Mosier and Coppleson (1968) and Unanue (1972) who noted that for an optimal *in vitro* antibody response cell clusters containing macrophages were required.

Together, these observations suggest that antigen-laden macrophages and interdigitating cells in the central PALS activate T cells to differentiate into T helper cells. Subsequently, immigrant antibody forming cell precursors contact antigen containing macrophages in the peripheral PALS and are induced to differentiate in antibody forming plasmablasts. Further proliferation and differentiation of antigen activated B cells is stimulated by antigen-activated T cells. This cellular cooperation may be initiated by cell-cell contacts (Mitchison et al., 1970; Feldman and Basten, 1972; Basten and Feldman, 1972) and/or by short range humoral factors secreted by the latter two cell types (reviewed by Basten and Mitchell, 1976).

The role of IDC in the immune response of normal mice is uncertain. Paper II has shown that IDC probably are involved in the generation of helper T cells. In T mice the formation of lymphoblasts started around IDC between day 2 and 3, which agrees well with the onset of the blast reaction in the central PALS in the present experiments. It seems therefore likely to assume that also in normal mice the early proliferation and differentiation of lymphocytes in the central PALS represents the generation of T helper cells. Whether IDC solely induce T lymphocytes to proliferate and differentiate into T helper cells, or whether they need the aid of antigen activated macrophages is unknown. It could be that IDC just act as non-specific proliferation stimulating cells and that the initial inducing signal is presented by antigen containing macrophages. On the other hand, IDC may be transformed macrophages and present antigen in immunogenic form to surrounding T lymphocytes. It is clear that further investigation is needed to enlighten the role of IDC during immune responses.

The differentiating antibody forming cell precursors migrate during the course of the immune response towards the red pulp and show, when inspected with the electronmicroscope, a characteristic cytoplasmic tail. *In vitro* studies have demonstrated that this configuration is specific for mobile lymphoid cells (Lewis 1931; Kondo and Yoshitake, 1976). McFarland et al. (1966) called this cytoplasmic tail "uropod" and showed with electronmicroscopy that most of the cell organelles were gathered in the uropod. Rosenstreich et al. (1972) found that in *in vitro* cultures of guinea pig lymphocytes all uropod bearing

lymphocytes were T cells and they proposed that this structure was unique for this lymphocyte subclass. Recently performed studies on anti-Ig induced capping of B lymphocytes (Schreiner and Unanue, 1976) have shown that B cells also form uropods. In careful immunofluorescence and phasecontrast studies the latter authors observed that 30 to 60 seconds after formation of the cap, the area under the cap was transformed into a uropod. As a result B cells started to move and endocytosed the capped receptors.

The present paper shows furthermore that the early induction of the immune response in the central and peripheral PALS results in the formation of IgM antibodies and preceeds the formation of germinal centers. Hence, germinal centers are not the site of early T-B cooperation during the formation of IgM antibody forming cells. Germinal center development coincidenced with IgG titers and it can therefore not be excluded that germinal centers are sites of T-B interaction during the formation of IgG antibody forming cells. However, we did not find large numbers of cells with a positive cytoplasmic fluorescence for Ig in germinal centers. The observed Ig positivity was confined to dendritic cells. These cells possibly have Fc receptors on their cell surface which retain Ag-Ab complexes (White et al., 1967; van Rooyen, 1973). Furthermore, IgG synthesis is strongly thymus dependent (Taylor and Wortis, 1968) and germinal centers appear not to contain T cells (Nieuwenhuis and Ford, 1976). However, Weissman et al., (1976) in membrane fluorescence studies with specific anti T cell antisera has shown the presence of small numbers of T cells in germinal centers.

The exact function of germinal centers in immune responses is as yet not clearly understood. However, when germinal centers during the peak of the immune response to SRBC are dissected, made into a cell suspension and transferred with SRBC into lethally irradiated hosts they give rise to antibody forming cells in a way characteristic for a secondary response (Wakefield and Thorbecke, 1968). These types of experiments led to the conclusion that germinal centers are involved in the generation of memory cells. Besides, germinal centers may also act as antigen dependent B cell amplification systems, (Nieuwenhuis and Keuning, 1974).

3.5 Paper V

Paper V introduces scanning electronmicroscopy as a tool to investigate the cell surface of lymphoid and non lymphoid cell types in lymphoid compartments. This study was initiated by the observations of Polliack et al., (1973) and Lin et al. (1973) providing evidence that the cell surface of T and B cells had a different topography: T cells were smooth, B cells showed many microvilli. These observations were obtained using suspensions of nucleated cells, isolated from human peripheral blood by Ficoll-Isopaque density gradient centrifugation. We tested their findings in lymphoid organs of mice either depleted of T cells (adult thymectomized, lethally irradiated, bone marrow reconstituted mice: B mice) and in mice depleted of B cells (adult, lethally irradiated, thymocyte reconstituted mice: T mice). Our technique had the advantage that lymphoid cells were fixed *in situ* by means of perfusion fixation within 30 seconds after onset of the perfusion. Polliack and Lin fixed

the cells after the isolation of the cells from the Ficoll Isopaque gradient, which takes about 1 hr.

We studied two types of areas in the mesenteric lymph node and spleen of B and T mice: (1) recirculation pathways and (2) homing areas.

Recirculation pathways in the lymph node are the high endothelial postcapillary venules (PCV) and the lymphatics (Gowans and Knight, 1964; Sedgley and Ford, 1976; Paper III) and in the spleen the marginal zone (Ford, 1969; Paper III). Homing areas for B cells are the outer cortex in lymph nodes and follicles in the spleen, for T cells the paracortex in lymph nodes and the central PALS in the spleen (Paper I, II, III, IV). From our experiments we concluded that in the mouse the surface structure of B and T lymphocytes was not different under the present experimental conditions. In fact, we found that both T and B cells were villous when recirculating, and smooth when homing in specific lymphoid compartments. Apparently, the cell membrane of lymphocytes is very fluid and microvilli may easily be retracted by the lymphocyte. The presence of both smooth and villous lymphocytes in PCV agrees with this idea.

Subsequently, we tested whether the difference between our results and those of Lin et al. (1973) and Polliack et al. (1973) could be explained by differences in preparative conditions by comparing the *in vitro* topography of lymphocytes suspended from different organs. Cell suspensions of the thymus of normal mice and of mice treated 2 days before with dexamethason (30 mg dexamethason per kg body weight) as well as cell suspensions of the mesenteric node of B mice and the mesenteric node of nude mice were washed with phosphate buffered saline and seeded into culture chambers. These culture chambers were equipped with a flat non-toxic teflon film as cell support. The cells were allowed to settle on the teflon film for 30 minutes and then fixed and dehydrated *in situ*. Details of this method have been reported elsewhere (van Ewijk and Hösli, 1975a, b; van Ewijk and Mulder, 1976). Pieces

SCANNING ELECTRONMICROSCOPY OF LYMPHOCYTES SEEDED INTO THE PLASTIC FILM DISH

CELL SOURCE	DIAMETER	n	NUMBER OF MICRO-VILLI/CELL	n	LENGTH OF MICRO-VILLI (μ)	n
THYMUS	3,9 ± 0.5μ	(94)	52 ± 48	(146)	0.47 ± 0.25μ	(107)
THYMUS of dexamethason-treated mice	4,3 ± 0.7μ	(190)	148 ± 60	(110)	0.16 ± 0.08μ	(225)
Mesenteric node of TxBM-mice	4,2 ± 0.5μ	(122)	48 ± 30	(120)	0.47 ± 0.13μ	(175)
Mesenteric node of nude mice	4,2 ± 0.5μ	(214)	68 ± 48	(116)	0.42 ± 0.26μ	(155)

of the film were cut out and processed for scanning electronmicroscopy as described in paper V. The results of these studies are summarized in Table I. As can be seen each type of lymphocyte is heterogeneous with regard to cell size, number and length of microvilli, and a reliable distinction between T and B cells is, in this *in vitro* system, not possible. Thymocytes are smooth surfaced when observed *in situ* (Weiss, personal communication) whereas the present experiment shows that the thymocytes, when brought into suspension expose various numbers of microvilli on their cell surface. This again supports the view that microvilli are cell organelles which are protruded or retracted from the lymphocyte membrane in relatively short periods.

As can be seen in Fig. 7, lymphocytes expose prolonged microvilli towards the teflon film which indicates that microvilli are involved in the settling of the lymphocyte on the supporting surface. Similar observations were made *in vivo* where lymphocytes were found to contact the high endothelial cells in PCV by means of microvilli. Supporting evidence for the retractibility of lymphoid microvilli came furthermore from experiments reported by Fagraeus (1974), who by immunofluorescence demonstrated the presence of actin in microvilli.

Anderson and Anderson (1976) confirmed our observations on the route of lymphocyte migration through the wall of PCV. In transmission electronmicroscopic studies of postcapillary venules they found that microvilli on lymphocytes contacted the endothelial cells in shallow pits. They observed electron-dense cross bridges between both types of cells, possibly the morphological

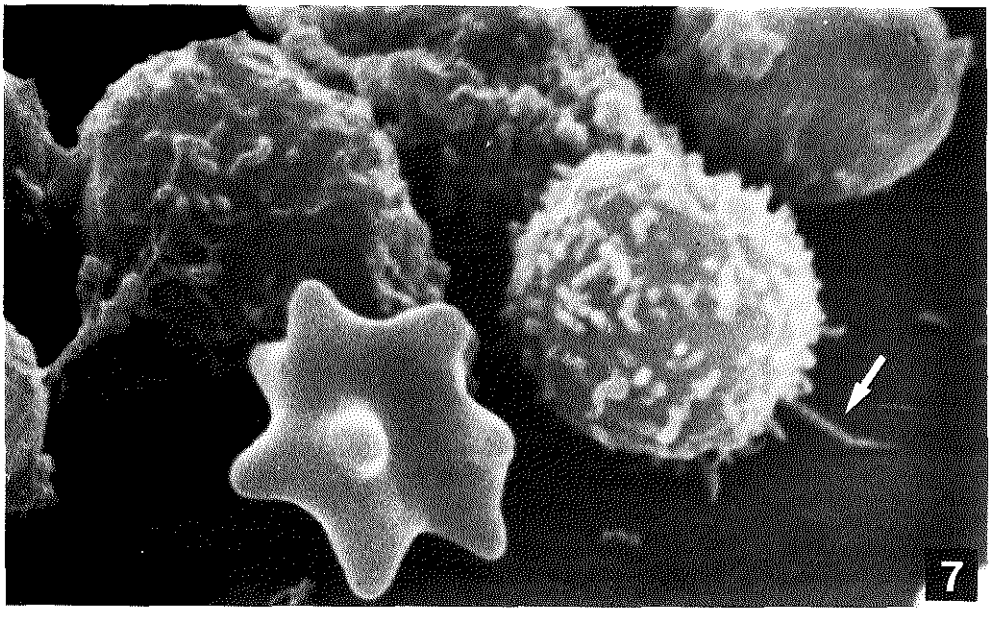

Fig. 7. Lymphocytes suspended from the mesenteric lymph node and seeded into a tissue culture chamber. They attach to the substrate with prolonged microvilli (arrow). The star-shaped cell in the center of the micrograph is a deformed erythrocyte.

substrate of interacting receptors. Stamper and Woodruff (1976) have recently shown that (recirculating) lymphocytes have specific receptors for high endothelial cells in PCV. They incubated frozen fixed sections of lymphoid tissue a.o. with freshly isolated thoracic duct lymphocytes and found that these lymphocytes adhered specifically to the wall of PCV, whereas lymphocytes suspended from thymus and bone marrow did not.

From our experiments and from the literature we conclude that microvilli can occur on both T and B cells and that they are contractile organelles which are probably involved in cell-cell recognition.

3.6 Paper VI

The last paper of this thesis is concerned with the ultrastructure of a "microenvironment" of the lymphocyte itself: the cell membrane. The preceeding paper has already shown that lymphocytes can change their membrane topography and we concluded that morphological distinction between mouse T and B lymphocytes was not possible using the scanning electronmicroscope. The purpose of this paper was twofold. Firstly, we wanted to investigate whether ultrastructural differences between cell membrane of T and B lymphocytes were detectable when observed with the transmission electronmicroscope. Secondly, we wanted to develop a labeling technique, which would enable us to distinguish between the cell membranes of T and B lymphocytes in both the transmission and scanning electronmiscroscope. To this purpose we used the freeze-etch technique, which is highly suitable for the demonstration of membrane structures in the transmission electronmicroscope. The preparation of freeze-etch replicas is relatively simple and is far less time consuming than the standard techniques for the preparation of ultrathin sections of biological specimens.

The important quality of this method is the fact that during fracturing of the frozen specimen the fracture plane preferentially follows the hydrophobic part of the bilayered membrane structures (Pinto da Silva and Branton, 1970), hence the hydrophobic fracture face of the membrane is exposed (Fig. 8). Subsequent etching exposes the outer surface of the cell in addition to the hydrophobic fracture face (Fig. 8, indicated by: 1-2 and 3-4). The fracture faces of membrane structures of lymphocytes show numerous intramembraneous particles (ranging from 70 Å to 90 Å in diameter). The inner face of the fractured membrane shows about 500 particles per μ^2 (Scott and Marchesi, 1972). The functional properties of intramembraneous particles is unknown however, in erythrocytes it has been demonstrated that they are associated with glycoproteins (Pinto da Silva et al., 1971). The outer etched face of the membrane usually has a smooth appearance.

Using this method we studied the cell membrane of thymocytes and of lymphocytes suspended from the mesenteric lymph nodes of nude mice. The inner and outer fracture faces of both cell types showed evenly distributed membrane particles. In addition to these particles we found pits of corresponding size to particles (about 100 Å in diameter). The even distribution of pits and particles is in contrast to findings reported by Mandel (1972) who noted

aggregates of particles in T lymphocytes. Later on, this particle aggregation was shown to be induced by cryoprotective agents (McIntyre et al., 1973). De Groot et al. (1975) has shown that the particle distribution in lymphocyte membranes is easily affected by experimental conditions, like changes in temperature, and the presence or absence of albumin in lymphocyte suspensions. We concluded from our experiments that under the present experimental conditions (rapid freezing of cells without cryoprotective agents in serum free phosphate buffered saline) the membranes of T and B cells in freeze-etch replicas are not different with respect to number and distribution of the particles and pits.

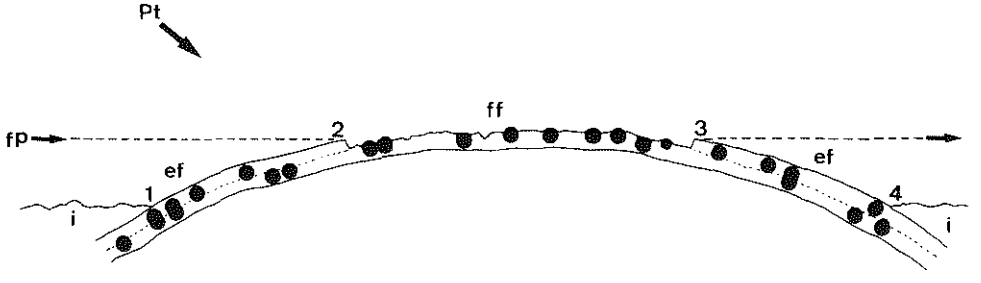

Fig. 8. Schematic presentation of the freeze-etched cell membrane of a lymphocyte. *fp* = fracture plane; *ff* = inner hydrophobic fracture face of the membrane; *ef* = etched, external face of the membrane; *i* = surrounding ice. The fracture face in the replica is exposed between 2 and 3, the etched face is exposed between 1 and 2, and between 3 and 4. The black dots represent the intramembranous particles. The arrow, indicated by *Pt*, indicates the direction of platinum shadow-casting.

The cell surface labeling methods for lymphocytes described in this paper are based on the use of specific antisera to detect cell surface determinants on lymphoid cells. The first method is a direct conjugation method of anti-Ig antisera or anti Θ antisera to Turnip Yellow Mosaic Virus (TYMV) particles by means of freshly distilled glutaraldehyde. TYMV particles are spherical and measure 280 Å. They are within the detection level of the freeze-etch technique and also within the detection level of the scanning electronmicroscope. The second method described is an indirect method in which Fc receptors on lymphoid cells are demonstrated with antigen-antibody complexes (Alphalpha mosaic virus (AMV) / anti alphalpha mosaic virus antibody complexes). With the present labeling method we found that the Θ antigens on the surface of thymocytes were randomly distributed. Ig molecules and Fc receptors on B cells, however, were as a result of the incubation with the conjugate not randomly distributed but were found in small clusters. These clusters are probably similar to "patches" as observed in immunofluorescence studies on suspensions of B lymphocytes, incubated with fluorescent anti Ig antisera (Raff and de Petris, 1973). Patches accumulate into a cap of label which is found in a part of the cell membrane opposite the Golgi region (de Petris and Raff 1972). Following capping the label is rapidly endocytosed. Patch formation and capping indicate the fluid nature of the lymphocyte membrane (see review by

Nicholson, 1976). Both labeling techniques presented are potential labeling methods for ultrastructural studies of freeze-etch replicated cell membranes since the virus particles are not only detectable on the etched surface of the labeled cell but also on the inner hydrophobic fracture face of the cell membrane. Therefore studies on the relation between membrane particles and antigenic determinants are now possible. Such studies may then provide further insight into the ultrastructure of lymphoid subpopulations.

Summary

Vertebrates possess an immunosurveillance system which defends the animal against foreign pathogenic invaders and cancer. It is composed of lymphoid cells, lymphoid organs and different types of humoral factors secreted by the cellular constituents of the immune system. Lymphocytes, like the other cell types in the peripheral blood, are the progeny of pluripotent haemopoietic stem cells (introduced in chapter 1). In adult mammals, haemopoietic stem cells are located in the bone marrow. There are two basic *lines* of differentiation leading to two distinct subpopulations of lymphoid cells: the T lymphocytes and B lymphocytes.

T lymphocytes are cells which during an intermediate stage have differentiated in the thymus. Upon maturation, these cells migrate to T cell regions (thymus dependent areas) in peripheral lymphoid organs. T cells are concerned with "cellular immunity", i.e. the specific immune reactivity which can be adoptively transferred by injecting immune donor cells into syngeneic recipient animals. Examples of cellular immunity are the delayed type hypersensitivity reactions to various types of antigens (a.o. tuberculin reaction), reaction against fungi and viruses, and cytotoxic killing of antigenic tumour cells and organ transplants.

B lymphocytes on the other hand, seed from the bone marrow directly into peripheral lymphoid organs and localize in distinct B cell compartments (thymus independent areas). Upon antigenic stimulation, B cells differentiate to plasma cells, which synthesize antibody molecules specific for the invaded or injected antigen. This type of systemic immune reactivity is known as "humoral immunity" and acts primarily against the extra-cellular phases of bacterial and viral infections. Depending on the type of antigen, B cells may need cooperation with antigen-activated T cells during early phases of the humoral immune response.

Lymphocytes differ in a number of aspects from the other cell types in the blood. They have the unique property to recognize "foreignness" by means of specific antigen receptors, they circulate between blood and lymph and they "home" in peripheral lymphoid organs in well defined compartments and microenvironments where they may exert their physiological function.

It is the aim of this thesis to provide insight in the structure of lymphoid organs and in the interrelations between lymphoid and non-lymphoid cell types in different lymphoid compartments and microenvironments, both at the light and electronmicroscopic level. Chapter 2 summarizes the complicated architecture of spleen and lymph nodes and describes briefly the different lymphoid compartments in these organs. Chapter 3 and the following papers report and discuss the experimental work. The first four papers concern light and transmission electronmicroscopic investigations of the spleen and lymph node under different experimental conditions. The last two papers show cell surface characteristics of lymphoid cells *in situ* and in cell suspension as observed with the scanning and the transmission electron microscope.

Paper I describes the delineation of lymphoid compartments in the white pulp, such as the peripheral arteriolar lymphoid sheath (PALS), follicles and

marginal zone. Lymphocytes in the different white pulp compartments form a heterogeneous population when studied with the electron microscope. There is no clear morphological distinction between lymphocytes in T and B cell compartments although lymphocytes in the PALS are generally larger than lymphocytes in follicles. Furthermore follicular lymphocytes often show nuclear inclusions.

It appears that two areas possess characteristic non-lymphoid cell types. The central PALS contains "interdigitating cells" (IDC) whereas the peripheral part of germinal centers contains "dendritic cells" (DC). Both types of cells show an intimate contact with surrounding lymphocytes and it is suggested that these cells contribute to microenvironments specific for T and B cells respectively. Paper I furthermore emphasizes two traffic routes in the splenic white pulp: the marginal zone, and the lymph vessels in the PALS.

Paper II describes the composition of the T cell microenvironment in the spleen. To this purpose mice were lethally X-irradiated and reconstituted with syngeneic thymocytes. These "T" mice have no B cells and develop within 6 days only thymus dependent compartments in peripheral lymphoid organs. We followed the reconstitution of the T cell compartment in the spleen at intervals of 24 hr after irradiation and injection of the thymocytes. We found that the i.v. injected thymocytes localized around IDC. Some of these cells, especially after antigen injection transformed into lymphoblasts in close relation with IDC. A peak frequency of lymphoblasts was noted on day 5. On day 6, most lymphocytes in the PALS were medium-sized and had a similar morphology as lymphocytes residing in the PALS of normal mice. We tentatively conclude from this study that IDC contribute to microenvironments specific for the differentiation of immunologically active T cells in the peripheral lymphoid organs.

Paper III reports an autoradiographic study on the migration of B cells and the reconstitution of B cell compartments in the spleen and mesenteric lymph node of T mice. B cells, *in vitro* labeled with ^{3}H-leucine were i.v. injected into T mice which had been prepared 6 days previously. We followed the localization of the radioactively labeled B cells at 6, 18, 24, 42 and 66 hrs after injection.

Already at 6 hrs after injection, B cells were present in the white pulp of T mice. They predominantly localized in the peripheral part of the PALS, however, some B cells were also found in the central PALS. This finding indicates that T cell compartments are not exclusively resident areas for T lymphocytes alone. B cells were found to accumulate in primary follicles in the peripheral PALS from 6 hrs onwards. By 24 hrs after injection, labeled cells were also noted in lymph vessels. It appears from the study that B cells follow different entry routes in the splenic white pulp of T mice. They may enter the peripheral PALS either directly from the surrounding marginal zone or along reticulin sheaths surrounding the terminal arterioles. Subsequently, most B cells migrate along the peripheral PALS to form primary follicles. A minor part of B cells migrated through the central PALS, supposedly on their way to efferent lymphatics in the central PALS.

B cells entered the mesenteric node through the wall of postcapillary

venules in the paracortex. They formed follicles from 18 hrs onwards. Again B cells were found to traverse T cell areas on their way to B cell compartments. This particular migration pathway may well be relevant to explain T-B cooperation during immune responses *in vivo* to those antigens which require cooperation between both classes of lymphocytes.

Paper IV reports the cellular events during the humoral immune response the sheep erythrocytes in the different lymphoid compartments in the spleen of germfree mice. This antigen is "T cell dependent", which means that during the immune response T cells cooperate with B cells and macrophages leading to the differentiation of B cells to antibody producing plasmacells. It appears from this study that antigen is captured by macrophages located in the periphery of the white pulp. Subsequently, antigen-laden macrophages migrate into the PALS. In the central PALS T cells start to proliferate and differentiate into large lymphoblasts by 48 hr after injection of the erythrocytes. This process occurs in close juxtaposition with macrophages and IDC. B cell differentiation into Ig-synthesizing plasmablasts was first noted in the peripheral PALS at 72 hrs after antigen injection. The plasmablasts migrated along the central and terminal arterioles towards the red pulp. Germinal center formation started at day 4 within primary follicles. We conclude from this study that microenvironments regulating the cooperation between T and B cells are initially situated in the PALS.

Paper V reports a study of the topography of T and B cells in their respective compartments of the spleen and mesenteric lymph node, as viewed with scanning electronmicroscopy (SEM). From *in vitro* studies reported in the literature it appeared that T cells are smooth surfaced whereas B cells expose many microvilli. We investigated the *in vivo* morphology of T and B lymphocytes and concluded that under the present experimental conditions a reliable distinction between the two lymphoid subclasses was not possible. However, we could, for both T and B cells, distinguish between recirculating and homing lymphocytes. The large majority of lymphocytes present in recirculation pathways showed microvilli whereas lymphocytes in specific homing areas were smooth surfaced. Apparently the surface structure of lymphocyte can change depending on the microenvironment in which the lymphocyte is present. *In vitro* studies of lymphoid cell suspensions seeded into tissue culture chambers support this view. We suggest that microvilli on lymphocytes are cell organelles involved in cell-cell recognition studies.

Paper VI reports a transmission electronmicroscopic study on cell membranes of T and B lymphocytes as revealed by the freeze-etch technique. Examination of the freeze etch replicas failed to reveal any differences in the membrane structure of T and B lymphocytes. From this and the previous study it is clear that for a reliable distinction between T and B cells in the electron microscope specific label techniques are required. Paper VI describes to this purpose two labeling techniques to detect theta antigens on T cells and immunoglobulin molecules and Fc receptors on B lymphocytes using specific antisera coupled to plant viruses as electronmicroscopic markers. These labeling techniques are suitable for both freeze-etch electronmicroscopic studies and SEM studies of lymphoid subpopulations.

The main conclusions from these studies are the following:

(1) T and B cells localize each in distinct compartments in peripheral lymphoid organs. However, these compartments are not exclusively accessible for the lymphoid cells determining its nature: B cells may traverse thymus dependent areas and T cells may traverse thymus independent areas. This particular migration pattern may be relevant to explain T-B cooperation during the humoral immune response *in vivo.*

(2) In the spleen, specific microenvironments involved in the antigen-induced activation of lymphoid cells are located in the central PALS, the peripheral PALS and in germinal centers. Their specificity is possibly defined by non-lymphoid cell types such as reticulum cells, interdigitating cells, macrophages and dendritic reticulum cells.

(3) T and B cells have no distinct morphological characteristics based on which they can be recognized in the light and electron microscope. Therefore, specific labeling methods are required to distinguish between lymphoid subpopulations.

Samenvatting

Gewervelde dieren beschikken over een immunologisch systeem dat hen verdedigt tegen pathogene micro-organismen en tumoren. Dit systeem is opgebouwd uit lymfocyten, lymfoide organen en verschillende typen humorale factoren welke gesecerneerd worden door de cellen van het immuun systeem. Lymfocyten zijn evenals de andere celtypen in het bloed afkomstig van pluripotente haemopoietische stamcellen (zie **hoofdstuk 1**). Deze stamcellen zijn in volwassen zoogdieren voornamelijk gelocaliseerd in het beenmerg. Er zijn twee hoofdlijnen bekend volgens welke de differentiatie van stamcel tot lymfocyt verloopt. Als gevolg hiervan ontstaan er twee populaties lymfocyten: de T lymfocyten en de B lymfocyten.

T lymfocyten zijn lymfocyten welke gedurende hun ontwikkeling een differentiatie periode in de thymus hebben doorgemaakt. Na maturatie verlaten de T lymfocyten de thymus en migreren naar "thymus afhankelijke gebieden" in perifere lymfoide organen. T cellen zijn betrokken bij de *cellulaire immuniteit,* een vorm van immuniteit welke passief overgedragen kan worden door middel van injectie van celsuspensies in isologe ontvangers. Voorbeelden van cellulaire immunologische reacties zijn de overgevoeligheid van het vertraagde type zoals de tuberculine reactie, reacties tegen fungi en virussen en de cytotoxische reacties tegen antigene tumor cellen en lichaamsvreemde transplantaten.

B lymfocyten zijn lymfocyten welke na differentiatie in het beenmerg direct naar de perifere lymfoide organen migreren. Zij localiseren in zgn. "thymus-onafhankelijke" gebieden. Na stimulatie met antigeen differentiëren B lymfocyten tot immunoglobuline producerende plasma cellen. De immunoglobulinen herkennen het antigeen en vormen hiermee een specifiek complex dat vervolgens door fagocytaire cellen verwijderd kan worden. Dit type immunologische reactie staat bekend als *humorale immuniteit* en speelt een rol bij de afweer tegen extracellulair aanwezige pathogene micro-organismen en virussen. Vaak kunnen B cellen de differentiatie tot plasma cel niet geheel zelfstandig uitvoeren, zij hebben in dat geval de "hulp" van T cellen nodig.

Lymfocyten bezitten een aantal kenmerken waarin zij verschillen van de andere celtypen in het bloed: (a) Zij bezitten, zoals reeds vermeld, de unieke eigenschap om door middel van antigeen-receptoren op specifieke wijze antigene determinanten te herkennen. Interactie tussen antigeen en antigeen-receptor kan leiden tot een specifieke immunologische reactie, wat tot gevolg heeft dat het antigeen geëlimineerd wordt. (b) Zij recirculeren tussen bloed, lymfoide organen en lymfe en hebben als zodanig een maximale kans om antigene structuren in het lichaam te herkennen. (c) Zij migreren in de lymfoide organen naar specifieke compartimenten en micro-omgevingen. De structuur van deze lymfoide compartimenten leidt ertoe dat de lymfocyten hun physiologische functie op efficiente wijze kunnen uitvoeren.

Het doel van dit proefschrift is inzicht te geven in de structuur van de lymfoide organen, de ultrastructuur van T en B lymfocyten en de relatie duidelijk te maken tussen lymfoide en niet lymfoide cellen in de verschillende compartimenten en micro-omgevingen van de lymfoide organen.

Hoofdstuk 2 dient als inleiding op het experimentele werk en behandelt op beknopte wijze de histologie van milt en lymfklieren. **Hoofdstuk 3** en de daarop volgende publicaties beschrijven het experimentele werk dat de basis vormt van dit proefschrift. De eerste vier publicaties behandelen lichtmicroscopisch en electronenmicroscopisch onderzoek over de structuur van compartimenten en micro-omgevingen in milt en lymfklieren, zoals waargenomen onder verschillende experimentele omstandigheden. De laatste twee publicaties behandelen de oppervlakte structuur van lymfoide cellen *in situ* en in celsuspensies van lymfoide organen.

Publikatie I beschrijft de structuur van verschillende lymfoide compartimenten in de witte pulpa van de milt in normale proefdieren, zoals de peri-arteriolaire lymfocyten schede (PALS), de follikels en de marginal zone. Lymfocyten in deze compartimenten vormen met betrekking tot hun ultrastructuur een heterogene populatie, er is daardoor geen duidelijk verschil aantoonbaar tussen lymfocyten in T en B cel compartimenten. In het algemeen zijn de lymfocyten in de PALS wat groter dan de lymfocyten in follikels. Deze laatste cellen vertonen vaak insluitsels in de kern.

Twee gebieden in de witte pulpa bezitten karakteristieke niet-lymfoide celtypen. Het centrale gedeelte van de PALS bevat *interdigiterende cellen* (IDC), terwijl het perifere gedeelte van follikelcentra gekenmerkt is door de aanwezigheid van *dendritische cellen* (DC). Beide celtypen staan in nauwe relatie met de er omheen liggende lymfocyten en het lijkt dat deze cellen deel uitmaken van micro-omgevingen welke specifiek zijn voor respectievelijk T cellen en B cellen. Publicatie I behandelt tevens de migratie van lymfocyten door de witte pulpa.

Publicatie II beschrijft de opbouw van de micro-omgeving van T lymfocyten in de milt. De T cel micro-omgeving werd in muizen experimenteel "geisoleerd" door middel van lethale röntgen bestraling gevolgd door intraveneuze (i.v.) injectie van isologe thymocyten. De aldus verkregen "T" muizen bezitten geen B cellen en ontwikkelen in de loop van de reconstitutie periode alleen T cel compartimenten in perifere lymfoide organen. De ontwikkeling van het T cel compartiment in de milt werd gedurende 6 dagen na reconstitutie gevolgd, in opeenvolgende perioden van 24 uur. Het bleek dat de i.v. geinjiceerde thymocyten binnen 24 uur in de witte pulpa rondom cytoplasmatische uitlopers van IDC gelocaliseerd waren. In de loop van de reconstitutie periode werd transformatie van lymfocyten in lymfoblasten waargenomen nabij IDC. Tevens werd celdeling in de PALS waargenomen. Het aantal lymfoblasten rondom IDC was verhoogd wanneer antigeen gelijktijdig met de thymocyten na de bestraling ingespoten werd. Een maximaal aantal lymfoblasten werd op 5 dagen na reconstitutie aangetroffen. Op dag 6 waren de meeste lymfocyten in de PALS getransformeerd tot middelgrote lymfocyten met een morfologie vergelijkbaar met die van lymfocyten welke onder normale omstandigheden in de PALS aanwezig zijn. Uit dit onderzoek werd geconcludeerd dat IDC in de milt een onderdeel vormen van de micro-omgeving welke de differentiatie van immunologisch actieve T cellen stimuleert.

Publicatie III behandelt de migratie van B lymfocyten in de lymfatische organen van T muizen. De localisatie van met (^{3}H)-leucine gelabelde, i.v.

geinjiceerde B lymfocyten werd bestudeerd in autoradiogrammen van de milt en de mesenteriale lymfklier op 6, 18, 24, 42 en 66 uur na injectie. Reeds 6 uur na injectie waren B cellen aanwezig in de witte pulpa van de milt van T dieren. Zij waren voornamelijk gelocaliseerd in het perifere gedeelte van de PALS, sommige B cellen werden echter ook in het centrale gedeelte van de PALS aangetroffen. 18 Uur na injectie waren primaire follikels van gelabelde B cellen aan de perifierie van de PALS duidelijk waarneembaar. B cellen migreerden via twee routes naar de witte pulpa: (a) via de marginale zone en (b) via reticulum scheden rondom terminale arteriolen. De meeste B cellen bereikten vervolgens (via de PALS) de primaire follikels; een klein gedeelte van de B cellen migreerde naar efferente lymfe vaten nabij de centrale arteriolen.

In lymfeklieren kwamen de B lymfocyten het lymfoide stroma binnen door de wand van post-capillaire venulen om vervolgens via de paracortex naar de follikels te migreren. De vorming van primaire follikels kwam pas 18 uur na injectie van de gelabelde B cellen op gang. Ook hier werd gevonden dat B cellen op weg naar follikels door T cel gebieden migreerden. Waarschijnlijk vormt dit migratie patroon de basis voor de interactie tussen T en B cellen gedurende de humorale immuun respons.

Het verloop van de humorale immuun respons werd geanalyseerd in een onderzoek beschreven in **publicatie IV**. Voor dit onderzoek werden *kiemvrije* muizen gebruikt omdat de lymfoide weefsels van deze dieren niet of nauwelijks door antigeen gestimuleerd zijn. Als antigeen werden schape-erythrocyten gebruikt welke i.v. werden ingespoten. Dit antigeen is "T cel afhankelijk" hetgeen wil zeggen dat gedurende de humorale immuun respons interactie tussen T cellen, B cellen en macrofagen plaatsvindt. Het verloop van de immunologische reactie werd gevolgd in de milt omdat dit orgaan na intraveneuze injectie van antigeen als eerste een immuun respons vertoont. In de witte pulpa van deze dieren werden de volgende cellulaire veranderingen waargenomen. Macrofagen in de periferie van de witte pulpa fagocyteerden antigeen en migreerden vervolgens in de PALS. Waarschijnlijk als gevolg hiervan kwam binnen 48 uur blastvorming en celdeling in de centrale PALS op gang. Lymfoblasten werden vaak waargenomen in nauwe relatie met cytoplasmatische uitlopers van IDC. De differentiatie van B cellen tot immunoglobuline synthetiserende plasmablasten werd 72 uur na antigeen injectie waargenomen in het perifere gedeelte van de PALS. Plasmablasten migreerden in de navolgende periode langs de centrale en terminale arteriolen naar de rode pulpa. Follikelcentra werden waargenomen op dag 4 na injectie in de vorm van kleine opeenhopingen van lymfoblasten.

Uit dit onderzoek werd geconcludeerd dat micro-omgevingen welke de interactie tussen T en B cellen gedurende de humorale immuun respons reguleren aanvankelijk gelocaliseerd zijn in het perifere gedeelte van de PALS. Deze interactie leidt tot de differentiatie van IgM secernerende plasmablasten.

Publicatie V behandelt de oppervlakte structuur van T en B cellen, zoals waargenomen met het scanning elektronenmikroscoop. Deze studie werd verricht naar aanleiding van publicaties in de literatuur, waarin vermeld werd dat T en B cellen *in vitro* op grond van hun oppervlakte struktuur van elkaar konden worden onderscheiden. Wij onderzochten de oppervlakte struktuur

van T en B cellen *in situ* in verschillende compartimenten van milt en lymfklieren. Op grond van deze studie werd geconcludeerd dat, althans *in vivo*, geen onderscheid gemaakt kon worden tussen T en B cellen. Wel werd gevonden dat lymfocyten in recirculatie kanalen microvilli op het celoppervlak vertoonden terwijl lymfocyten in de specifieke compartimenten altijd glad waren.

In vitro studies, door ons uitgevoerd aan celsuspensies van T en B lymfocyten van verschillende organen toonden evenmin een karakteristiek verschil in oppervlak aan. Microvilli op lymfocyten zijn blijkbaar contractiele strukturen en spelen mogelijk een rol bij herkenningsmechanismen.

Publicatie VI beschrijft eveneens een onderzoek aan de celmembraan van T en B cellen, nu bekeken in het transmissie elektronenmikroskoop. Tijdens dit onderzoek werd de vries-ets techniek toegepast, een techniek welke bij uitstek geschikt is om membraan structuren in het transmissie elektronen mikroskoop te bestuderen. Replica's van gevries-etste T en B lymfocyten toonden geen verschillen aan tussen de membraan strukturen van T en B cellen. Uit dit onderzoek en uit het onderzoek gepresenteerd in publicaties I en V blijkt dat T en B lymfocyten niet op grond van morfologische kenmerken van elkaar kunnen worden onderscheiden. Onderzoek aan de ultrastruktuur van lymfoide subpopulaties vereist derhalve specifieke labelings methoden. Publicatie VI beschrijft twee methoden welke geschikt zijn om receptoren op T en B cellen op elektronenmikroskopisch niveau specifiek te labelen.

Samenvattend kan uit het hier beschreven experimentele werk geconcludeerd worden:

(1) T en B cellen localiseren elk in specifieke compartimenten in perifere lymfoide organen. Deze compartimenten zijn echter niet exclusief toegankelijk voor de celpopulatie welke het karakter ervan bepaalt: B cellen kunnen door T celgebieden migreren en T cellen kunnen door B celgebieden migreren. Dit migratie patroon vormt waarschijnlijk de basis voor de interactie tussen T en B cellen tijdens de (humorale) immunologische reaktie.

(2) In de milt is de micro-omgeving betrokken bij de (antigeen afhankelijke) differentiatie van T cellen gelocaliseerd in het centrale gedeelte van de PALS. De micro-omgevingen betrokken bij de antigeen afhankelijke differentiatie van B cellen zijn gelocaliseerd in de perifere PALS en in follikelcentra. De specificiteit van de micro-omgevingen wordt waarschijnlijk bepaald door niet-lymfoide celtypen, zoals reticulum cellen, interdigiterende cellen, macrofagen en dendritische cellen.

(3) T en B cellen bezitten geen duidelijke morfologische kenmerken op grond waarvan ze van elkaar kunnen worden onderscheiden. Derhalve is specifieke immunologische labeling van membraan receptoren nodig om T en B cellen op elektronenmikroskopisch niveau te kunnen herkennen.

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White Pulp Compartments in the Spleen of Rats and Mice

A Light and Electron Microscopic Study of Lymphoid and Non-Lymphoid Celltypes in T- and B-Areas

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Summary. The spleen of rats and mice was studied with the light and electron microscope. Special attention was paid to the delineation and composition of the white pulp compartments: periarteriolar lymphatic sheath (PALS), follicles and marginal zone. These three compartments each have their specific lymphoid and non-lymphoid cells.

Reticulum cells and reticulin fibres, although occurring in all three compartments, form a characteristic pattern in each compartment. In the PALS two areas can be distinguished: a central area, largely devoid of reticulum cells, and a peripheral area where reticulum cells are arranged in cylindrical shells. The central PALS forms the thymus dependent area of the spleen, the peripheral PALS contains both T- and B-lymphocytes. T-B-interactions requiring cell contact could take place in the latter area.

Lymph vessels originate from the shells of reticulum cells around the smaller arterioles; these vessels follow the central arteriole to the hilus of the spleen. Circumstantial evidence suggests that the lymph vessels form a recirculation pathway for T-cells and possibly also for B-cells.

In two areas of the splenic white pulp characteristic non-lymphoid cells are present. The central PALS contains interdigitating cells (IDC), which show a close contact with surrounding T-lymphocytes. The light zone of the follicle centre exhibits dendritic cells(DC). B-cells are found between the ramifications of the DC. It is conceivable that these cells play a role in the homing of T-cells and B-cells respectively. In addition they might create a microenvironment supporting differentiation and proliferation of T- and B-cells. The marginal zone does not contain a characteristic non-lymphoid cell type. However, in this compartment B-cells are directly exposed to the circulating blood. It is suggested that this factor constitutes one of the essentials of the microenvironment in the marginal zone.

Key words: Spleen (Rat, mouse) — T-cell, B-cell — Microenvironment — Cell traffic — Light and Electron Microscopy.

Introduction

In the spleen of rodents the white pulp constitutes the immunologically active compartment (Langevoort, 1963; Movat and Fernando, 1964, 1965). In the white

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pulp three (sub)compartments can easily be recognised at the light microscopical level: (1) the periarteriolar lymphatic sheaths (PALS), closely associated with branches of the splenic artery; (2) the follicles, localised at the periphery of the PALS; and (3) the marginal zone, surrounding PALS and follicles.

Different types of immunocompetent cells are present in different parts of the white pulp. Waksman *et al.* (1962), Parrott *et al.* (1966, 1971) and others have shown that the PALS is a thymus dependent area: thymus-derived lymphocytes (T-lymphocytes or T-cells) are predominantly localised in this part of the white pulp. Bone marrow-derived lymphocytes (B-lymphocytes or B-cells) populate the follicles (Sprent, 1973) and the marginal zone (Nieuwenhuis *et al.*, 1974a, b).

In two previous reports we have mainly dealt with electron microscopical aspects of the T-cell microenvironment in the spleen (van Ewijk *et al.*, 1974; Veerman, 1974a). The aim of the present study is to delineate the different compartments in the spleen of rats and mice and to define the essential features of lymphoid and non-lymphoid cells in these areas at the ultrastructural level. Some functional implications of the organisation of the white pulp are discussed.

Materials and Methods

Animals. Twelve rats of the Wistar strain, approximately 3 month sold, obtained from the Centraal Proefdierenbedrijf TNO (Zeist, the Netherlands) and 12 (DBA/2 × C57BL/Rij)F1 mice obtained from the Medical Biological Laboratories TNO (Rijswijk, the Netherlands) were used for this study. The animals were of both sexes; they were kept under routine laboratory conditions with free access to food and water.

Fixation and Staining Procedures. For light microscopic examination the animals were killed by cervical dislocation or by administration of an overdose of pentobarbital (Nembutal, Abbott). The spleen was then excised immediately and fixed by immersion. Routinely fixation took place for 5 hours in Zenker/Formol 40%/trichloroacetic acid 2% (18/1/1). Paraffin sections of these spleens were stained with methylgreen-pyronin (Brachet, 1953). To study the reticulin fibre pattern the spleen was fixed in Bouin fixative; this material was stained with Wilders reticulin stain or with Heidenhein's Azan-stain (Romeis, 1968).

For combined light- and electron microscopic examination animals were killed by perfusion fixation, under Nembutal anaesthesia (for details see Veerman *et al.*, 1974; van Ewijk *et al.*, 1974). From the spleen 200 μ sections were made using a Smith and Farquhar tissue chopper (Sorvall TC-2); sections containing suitable white pulp structures were selected for osmification and were embedded flat in Epon 812. Semithin sections were stained with toluidine blue in borax buffer (pH 8.5) and used for light microscopic examination. Ultrathin sections were stained with leadcitrate and uranylacetate.

For the detection of glycoproteins the periodic acid silvermethenamine method was used on non-osmificated sections (Rambourg and Leblond, 1967). Control sections were not treated with periodic acid.

Ultrathin sections were examined in a Philips EM200 or EM300 electron microscope.

Observations

Light Microscopy

The white pulp in the spleen of mice and rats is arranged around central arterioles, branching from the splenic artery (Figs. 1—5). The reticulin fibre pattern shows characteristic differences for each compartement (Figs. 1, 2). The central part of the periarteriolar lymphatic sheath (PALS) shows only a few reticulin fibres, while the peripheral PALS has more, coarser reticulin fibres, forming concentrically arranged cylindrical shells (Figs. 1, 2, see also Sainte-Marie and Sin,

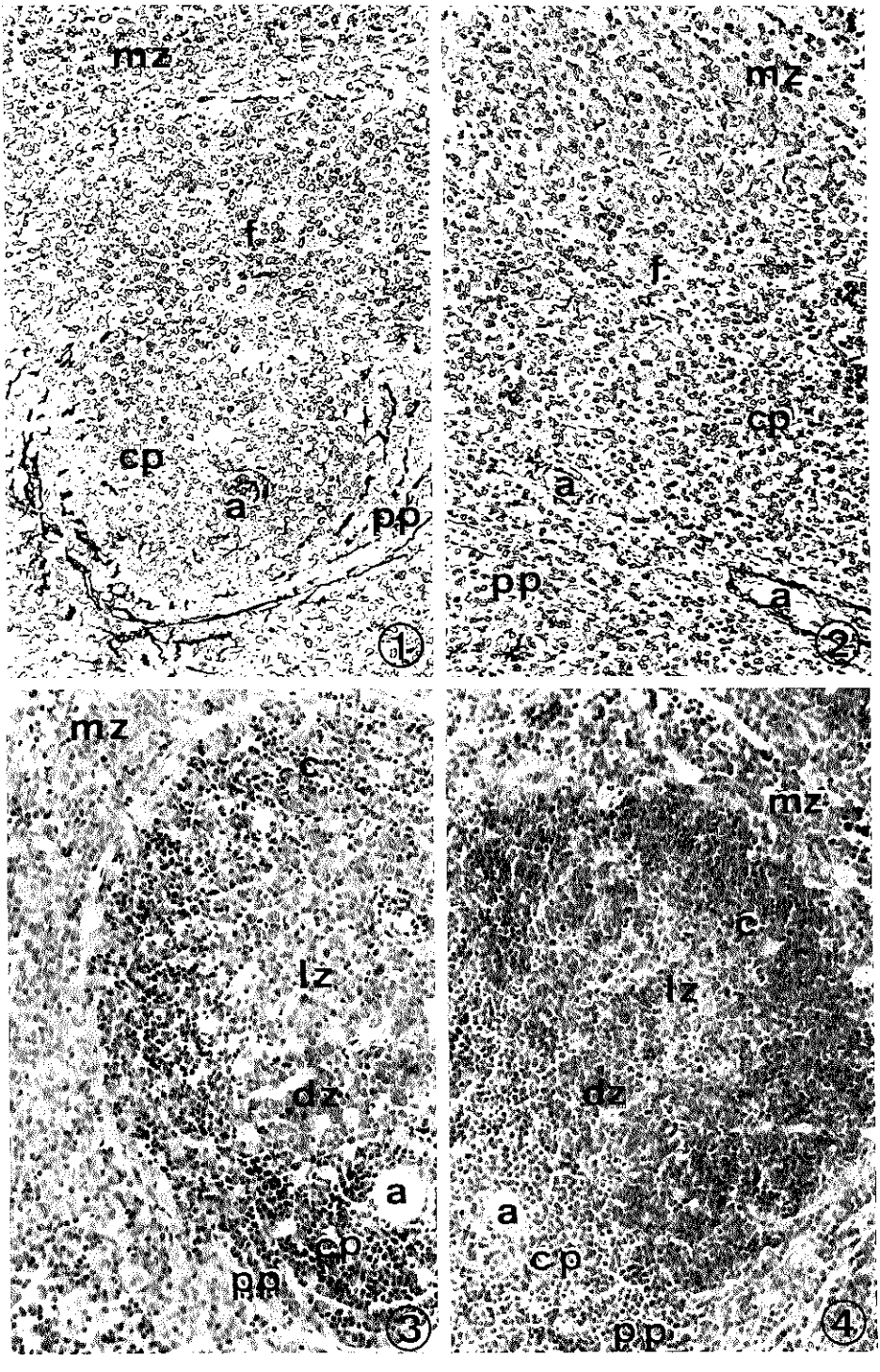
1968). This pattern is especially evident in rats; mice show finer reticulin fibres. Around terminal ramifications of the central arteriole only the equivalent of a peripheral PALS is present. Follicles mainly show reticulin fibres as an outer boundary. The very fine reticulin fibres in the marginal zone, evident by electron microscopical pictures, are not easily demonstrable in light microscopical preparations.

In the central PALS two characteristic cell types occur: small lymphocytes and non-lymphoid cells, designated as interdigitating cells: IDC (Veerman, 1974a; van Ewijk *et al.*, 1974). IDC show lightly stained cytoplasm due to low affinity for basic dyes. Cytoplasmic processes of these cells extend for considerable distances between surrounding lymphocytes. These processes, however, are not accompanied by reticulin. In the central area of the PALS a lymph vessel may be observed, parallel to, and in close association with the central artery (Figs. 7, 8). The lymph vessel gradually emerges from the concentric sheaths of reticulum cells around the terminal ramifications of the central arteriole (Fig. 7). Macrophages occur in moderate numbers both in central and peripheral PALS.

The peripheral PALS has a lamellar structure due to the arrangement of reticulum cells. Between the sheaths of reticulum cells one or two layers of lymphocytes are present. Plasma cells and plasmablasts may also be observed in the peripheral PALS. IDC, however, do not occur in this area. The outermost shell of reticulum cells forms the boundary of the PALS with the marginal zone. In this area capillaries extending from the central arteriole and traversing the PALS open freely into the marginal zone (cf. van Rooijen, 1972).

At some places in the peripheral PALS follicular structures are present. On the average mice show larger numbers of follicles per white pulp section than rats; conversely, the peripheral PALS is more evident in the spleen of rats. Follicles may consist of a homogeneous population of small, darkly staining lymphocytes (primary follicles, Fig. 5) or exhibit a follicle centre (germinal centre) surrounded by a corona of small, darkly staining lymphocytes (secondary follicle, Figs. 3, 4). Follicle centres may show two zones: a relatively dark and pyroninophilic one, the so-called dense zone, situated adjacent to the central PALS, and a less pyroninophilic one, or light zone, overlying the dark zone (Fig. 3, 4). The dark zone is occupied by lymphoblasts with high mitotic activity. In the light zone smaller lymphoblasts can be observed, together with macrophages. The latter cells are characterised by ingested lymphocytes; these phagocytes are known as tingible body macrophages. Follicle centres are demarcated from the corona by loosely arranged reticulum cells and small capillaries.

The capillaries of the follicle terminate in the marginal sinus (Fig. 5), which has a continuous endothelial lining on the follicular side, but is open at the marginal zone side (Fig. 6). Here, the layer of endothelial cells is continuous with reticulum cells. The reticulum cells are accompanied by fine reticulin fibres. In the interstices erythrocytes, thrombocytes, granulocytes, monocytes and macrophages are present. Perfusion fixation reveals large intercellular spaces (Fig. 5, 6). The circulation passes freely between the cells and is not limited by an endothelial lining, i.e. the circulation in the marginal zone is of the open type. At the periphery the marginal zone is bordered by red pulp sinuses, in some places it is continuous with cords of Billroth.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Electron Microscopy

1. Lymphoid Cells

In the PALS two types of lymphocytes can be observed (Figs. 9, 10): small electron dense lymphocytes, and somewhat larger and less electron dense cells. The former are not frequent in normal animals, the latter constitute the bulk of the lymphocytes in the PALS. Both have a nucleus with a condensed chromatin pattern. In the more electron dense lymphocytes the cell organelles are distributed randomly. In the less electron dense lymphocytes the nuclei frequently show indentations; at this side of the nucleus in the cytoplasm the centrioles, a small Golgi complex and most of the mitochondria are gathered. Both types of lymphocytes show mostly free ribosomes; in the electron dense lymphocytes there is a high concentration of ribosomes.

The lymphocytes in the central area of the PALS are closely packed (intercellular distance about 100–200 Å; Fig. 9). They are frequently found to be in close contact with IDC: fingerlike protrusions, emerging from the lymphocytes, indent the surface of the IDC (Figs. 10, 23). Occasionally tight junctions are observed between both types of cells (Fig. 12). Lymphoblasts which may also occur in this area, are often seen alongside IDC (Fig. 14). They show rosette-like polyribosomes and only scanty amounts of rough endoplasmic reticulum.

The lymphocytes in the peripheral PALS are less tightly packed (intercellular space more than 200 Å wide, Fig. 15). Their general features are comparable to those of the less electron dense lymphocytes in the central PALS. Lymphoblasts occurring in this particular area show, in contrast to those in the central PALS, a distinct rough endoplasmic reticulum. They have been found to differentiate into plasma cells which are characterised by a prominent rough endoplasmic reticulum and a well developed Golgi region (Fig. 16). On the membranes of the endoplasmic reticulum coiled polyribosomes are found consisting of up to 18 ribosomes (Fig. 17).

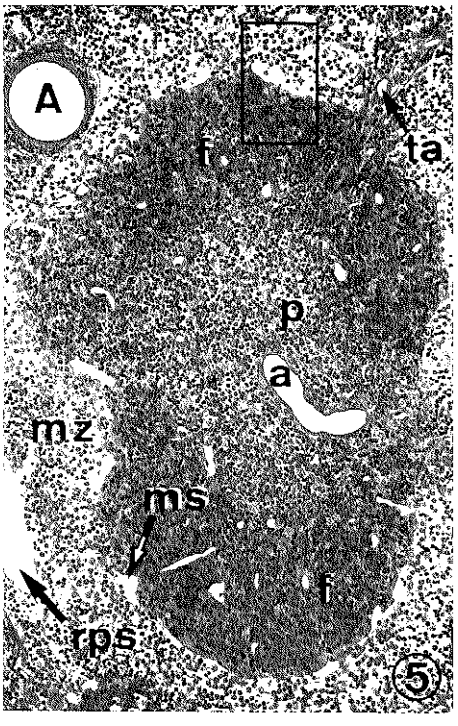
In the primary follicles and in the corona of the secondary follicles the small lymphocytes (Fig. 18) differ in several aspects from those in the PALS: (1) they are generally smaller; (2) follicular small lymphocytes have a more irregular cell surface with microvilli especially at sites where they are adjacent to dendritic cells (Fig. 25); (3) in the nucleus of the follicular small lymphocytes a nuclear body

Fig. 1. The reticulin pattern in the spleen of the rat is most clearly seen in the periarteriolar lymphatic sheath (PALS). A central area in the PALS (*cp*) is nearly devoid of reticulin. The peripheral area (*pp*) is layered. The follicle (*f*) shows very little reticulin. The marginal zone (*mz*) contains very fine reticulin fibres, these are only seen with difficulty, between the cells. *a* Central arteriole. Rat. Wilder stain, ×400

Fig. 2. In the mouse spleen the reticulin fibre pattern is comparable to that in the rat, but the reticulin fibres are finer. Abbreviations as in Fig. 1. Mouse. Wilder stain, ×400

Fig. 3. In the spleen of most normal rats follicle centres occur, with a dark zone (*dz*), situated immediately against the central PALS (*cp*) and a light zone (*lz*). The peripheral PALS (*pp*) surrounds the central PALS. *a* Central arteriole. *c* corona; *mz* marginal zone, Rat. Methylgreen-pyronin, ×400

Fig. 4. Abbreviations as Fig. 3. Mouse. Methylgreen-pyronin, ×400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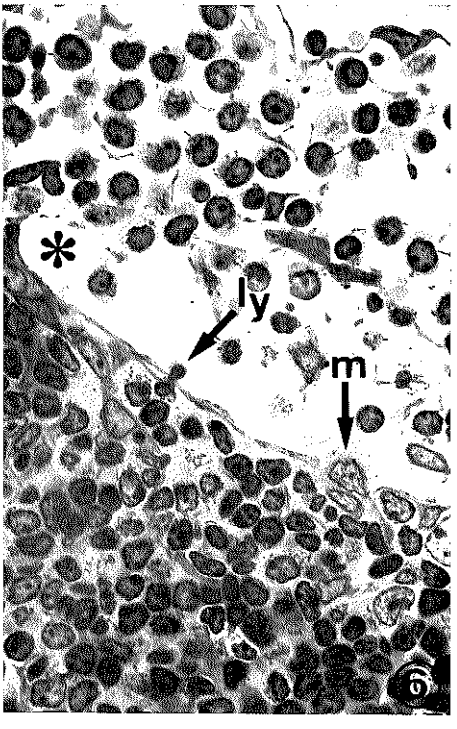
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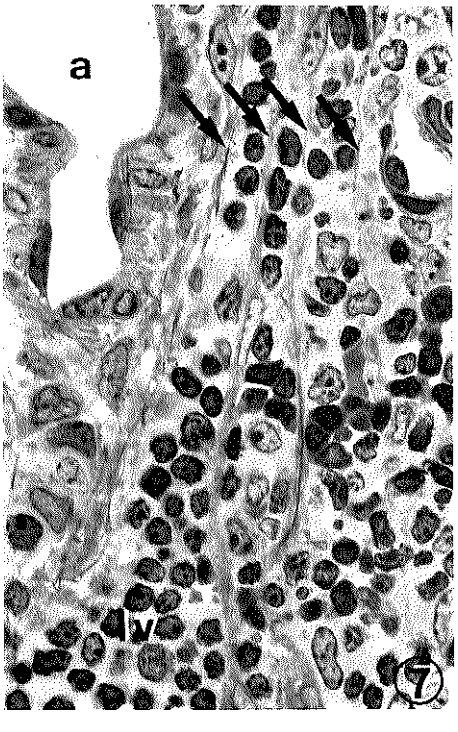
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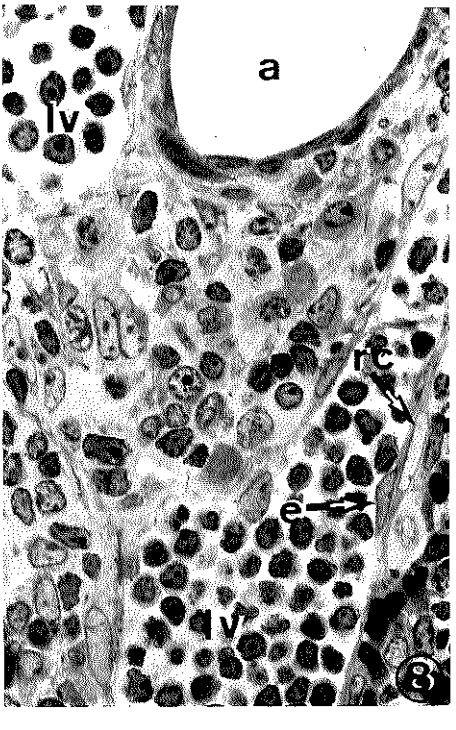
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(sphaeridium) is more often observed than in lymphocytes in the PALS (Figs. 18, 25).

The follicle centre contains lymphoblasts studded with polyribosomes (Fig. 19). They show, however, only a few strands of rough endoplasmic reticulum.

The marginal zone contains predominantly medium-sized lymphocytes. The nucleus of these cells shows a disperse chromatin pattern (Fig. 20). The ribosomes in the cytoplasm are mainly present as monoribosomes. These cells are frequently found to be in contact with reticulum cells (Fig. 20). Their free cell surface shows irregular blunt protrusions. It is this type of cell that is seen crossing the border between follicle and marginal zone. During this process the cytoplasmic tail of these cells is generally directed towards the follicle (Fig. 20).

2. Non-lymphoid Cells

a) Reticulum Cells. Reticulum cells form the basic framework of the splenic white pulp. Their general appearance is the same in all compartments (Figs. 15, 18, 20): they are spindle-shaped and have long, slender cytoplasmic extensions which are accompanied by reticulin fibres. Most often the reticulin fibres are totally ensheathed by cytoplasm of reticulum cells. The nucleus of these cells shows dispersed chromatin. Its cytoplasm contains rough endoplasmic reticulum, often arranged parallel to the longitudinal axis of the cell. Microfilaments are conspicuous, particularly underneath the cell surface. In places where the cells are in contact with reticulin fibres, an electron dense filamentous substance lies on the inside of the cell membrane (Fig. 15). Although the general morphology of all reticulum cells is comparable, their number and spatial orientation in the various white pulp compartments is different and often quite characteristic. This is most obvious in the peripheral PALS and around the terminal ramifications of the central arteriole. Here the reticulum cells are arranged on cylindrical shells. In the marginal zone they form a three-dimensional meshwork.

b) Macrophages. Macrophages occur in all white pulp compartments. They are most easily recognised by the presence of phagocytised material (Figs. 18, 21, 22). Their nucleus is mostly round to oval. The chromatin is dispersed and mostly

Fig. 5. The splenic artery (*A*) branches into central arterioles (*a*), associated with lymphoid tissue. Fine capillaries extending from the central arteriole (*a*) traverse the PALS (*p*) and follicles (*f*) and open into the marginal zone (*mz*). Terminal ramifications of the central arteriole (*ta*) traverse the marginal zone and terminate in the red pulp. *ms* marginal sinus; *rps* red pulp sinus. Mouse. Perfusion fixation. Toluidine blue. ×110

Fig. 6. Higher magnification of the boxed area in Fig. 5. A lymphocyte (*ly*) is passing the border between the marginal sinus (asterisk) and the follicle (*f*). The marginal sinus is open at the side of the marginal zone. *M* macrophage. Mouse. Perfusion fixation. Toluidine blue. ×920

Fig. 7. Lymph vessels (*lv*) in the PALS originate in the sheaths of reticulum cells (arrows) which are especially prominent around the smallest arterioles (*a*). Mouse. Perfusion fixation. Toluidine blue. ×920

Fig. 8. The lymph vessels (*lv*) closely follow the central arteriole (*a*). Note the difference in the nuclear morphology of the endothelial cells (*e*) of the lymph vessels and the surrounding reticulum cells (*rc*). Mouse. Perfusion fixation. Toluidine blue. ×920

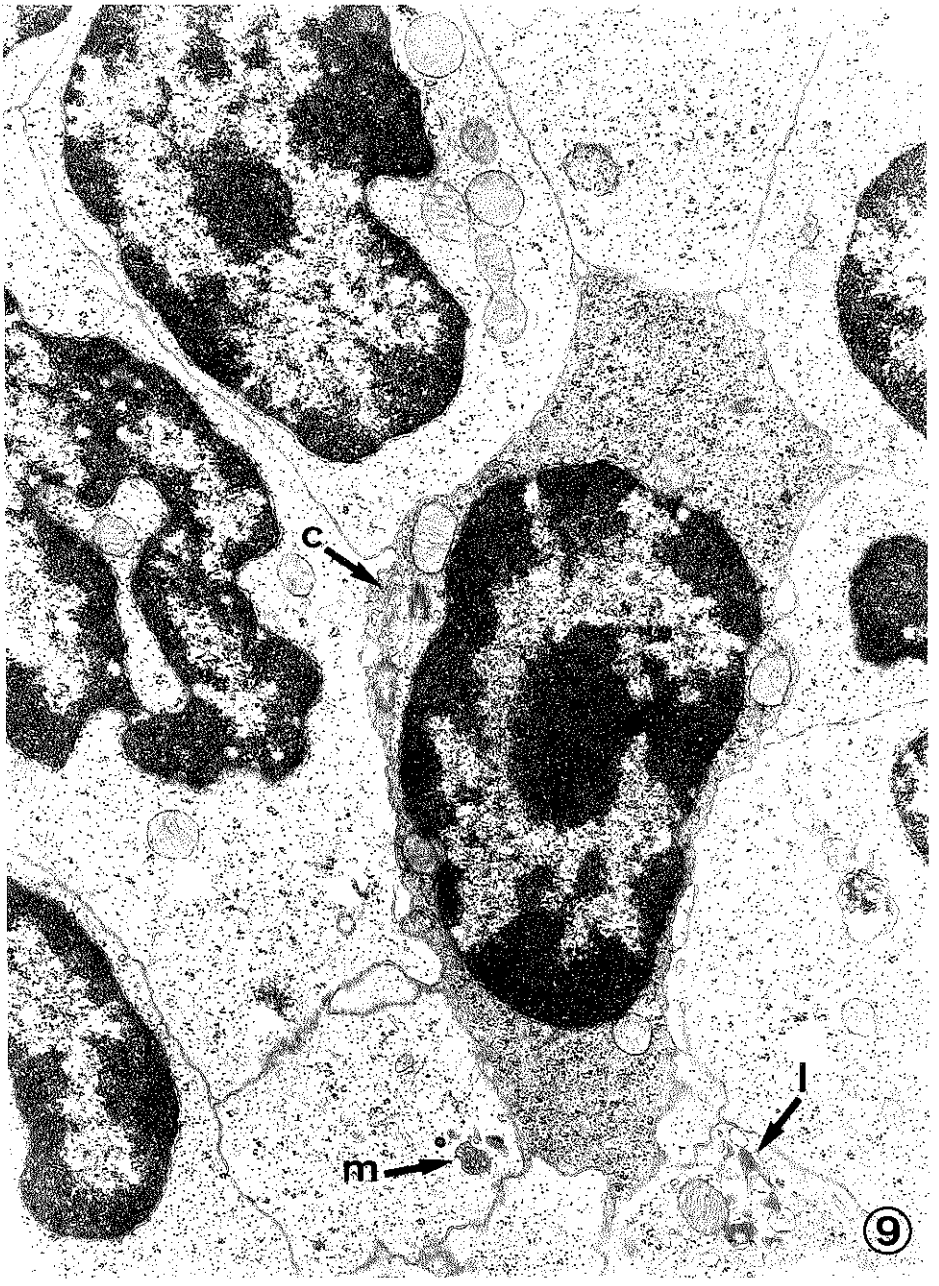


Fig. 9. The central PALS contains two types of lymphocytes: small electron dense lymphocytes, exhibiting a centriole (*c*), randomly distributed mitochondria and many ribosomes, and larger, less electron dense lymphocytes, which show frequently a nuclear indentation where most of the cell organelles are gathered. The cytoplasm of the latter shows less ribosomes. Multivesicular bodies (*m*) and lysosome-like bodies (*l*) frequently occur. Rat. ×18000

marginally located. In the relatively extensive cytoplasm numerous phagosomes and lysosomes are present. The cell surface shows signs of active endocytosis. Although they occur in all white pulp compartments, macrophages are most prominent in the marginal zone, in the peripheral part of the follicles and in the light zone of the follicle centre. The contents of their lysosomes are often characteristic of the region where the macrophage is found: in the marginal zone they may contain remnants of erythrocytes, those occurring peripherally in the follicles are characterised by telolysosomes with homogeneous slightly granular contents (Fig. 18; cf. Streefkerk and Veerman, 1971), in the germinal centre they contain lymphocytes in all stages of degradation (tingible bodies; Fig. 22).

c) Interdigitating Cells. Interdigitating cells (IDC) are only found in the central PALS. In general they show a strikingly electron lucent cytoplasm (Figs. 10, 11, 14, 23). Rather broad cytoplasmic extensions, largely devoid of cell organelles, interdigitate with the extensions of neighbouring IDC. The nucleus of the IDC is frequently irregularly shaped and contains a thin layer of marginally located chromatin. A small nucleolus is often present. Most of the cell organelles occur in the cytocentre: these include a number of small mitochondria, several Golgi complexes and smooth endoplasmic reticulum. Varying amounts of small electron dense lysosome-like bodies are present, but phagosomes are not frequently observed. The cytocentre exhibits a complex of small vesicles, closely packed together, which we designated as vesicular complex (Fig. 23). They show a positive reaction with the periodic acid-silvermethenamine reaction (Fig. 24). In more peripheral regions of the cell tubular structures may be observed in contact with the cell surface (Fig. 11). Between neighbouring IDC tight junctions are observed (Fig. 13); they also occur between IDC and lymphocytes (Fig. 12).

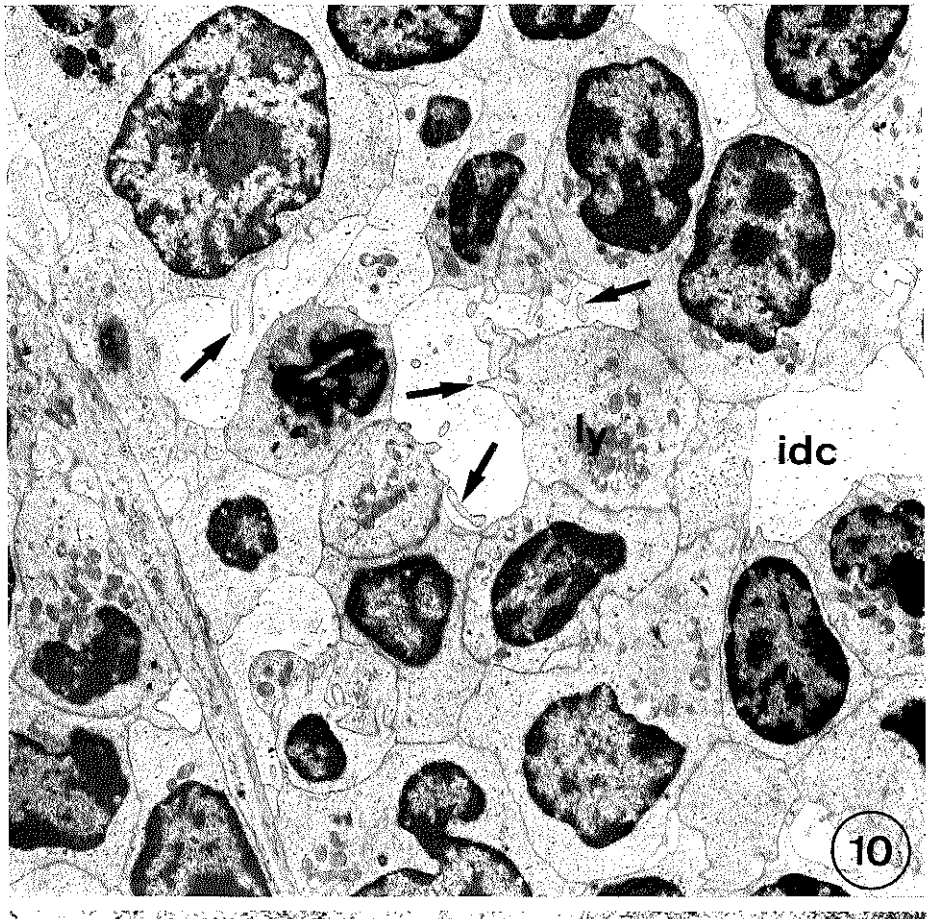
d) Dendritic Cells. Dendritic cells have been found exclusively in the light zone of the follicle centre where they are often associated with capillaries. They show a nucleus with a light, granular chromatin pattern, concentrated in a narrow rim at the periphery of the nucleus (Fig. 25). Around the nucleus only a scanty cytoplasm is present. The majority of the cell organelles is situated in cytoplasmic processes adjacent to the nucleus. These processes show very fine ramifications and extend for considerable distances between the follicular lymphocytes.

In the extracellular space between the ramifications electron dense material may be present, often containing globular material (cf. Abe and Ito, 1973) and virus-like particles (cf. Szakal and Hanna, 1968).

Discussion

I. Delineation of Splenic White Pulp Compartments

The splenic white pulp consists of three distinct areas (Fig. 26): the PALS, the follicles at the periphery of the PALS and the marginal zone, surrounding both PALS and follicles except for the most distal branchings of the central arteriole. It has been shown that these areas each have their own characteristic reticulin fibre pattern, vascularisation pattern and cell population. Comparison of the reticulin fibre pattern in the compartments of the white pulp shows that the PALS has the most coarse reticulin fibres, they are especially evident in the peripheral PALS (cf. Weiss, 1972). In the follicles a few thin reticulin fibres occur (cf. Parrott and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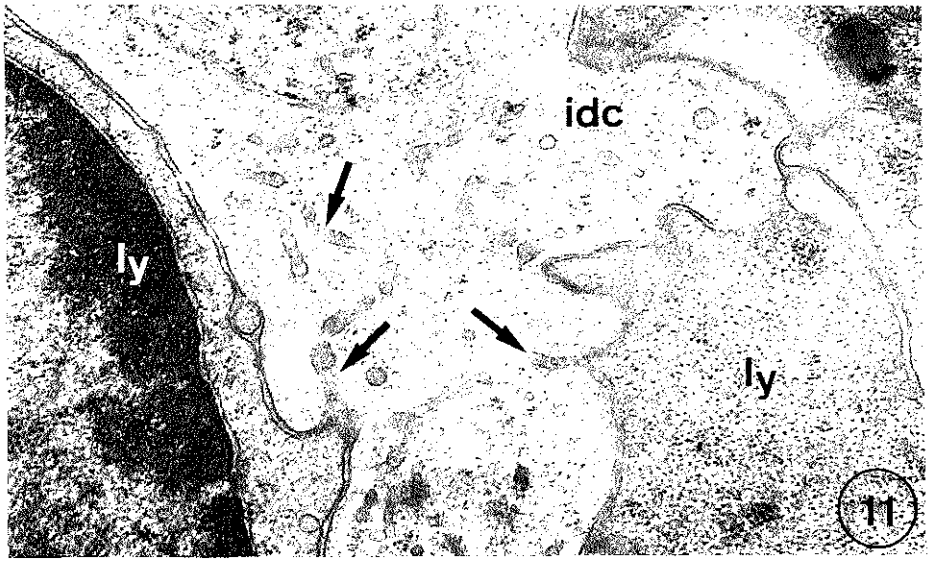
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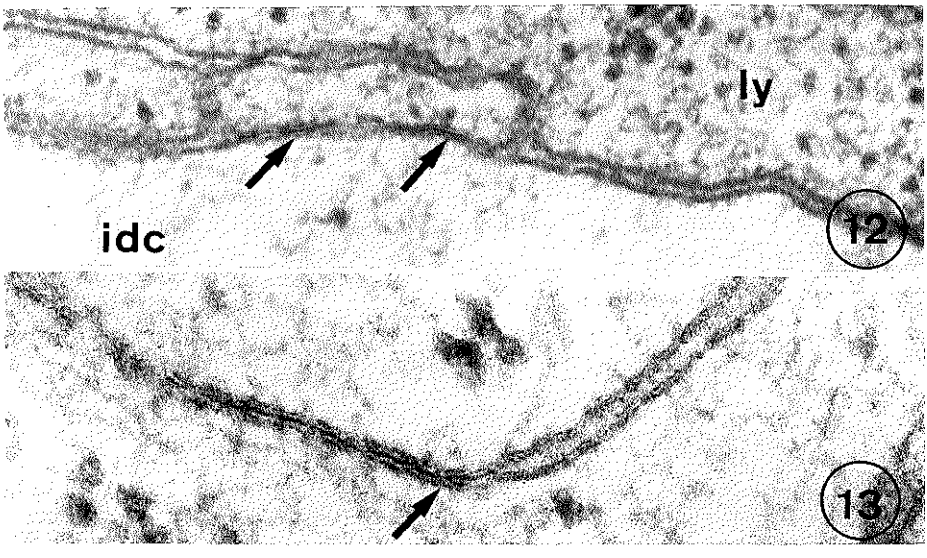


Fig. 12. Central PALS. Tight junctions (arrows) between a lymphocyte (*ly*) and an IDC. Rat. ×150000

Fig. 13. Central PALS. Tight junction (arrow) between cytoplasmic extensions of IDC. Rat. ×200000

de Sousa, 1966; Sainte Marie and Sin, 1968). The marginal zone has a dense meshwork of thin reticulin fibres. These are more easily demonstrated by electron microscopy than by reticulin stains. Comparison of the reticulin pattern in the white pulp of rats and mice reveals that in mice the peripheral PALS is largely occupied by follicles, and that in rats the reticulin fibre pattern is coarser and more prominent than in mice, especially in the peripheral PALS.

Due to the perfusion fixation the vascularisation pattern is easily visible. Capillaries branching from the central arteriole traverse the peripheral PALS often in an oblique fashion. In the follicles the capillaries form a demarcation between the follicle centre and the surrounding lymphocyte corona. They pass the corona and open freely in the marginal zone. Around the follicles they form the marginal sinus. This observation confirms studies of van Rooijen (1972) in the rabbit.

There exists a continuous pathway from the central arterioles via terminal arterioles to the red pulp sinuses (Murakami *et al.*, 1973); this is a closed circulation. Since capillaries open into the marginal zone, in the spleen of rats and mice obviously both closed and open circulation types occur. The relative importance of

Fig. 10. Central PALS. Lymphocytes of the less electron dense type (*ly*) contact cytoplasmic extensions of interdigitating cells (*idc*) by means of finger-like protrusions (arrows). Mouse. ×5000

Fig. 11. Central PALS. Lymphocytes (*ly*) in contact with IDC. The cell membrane of the IDC shows tubular invaginations (arrows) in the neighbourhood of these contact regions. Rat. ×28000

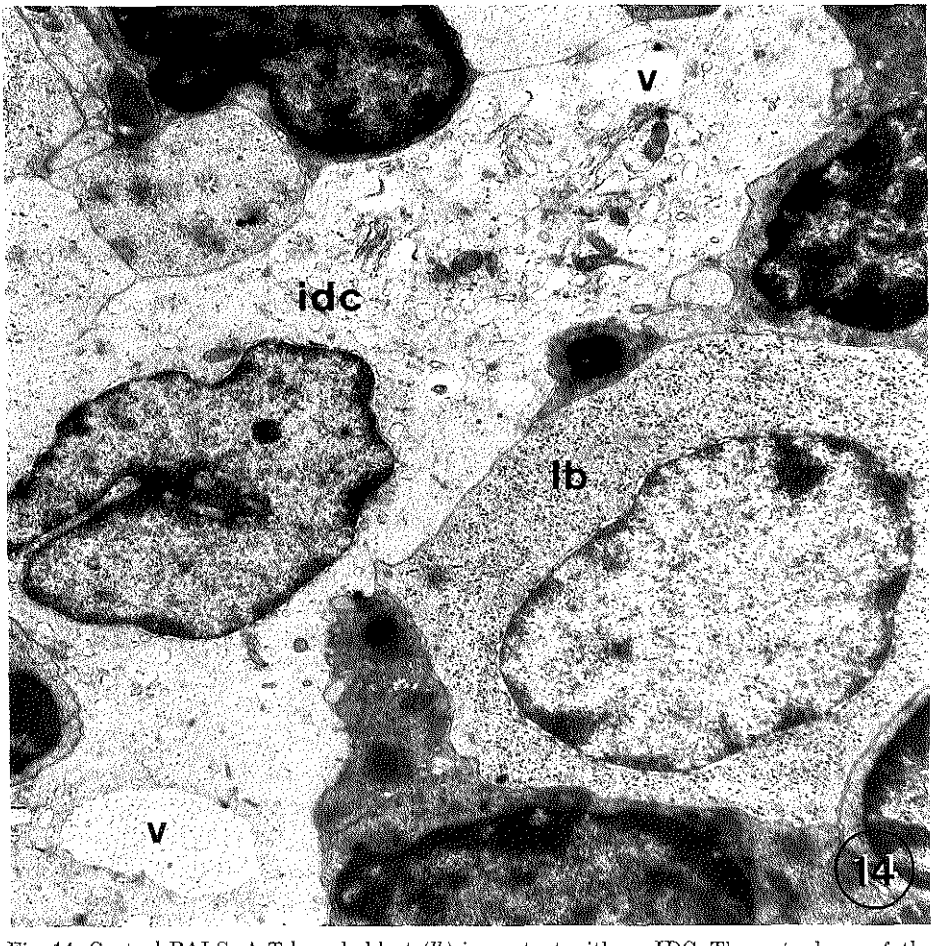


Fig. 14. Central PALS. A T-lymphoblast (*lb*) in contact with an IDC. The cytoplasm of the lymphoblast contains numerous rosette-like ribosomes. The IDC contains several large vacuoles (*v*), filled with fibrillar material. Mouse. ×9000

these two path-ways can only be speculated upon (cf. Pictet *et al.*, 1969). In the marginal zone the cells constituting this zone are in direct contact with blood; they are therefore in a favourable position to contact foreign material in the circulation. It has been shown that the first trapping of intravenously injected antigen (Mitchell and Abbot, 1971; Nossal and Ada, 1971) and antigen-antibody complexes (Brown *et al.*, 1973; van Rooijen, 1973) occurs in the marginal zone. It is supposed that free contact of lymphocytes with blood constitutes an essential feature of the microenvironment in the marginal zone.

II. Non-lymphoid Cells

It appears that two areas in the white pulp of the spleen possess a characteristic non-lymphoid cell type: interdigitating cells (IDC) are exclusively present in the central PALS; dendritic cells (DC) occur in the light zone of the germinal centre.

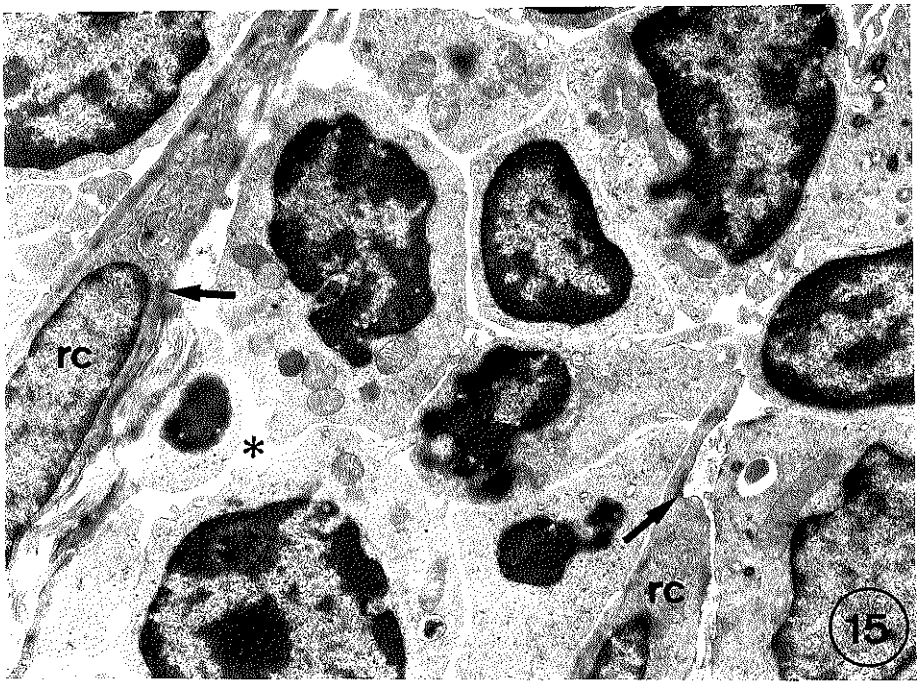
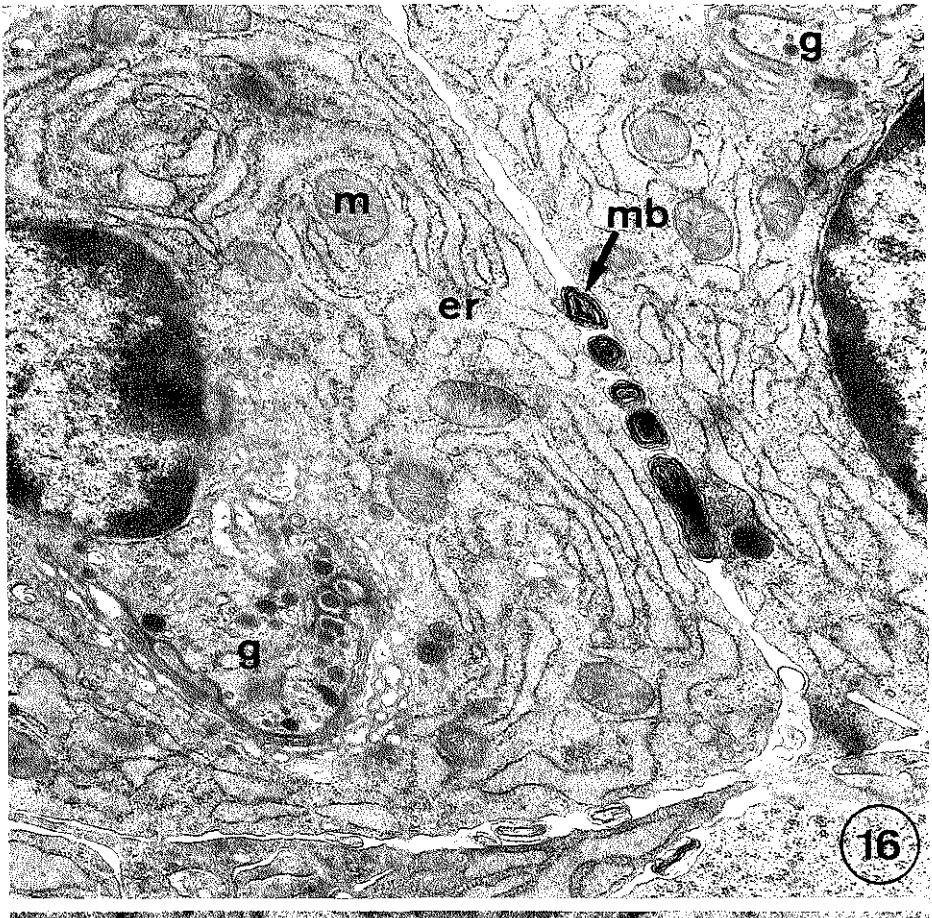


Fig. 15. Peripheral PALS. Lymphocytes are situated between sheaths of reticulum cells (*rc*). Note the relatively large intercellular space (*) between the lymphocytes. Reticulin fibres are seen in contact with reticulum cells. At this site an electron dense substance (arrows) is found in the cytoplasm against the cell membrane. Rat. ×8750

IDC bear resemblance to macrophages. They are probably derived from monocytes (Veerman, 1974a). Differences from "typical" macrophages, such as those in the marginal zone, are: the very translucent cytoplasm of IDC; the elongated nucleus; the abscence of ruffled membrane surface; the paucity of phagosomes; the presence of a vesicular complex, filled with glycoproteins; the contact with T-lymphocytes. Moreover, in IDC acid phosphatase is not demonstrably present, while macrophages show distinct acid phosphatase activity in lysosomes and Golgi complex (Veerman, 1974b). It can be tentatively concluded that IDC are members of the mononuclear phagocyte system in another stage of development than "typical" macrophages.

In a previous study it has been shown that the central PALS is the splenic thymus dependent area, whereas the peripheral PALS, the follicles and the marginal zone are non-thymus dependent (Veerman, 1974a). Veldman (1970) has shown that IDC occur in the paracortex of rabbit lymph nodes which are also thymus dependent. It is therefore supposed that IDC are characteristically present in thymus dependent areas. Between IDC and T-cells intimate cell contacts exists. Finger-like protrusions emerge from T-cells and indent the surface of IDC. The present investigation has revealed the existence of tight junctions between both types of cells. Both structures suggest that T-cells may be retained at the surface



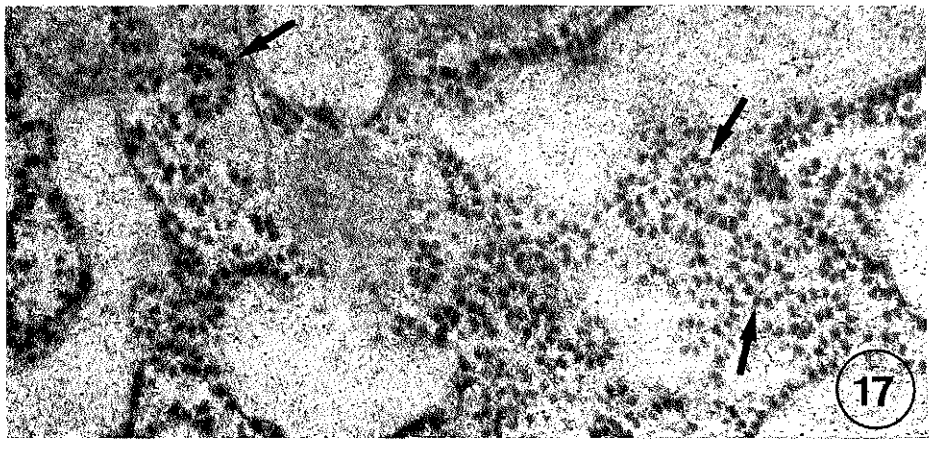


Fig. 16. Peripheral PALS, plasma cells. The cytoplasm shows numerous cisternae of rough endoplasmic reticulum (*er*), a well developed Golgi area (*g*) and several mitochondria (*m*). Between the plasma cells myelin bodies (*mb*) occur. Mouse. ×16000

Fig. 17. Higher magnification of the cytoplasm of a plasma cell. Coiled polyribosomes (arrows), consisting of up to 18 ribosomes, are associated with dilated cisternae of the endoplasmic reticulum. Mouse. ×60000

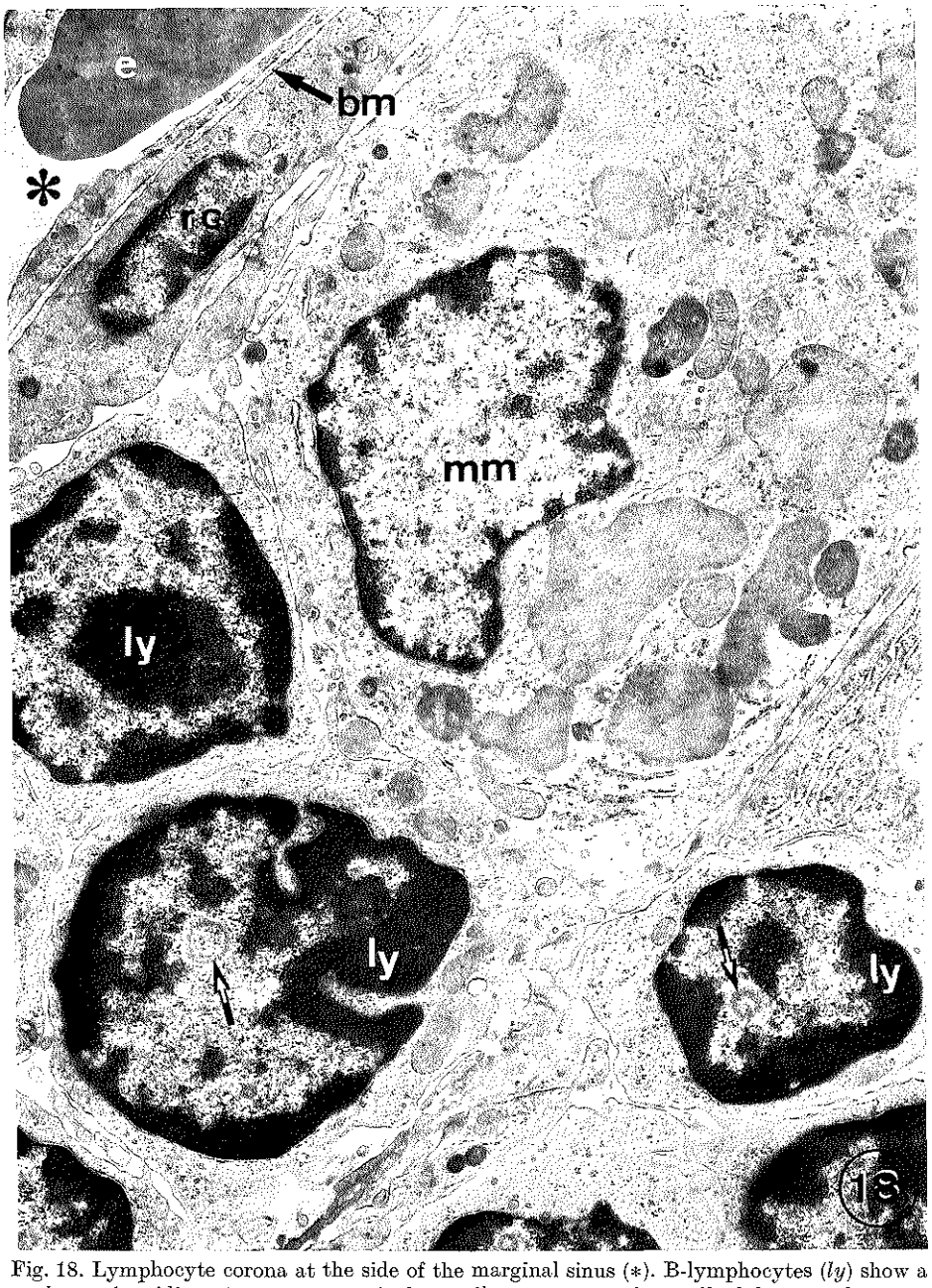


Fig. 18. Lymphocyte corona at the side of the marginal sinus (*). B-lymphocytes (*ly*) show a nuclear sphaeridium (arrows). *rc* reticulum cell; *mm* marginal metallophil; *e* erythrocyte; *bm* basement membrane. Rat. ×17500

of IDC. A similar situation has been described by Leene (1971, 1972) in the developing palatine tonsil of the rabbit: lymphoid cells, upon entering the palatine tonsil contacted mesenchymal cells with fingerlike extensions and close membrane

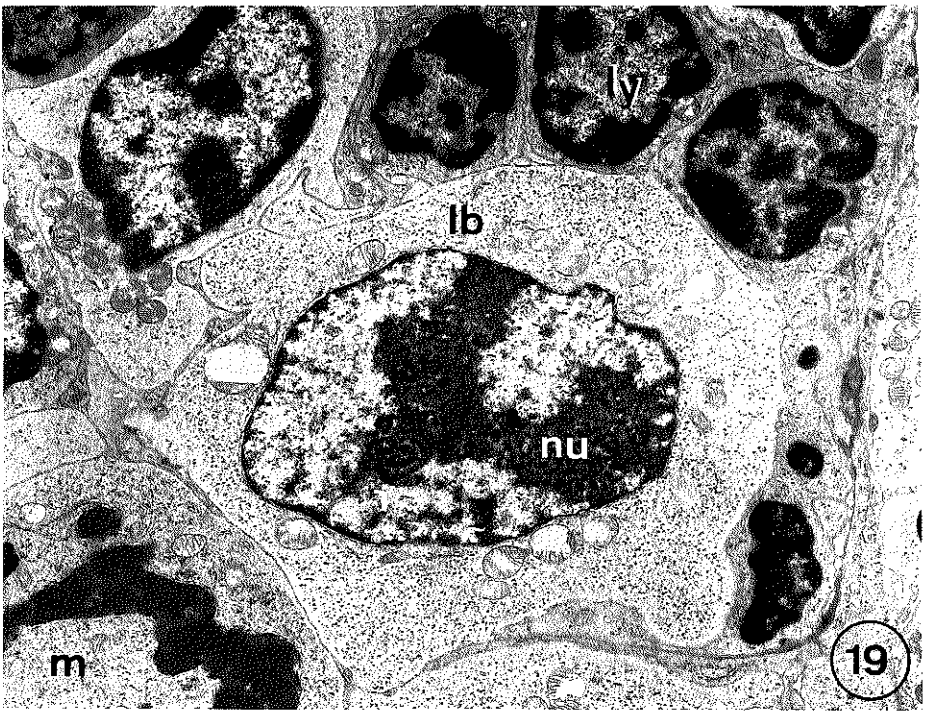


Fig. 19. Follicle centre. The cytoplasm of the lymphoblast (*lb*) contains numerous rosette-like polyribosomes, several mitochondria and slender strands of endoplasmic reticulum. The nucleus shows a prominent nucleolus (*nu*). *ly* small lymphocyte; *m* cell in mitosis. Rat. ×7300

junctions. These lymphocytes then underwent a differentiation process. Blast transformation of T-cells also occurs at the surface of IDC (van Ewijk *et al.*, 1974). As it has been shown that tight junctions (gap junctions) in general offer a means of intercellular communication (Loewenstein, 1973; Bennett, 1973), the idea that the tight junctions reported here reflect a pathway by which IDC influence T-cells must be considered. These data, taken together suggest that IDC contribute to the microenvironment for T-cells.

Dendritic cells (DC) were not observed in all rats and mice studied. If present they were found in the light zone of the follicle centres. Since germinal centres are an expression of immunological activity (Thorbecke, 1959; Langevoort, 1963), it seems that the presence or absence of DC also may be related to the individual immunological background of the animal.

Obviously IDC form an essential constituent of the microenvironment in the thymus dependent area, and DC do so for the (thymus independent) light zone of the germinal centre. IDC possibly play a role in the homing of T-cells (van Ewijk *et al.*, 1974), perhaps the DC are involved in the homing of B-cells, especially B-cells carrying antigen-antibody complexes (Brown *et al.*, 1973; van Rooijen, 1973) and possibly antigen-binding B-cells (Durkin and Thorbecke, 1972).

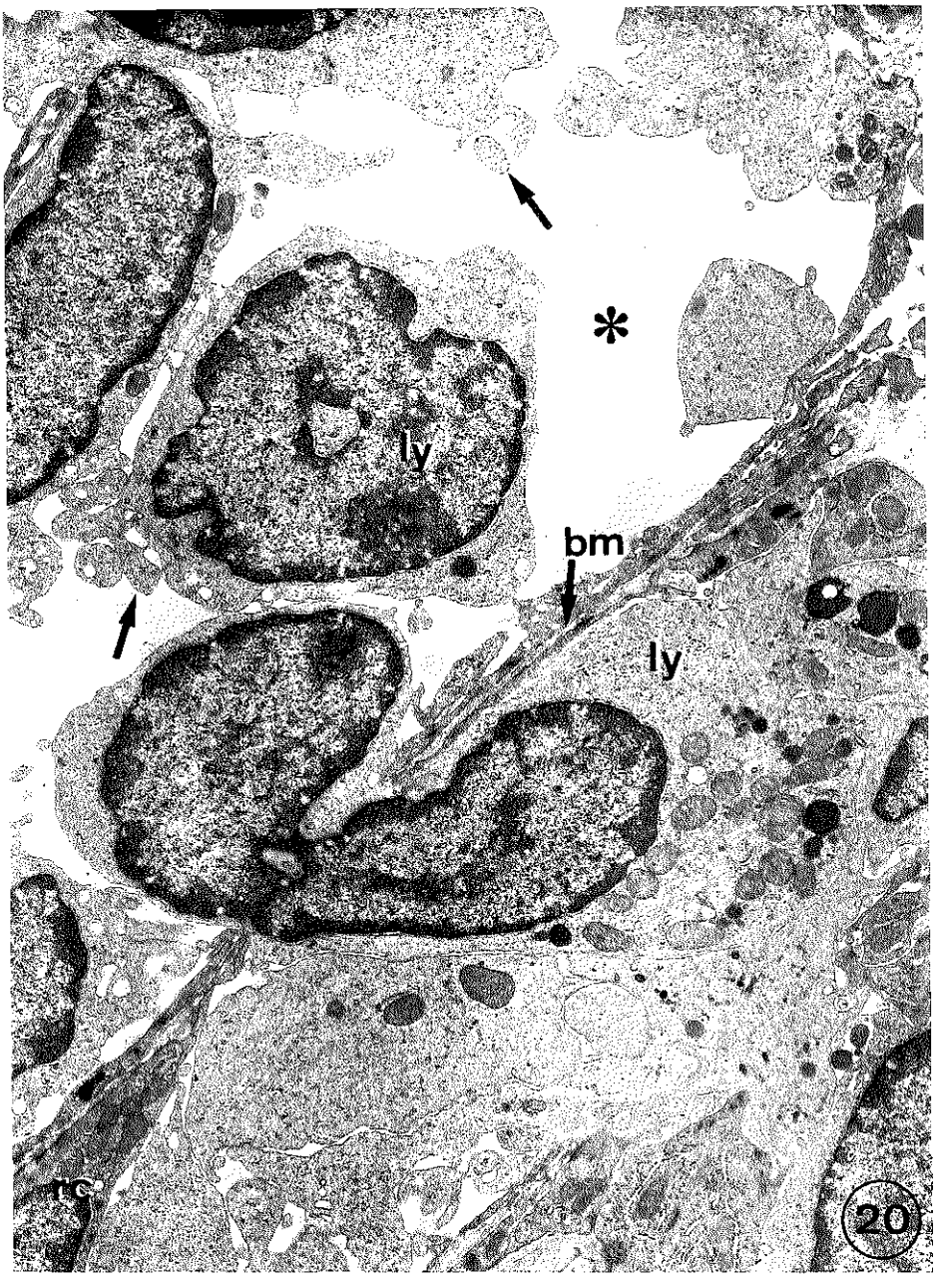


Fig. 20. Marginal sinus (*). A medium sized lymphocyte (*ly*) passes the border between the follicle and the marginal sinus. The cytoplasmic tail is directed towards the follicle. Arrows indicate microvilli of marginal zone lymphocytes. *bm* basement membrane; *rc* reticulum cell. Rat. ×10000

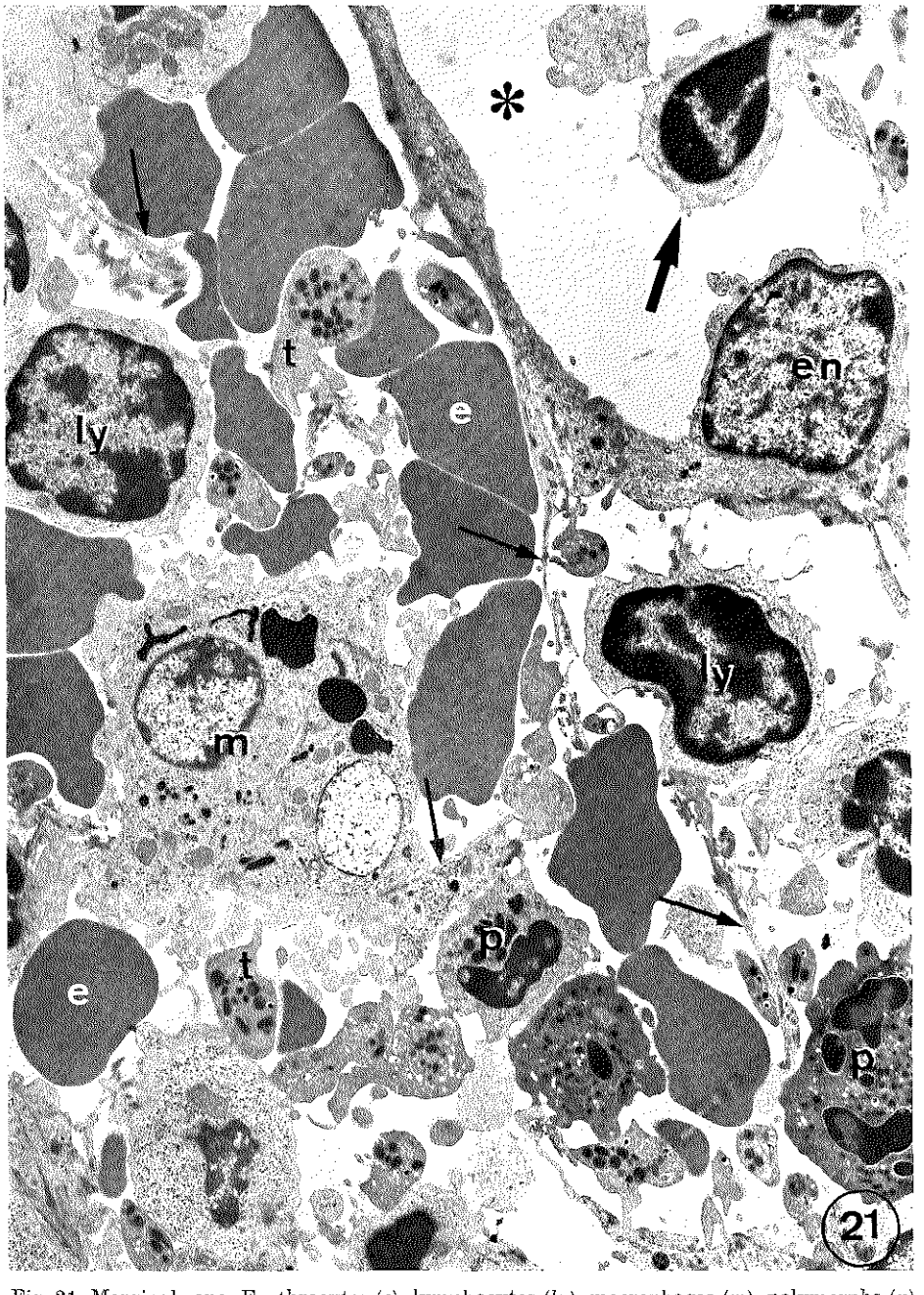


Fig. 21. Marginal zone. Erythrocytes (*e*), lymphocytes (*ly*), macrophages (*m*), polymorphs (*p*) and thrombocytes (*t*) are present between the reticulum cells (small arrows). Note the large extracellular space. A lymphocyte (large arrow) is passing between the endothelial lining of a red pulp sinus (*). Mouse. ×6000

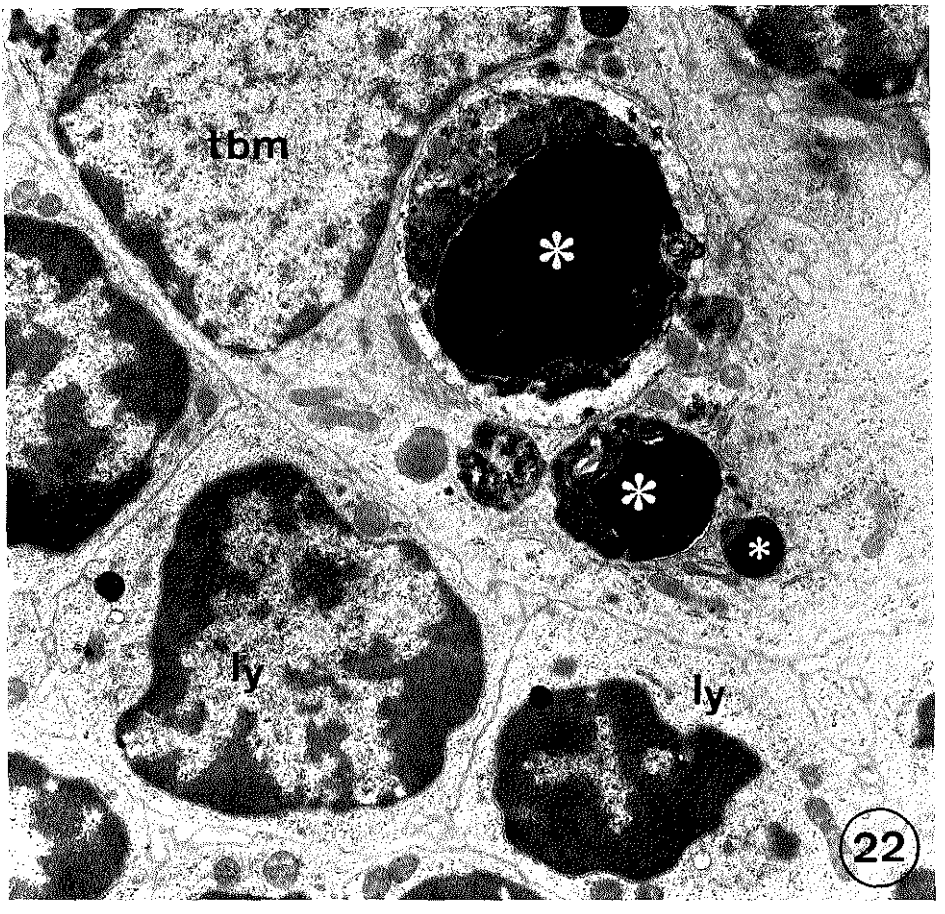
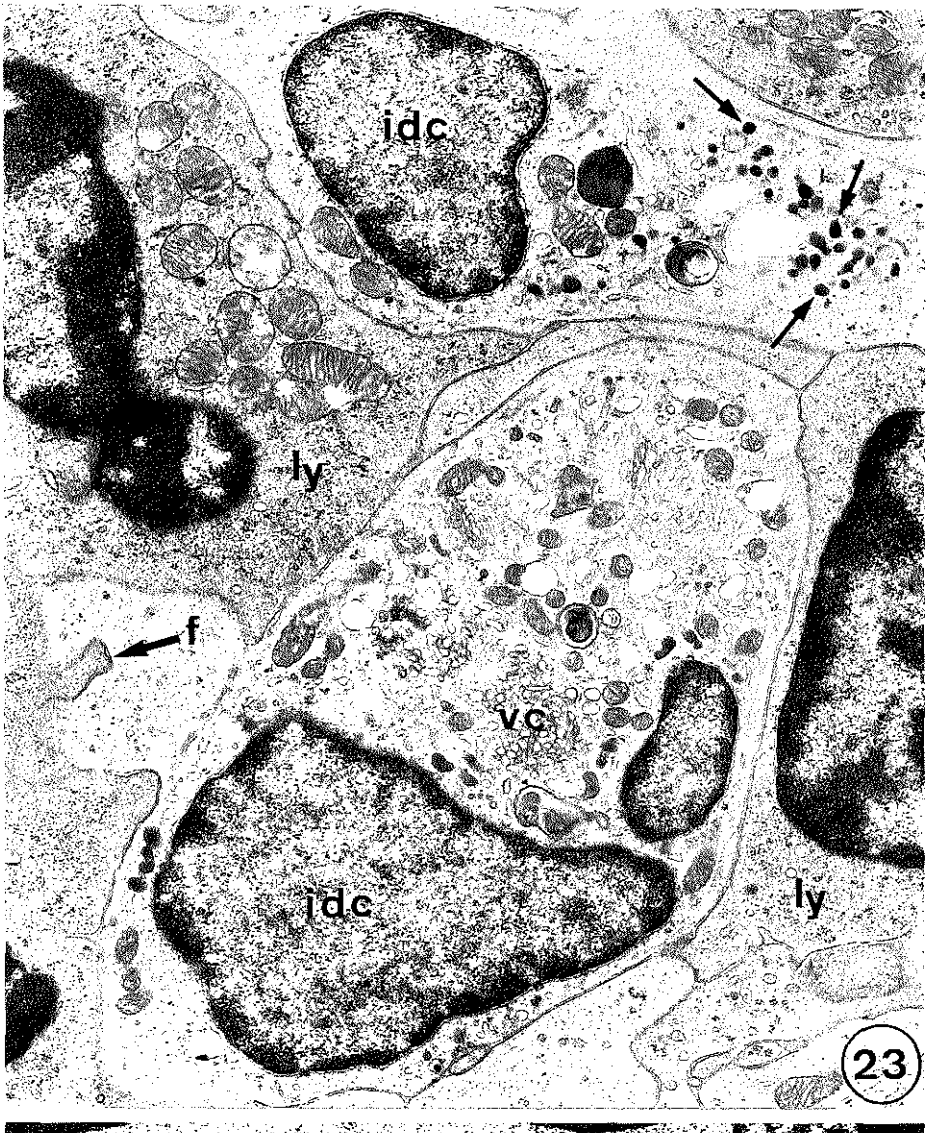


Fig. 22. Follicle centre. A tingible body macrophage (*tbm*) contains phagosomes with lymphocytes in several stages of degradation (*). *Ly* small lymphocyte. Mouse. ×10000

III. Lymphoid Cells

In the PALS two types of small lymphocytes occur. The small electron dense lymphocytes are only infrequently seen in normal animals. They are more often present in the PALS of irradiated animals injected with syngeneic thymocytes (van Ewijk *et al.*, 1974). The electron dense lymphocytes are also more often seen in antigenically stimulated animals (Veerman, 1974b). It seems, that both cell types are T-cells in different stages of differentiation.

Comparison of the less electron dense lymphocytes in the central PALS (T-cells) with the small lymphocytes in the follicles (B-cells), reveals three distinctions: 1. B-cells in the follicles are generally smaller than T-cells. This difference was earlier observed by Abe and Ito (1970). 2. The surface structure of B-lymphocytes both in the marginal zone and in the follicles, especially where they are adjacent to DC, is irregular: they show many microvillous projections. T-cells appear to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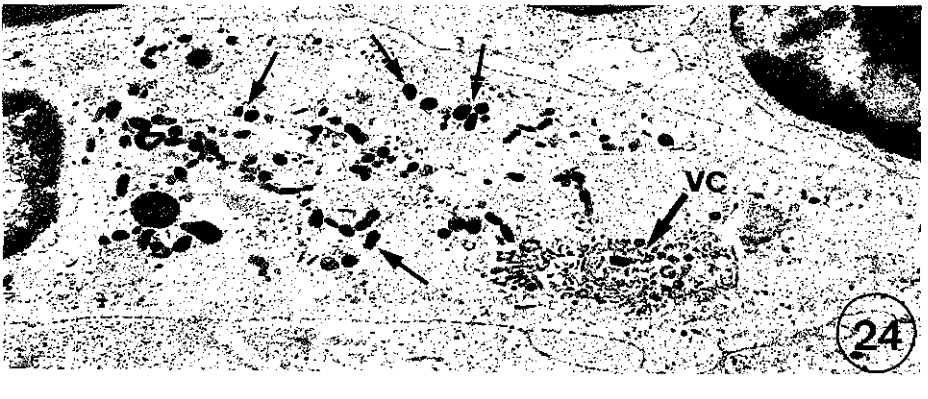
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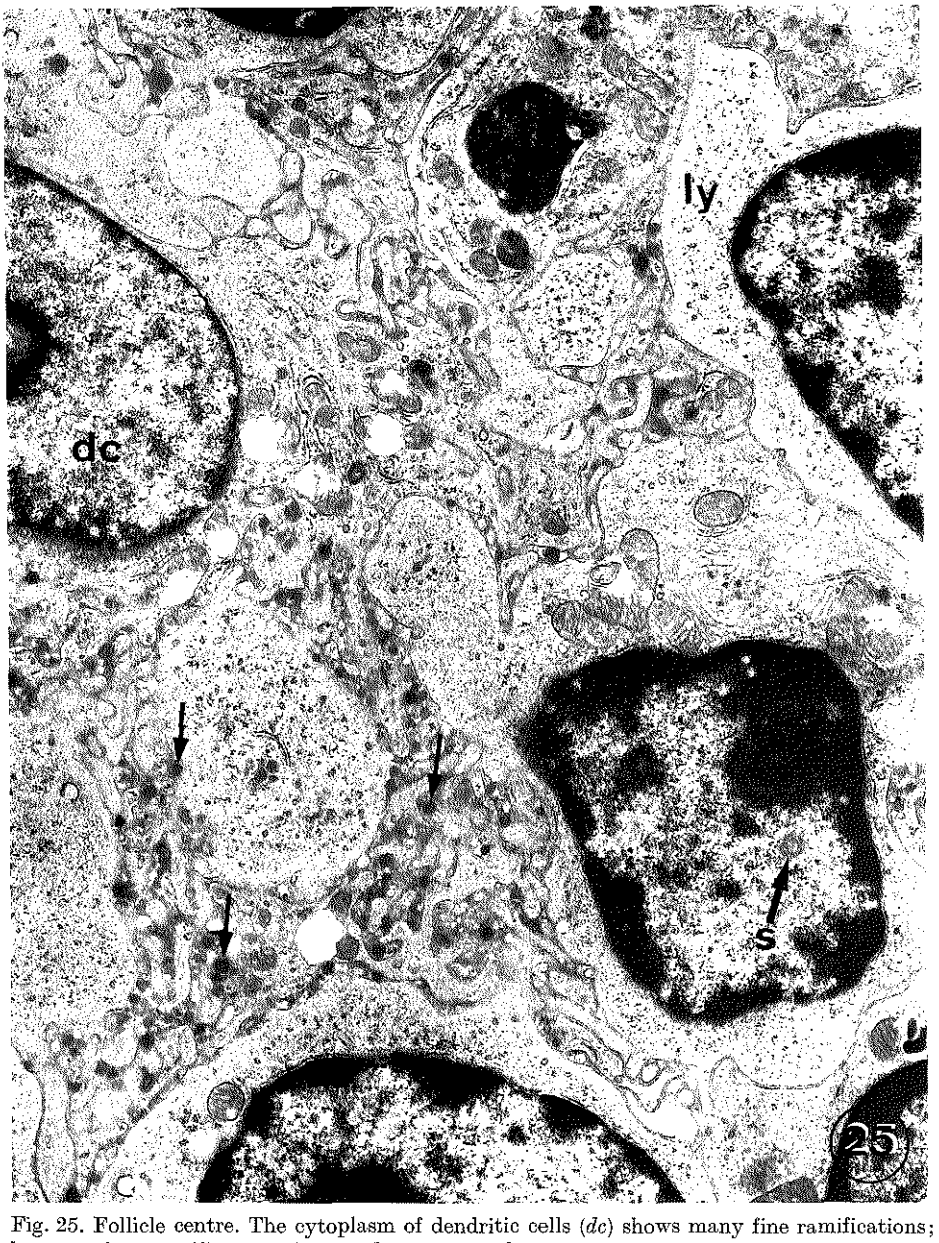

Fig. 25. Follicle centre. The cytoplasm of dendritic cells (*dc*) shows many fine ramifications; between these ramifications electron dense material is present, frequently in the form of small globules (arrows). *ly* lymphocyte; *s* sphaeridium. Rat. ×13000

Fig. 23. Central PALS. Interdigitating cells (*idc*). The cytocentre contains several mitochondria, a vesicular complex (*vc*) and small lysosome-like bodies (arrows). IDC do not show active phagocytosis. Surrounding lymphocytes (*ly*) indent the membrane of the IDC by means of fingerlike protrusions (*f*). Rat. ×17500

Fig. 24. Central PALS. Glycoproteins in the cell coat, the lysosomes (arrows) and in the vesicular complex (*vc*) are stained. Nuclear staining also occurs in control sections and is aspecific. Rat. Periodic acid silvermethenamine stain. ×20000

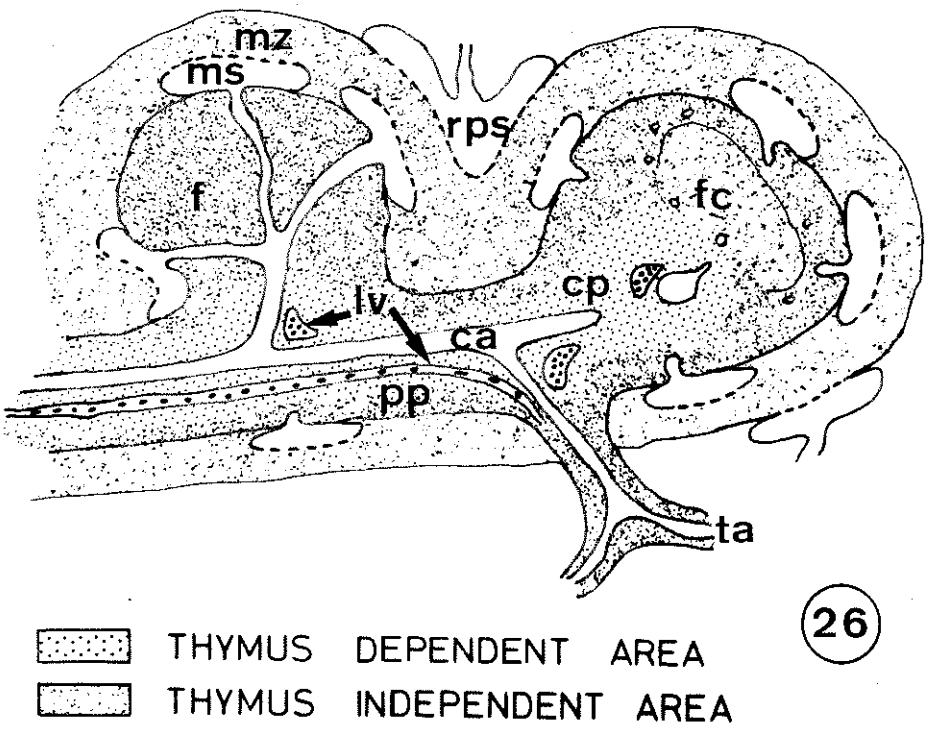

Fig. 26. Schematic diagram of the splenic white pulp in rats and mice. Around a central arteriole the periarteriolar lymphatic sheath (PALS) is situated. The PALS has a central, thymus-dependent, area (*cp*) and a peripheral area (*pp*) where both T- and B-cells occur. Follicles (*f*) are situated in the peripheral PALS, and may exhibit a follicle centre (*fc*). The follicles with the marginal zone (*mz*) constitute the thymus independent area. Close to the central arteriole a lymph vessel (*lv*) occurs which originates in the white pulp around the terminal arterioles (*ta*). *Ms* marginal sinus; *rps* red pulp sinus

have a smooth cell surface. This difference in surface structure was also observed by Polliack *et al.* (1973) on B- and T-cells in suspension. The view, however, that cell surface structure would reflect a characteristic difference between B- and T-cells has recently been challenged (Scott Linthicum and Sell, 1974; van Ewijk and Brons, manuscript in preparation). 3. Sphaeridia or nuclear bodies are often present in the nucleus of B-cells (cf. Suter *et al.*, 1972; Dukor *et al.*, 1972), but are only very rarely seen in the nucleus of T-cells. Their function is not yet understood. Simar (1969) has shown that they may contain both DNA and RNA, he noted that the number of cells containing sphaeridia rose following immunisation and concluded that they had some function in protein synthesis.

The peripheral PALS, containing several types of lymphocytes and plasmablasts, is not part of the thymus dependent area. It is an area where both T- and B-cells are present. It may thus be an important zone for T-B interaction.

IV. Cell Traffic

The circulation and immigration of lymphocytes into the spleen of rats has been extensively studied by Ford (1969a, b). From his experiments it was concluded that the marginal zone has a major function in the trapping of lymphocytes which entered the spleen. T-lymphocytes were found to migrate from the marginal zone to the PALS in 4 to 10 hours. Recently, Nieuwenhuis and Ford (manuscript in preparation) have studied B-cell migration in the spleen of rats. They found evidence for a migration pattern of B-cells from the marginal zone via the PALS around the smaller arterioles and the peripheral PALS, to the follicular lymphocyte corona.

Our morphological studies indicate two possible efferent pathways for lymphocytes from the splenic white pulp. Firstly, lymphatics in the central PALS indicate a lymphocyte outflow, secondly, lymphocytes are seen in the process of passing through the endothelial lining of the follicles. The lymphatics in the PALS are sometimes designated as pseudo-lymphatics (Mitchell, 1973). Since they are lined by endothelial cells it is evident that these structures are indeed real lymph vessels. The deep lymphatics of the spleen drain to the para-pancreatic lymph nodes (Tischendorf, 1969), thus suggesting an efferent pathway for recirculating lymphocytes. In T-cell depleted rats and mice these lymph vessels contain fewer lymphocytes than in intact animals (Veerman, van Ewijk, unpublished observations). Hence, T-cells apparently use this route. However, it remains possible that B-cells also leave the spleen via these lymph vessels. It seems that the unidirectional flow, via the terminal arterioles to the red pulp, as suggested by Mitchell (1973), has to be supplemented with a second efferent pathway for lymphocytes, using the lymphatics parallel to the central arteriole (cf. WHO-report, 1970).

The lymphocytes passing the border between follicles and marginal zone are most probably B-cells, since both follicles and marginal zone contain B-cells (Nieuwenhuis and Keuning, 1974). In vitro experiments have shown that moving lymphocytes display a cytoplasmic tail, a so called uropod, in which most cell organelles are gathered (McFarland, 1969). The lymphocytes migrate in a direction opposite to this uropod (Bessis, 1973). Lymphocytes passing the follicular lining show a cytoplasmic tail oriented towards the follicle. This would indicate therefore that they are moving out of the follicle. It has to be mentioned however, that following antigenic stimulation an influx of marginal zone cells into follicles has been demonstrated (Petterson *et al.*, 1967; Veerman and de Vries, manuscript in preparation). It has therefore to be considered that migration both into and out of the follicle may take place at the marginal sinus.

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Reconstitution of the Thymus Dependent Area in the Spleen of Lethally Irradiated Mice

A Light and Electron Microscopical Study of the T-cell Microenvironment

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Summary. To study the submicroscopical morphology of the microenvironment for T-lymphocytes in the spleen, mice were lethally X-irradiated and injected intravenously with syngeneic thymocytes.

24 hours after cell transfer, small lymphocytes occurred in the thymus dependent area of the spleen: the periarteriolar lymphatic sheath (PALS).

They localized preferentially around a special type of mononuclear phagocyte, the Interdigitating Cell (IDC), which is considered to be characteristic for thymus-dependent areas in peripheral lymphoid organs.

A close cell contact between both cell types was observed: small lymphocytes protruded into the cytoplasm of the IDC by means of fingerlike protrusions. This type of cell contact seems to induce blast transformation of the lymphoid cells which resulted in the formation of medium sized T-cells.

In a control experiment, spleen cells from thymectomized, X-irradiated and bone marrow reconstituted mice were injected intravenously into lethally X-irradiated recipients. These B-lymphocytes, however, were not found to be localized around IDC. They preferentially formed primary follicles at the periphery of lymphocyte-depleted thymus dependent areas.

Key words: Spleen — Thymus dependent area — Periarteriolar lymphatic sheath (PALS) — Interdigitating cell (IDC) — T-lymphocyte — Electron microscopy.

Introduction

Intravenous antigenic stimulation of mice with sheep red blood cells evokes a plasma-cellular reaction in the spleen, which requires in addition to macrophages, the cooperation of two types of lymphocytes: T-cells and B-cells (for reviews see Miller, 1972; Feldmann 1973).

To study this cooperation phenomenon in histological and electronmicroscopical preparations of the spleen, a careful examination of the microenvironment of T- and B-cells respectively is necessary.

At the light microscopical level, two areas can be distinguished in the white pulp of the spleen, i.e. the periarteriolar lymphatic sheath (PALS), localized around branches of the splenic artery, and the follicular structures or malpighian bodies, which are lodged at the periphery of the lymphatic sheath (Langevoort 1963, Moore 1964, Weiss 1964, 1972).

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From the light microscopical investigations of Waksman *et al.* (1962), Howard *et al.* (1972) in rats and from Parrot *et al.* (1966, 1971), de Sousa *et al.* (1969), de Sousa (1971), Mitchell (1972), Gutman and Weissman (1972) and Sprent (1973) in mice, it is known that the PALS and the follicular structures are distinct compartments in the spleen where T- and B-cells home respectively.

However, submicroscopically these areas have been poorly investigated. In contrast to this, microenvironments for T- and B-cells in lymph nodes were extensively investigated by Veldman (1970).

In the present investigation the T-cell microenvironment is analyzed in the spleen of mice, and an attempt is made to identify the T-lymphocyte at the electron microscopical level. To differentiate between T- and B-cell microenvironments, animals were X-irradiated and injected with "pure" T- or B-cell suspensions. Subsequently the localization of both donor cell types was studied during 6 days after irradiation and cell transfer.

Material and Methods

Animals

Male ($DBA_2 \times C_{57}Bl/Rij$) F_1 mice approximately 15–20 weeks old were used. They were obtained from the Medical Biological Laboratory TNO, Rijswijk, The Netherlands.

X-Irradiation

The recipient mice received 850 rad whole body irradiation generated in a Philips Müller MG 300 X-ray machine. Animals were irradiated in well-aerated circular Perspex cages. Physical constants of the irradiation were: 250 kV (constant potential); 11 mA; added filtration of 1.0 mm Cu; irradiation was corrected for field inhomogeneity; focus-object distance 53 cm; animals were irradiated at a dose rate of 30 rad/min. Maximal back scatter was achieved by placing the cage on a layer of 11 cm hardboard. During irradiation the dose was measured with a Baldwin Ionex dosimeter. Radiation control mice died in 9–16 days.

Cell Suspensions

A. Thymocytes. Thymuses from 6 weeks old animals were minced with scissors and gently pressed through a nylon sieve. The cells were washed three times in a balanced salt solution (BSS) (Mishell and Dutton, 1967) containing 5 per cent fetal calf serum; viable cells were counted with the trypan blue dye exclusion test, final cell concentration was adjusted to 10^8 cells/ml; 0.5 ml of the cell suspension was injected intravenously into irradiated recipients.

B. Spleen Cells. Lymphocytes were collected from the spleen of adult thymectomized, lethally irradiated mice, reconstituted with anti-Θ-serum treated bone marrow (Van Muiswinkel *et al.*, in preparation), 4–6 weeks after reconstitution. Before the spleen was removed, the thoracic cavity was checked for the presence of thymic remnants. When no remnants were found, the spleen was removed and the cell suspension was prepared as described at A.

Cell suspensions were made at a concentration of 2×10^8 nucleated cells/ml, 0.5 ml was injected intravenously into irradiated recipients.

Perfusion Fixation Technique, Embedding, Staining

To study the localization of thymocytes and spleen cells in the irradiated host, groups of 4 lethally irradiated animals reconstituted with thymocytes, 4 lethally irradiated animals reconstituted with spleen cells and 2 lethally irradiated control animals were killed on successive days, up to 6 days after administration of donor cells by means of perfusion fixation. The perfusion device is schematically presented in Fig. 1.

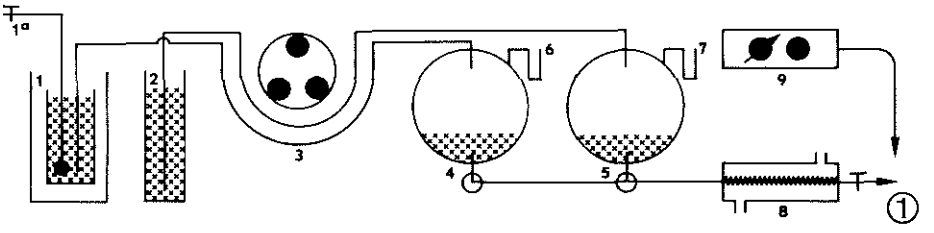


Fig. 1. Schematic presentation of the perfusion fixation device. *1* BSS, cooled at 4°C, *1a* admittance of oxygen, *2* fixation solution, *3* roller pump (Watson & Marlow, type MHRE 22), *4*, *5* cushioning chamber to maintain a constant pressure, *6*, *7* manometer, *8* thermostatic water bath, *9* Amsterdam Infant ventilator

Since the abdominal aorta in mice is very small in diameter, perfusion was carried out via the heart. Animals were anaesthetized with Nembutal (70 mg/kg body weight). Before opening the thorax, the trachea was connected to an Amsterdam Infant ventilator (Loos & Co., Amsterdam, Holland); respiration was maintained with moistured air containing 5 per cent CO_2. The perfusion cannula was inserted into the left ventricle and the right atrium was opened. Subsequently the perfusion was started with oxygenated BSS containing 0.1 per cent procaine and 0.2 per cent dextran-40, pH = 7.2; ±300 milliosmols (flow rate 0.4 ml/sec., pressure measured in cushioning chamber 50 mm Hg). Respiration was stopped after bleaching of the liver. After 2 minutes perfusion was switched to the fixation solution consisting of 1.5 per cent glutaraldehyde in 0.1 M sodium cacodylate, pH = 7.2; ±385 milliosmols, and continued for another 6 minutes. Before taking out the spleen, perfusion efficiency was checked by examining the splenic artery under a dissecting microscope. When the splenic artery appeared to be free from blood, the spleen was taken out, cut into thin slices and fixed for an additional period of 2 hours in the perfusion fixation solution at 4°C. Subsequently the slices were rinsed in 0.14 M sodium cacodylate buffer at pH 7.2 and post-fixed in 1 per cent osmium tetroxide in 0.1 M sodium cacodylate buffer at 4°C for 2 hours. Specimens were rapidly dehydrated in graded series of alcohol, rinsed in propylene oxide and embedded in Epon 812.

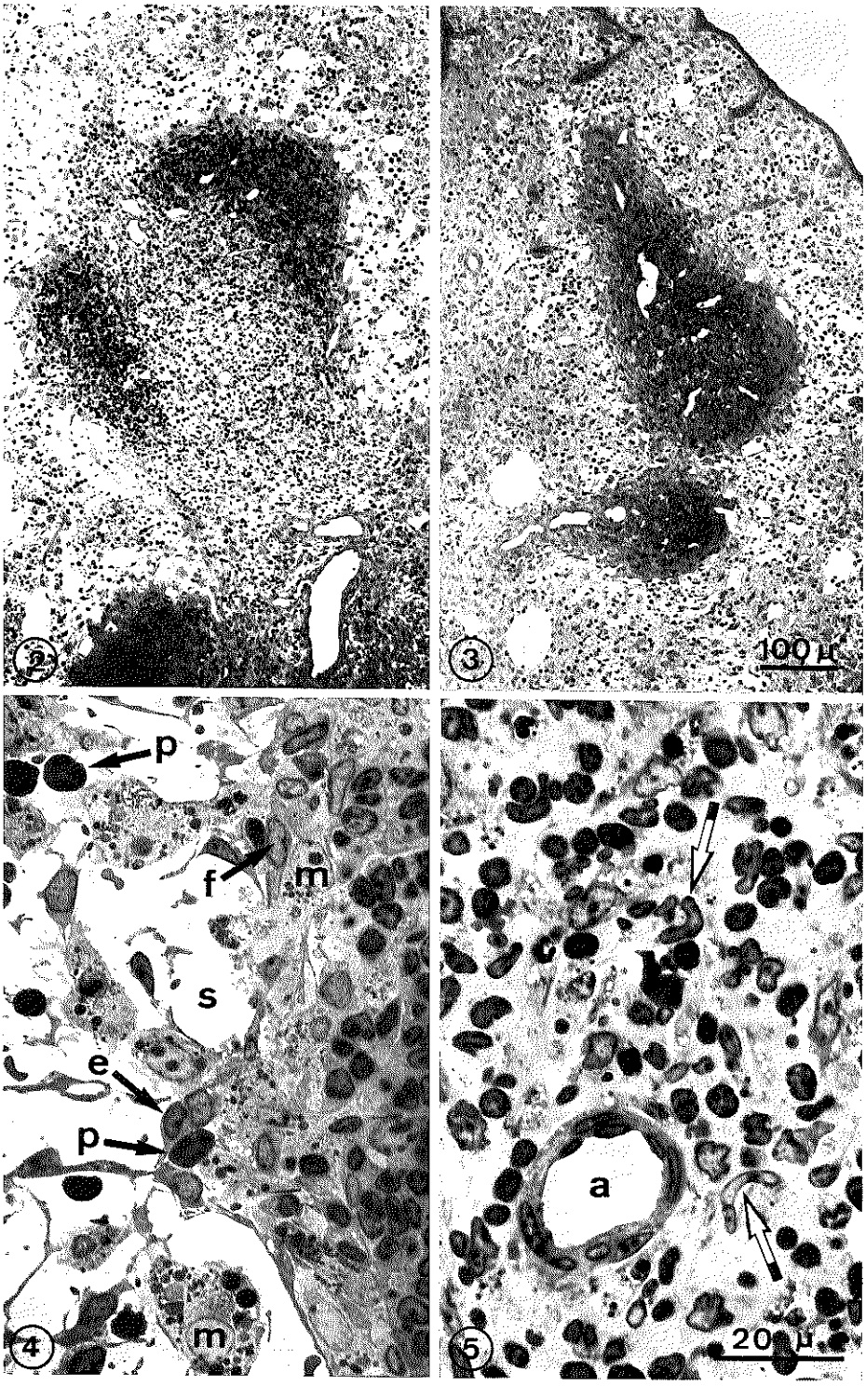
Light- and Electron Microscopical Specimen Preparation

For light microscopical studies, series of about 1 μ thick sections were cut on a Reichert Om U3 microtome. The sections were stained with an aqueous solution of toluidin blue adjusted to pH 8.6 with 0.1 M Borax buffer. Coverslips were mounted with Epon instead of conventional mounting media in order to avoid bleaching of the stained sections. Before ultrastructure studies were performed, the white pulp of the spleen was visually selected in the last 1 μ section. The desired area was relocated in the corresponding Epon block under a dissecting microscope and marked with a razor blade. The surrounding material was trimmed off with the "mesa technique" according to De Bruijn and McGee Russell (1966). Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined with a Philips EM 300 electron microscope operated at 60 kV, with a 40 μ objective aperture.

Results

Perfusion Fixation

As a result of the perfusion fixation the peripheral blood in arterioles, sinuses and venules had been removed. In this way in the spleen a clear topographical distinction between red and white pulp was obtained. This greatly facilitated light microscopical investigation of both areas (Fig. 2, 3, 4).



Figs. 2—5

Lethally X-irradiated Control Animals, Light Microscopical Observations

24 Hours after irradiation erythropoietic and granulopoietic islets were absent in the red pulp; many dead cells being phagocytized were found. The marginal zone, bordering the white pulp, was infiltrated with large amounts of polymorphs; also penetration of polymorphs into the white pulp was observed (Fig. 4). Malpighian bodies and periarteriolar lymphatic sheathes (PALS) were markedly depleted of lymphocytes, also here many dead cells were removed by macrophages.

During the next days most macrophages and polymorphs disappeared from the white pulp; in contrast, the red pulp showed an increase in the number of actively phagocytizing cells. The decrease in the number of lymphoid cells and macrophages caused a reduction of the total volume of the white pulp. 6 Days after irradiation the white pulp consisted only of fibroblasts, a few macrophages and some mature plasma cells. It should be noted, however, that by that time in some parts of the splenic white pulp small accumulations of lymphoid cells were found around branches of the splenic artery. These cells probably survived the lethal dose of X-irradiation.

Lethally X-irradiated Animals Reconstituted with Syngeneic B-cells, Light Microscopical Observations

Animals irradiated and injected with 10^8 spleen cells from adult thymectomized, lethally irradiated and bone marrow reconstituted mice showed a distinct localization pattern of lymphoid cells in the white pulp at 24 hours after cell transfer.

At the periphery of a largely depleted PALS, accumulations of small dark-staining lymphocytes, 3.5–5.5 μ in diameter, could be observed (primary follicles, Fig. 2). These primary follicles were surrounded by a meshwork of endothelial cells, macrophages and fibroblasts (Fig. 4). In the central part of the primary follicles also a few macrophages were found, characterized by ingested material and large round nuclei.

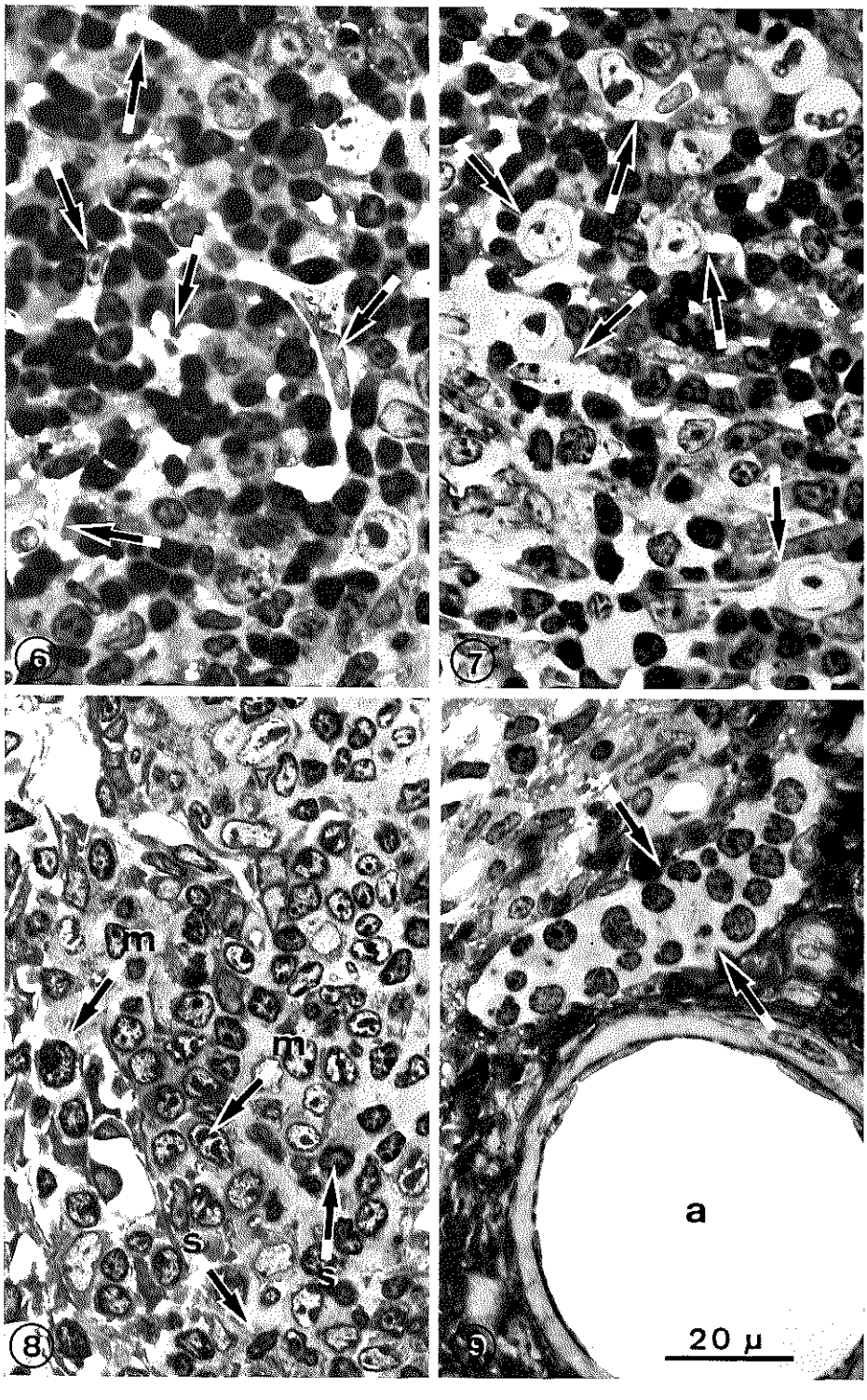
As is shown in Fig. 5, the thymus dependent area of these animals remained largely depleted of lymphoid cells. However, a few lymphocytes were always found in this area. In addition, lymphoid cells also occurred in small amounts in lymphatic vessels, parallel to branches of the splenic artery.

Fig. 2. Low magnification of the white pulp of the spleen, 24 hours after X-irradiation and reconstitution with B-lymphocytes. Primary follicles are populated with lymphocytes; the PALS remains largely depleted of lymphocytes ($\times 110$)

Fig. 3. Low magnification of the white pulp of the spleen, 24 hours after X-irradiation and reconstitution with thymocytes. The PALS is populated with lymphocytes; no primary follicles are present ($\times 110$)

Fig. 4. Marginal zone of a primary follicle, 24 hours after X-irradiation and reconstitution with B-lymphocytes. Macrophages (*m*) occur in the marginal sinus (*s*) and the outer zone of the primary follicles. Polymorphs (*p*) infiltrate into the white pulp. *e* endothelial cell, *f* fibroblast ($\times 920$)

Fig. 5. PALS, 24 hours after X-irradiation and reconstitution with B-lymphocytes. A few lymphocytes can be observed in this area. Interdigitating cells (IDC) show a peculiarly formed nucleus (arrows). The cytoplasm of these cells is toluidin blue-negative and contains few organelles. *a* arteriole ($\times 920$)



Figs. 6—9

Macrophages and fibroblasts were the predominant cell types; furthermore a special mononuclear phagocyte was found in the PALS. The cytoplasm of this latter cell type did not show affinity for toluidin blue, the nucleus of these cells was elongated and irregular in shape (Fig. 5, arrows). Veldman (1970) reported similar mononuclear phagocytes in the paracortex of lymph-nodes in the rabbit. According to his proposed nomenclature for this type of cells the term Interdigitating Cell (IDC) will also be used in the present report.

Again, like the control group, during the following days a decrease in the volume of the thymus-dependent area was observed.

In the meantime primary follicles showed an increase in the number of medium sized lymphocytes, 6–8 μ in diameter, and 4 days after cell transfer plasmablasts were present in the white pulp. After another 2 days large numbers of plasma cells were found both in the white pulp closely associated with branches of the splenic artery, as well as in the red pulp. These observations suggest that part of the injected spleen cells transformed into plasma-cells during the first 6 days of reconstitution.

Lethally X-irradiated Animals, Reconstituted with Syngeneic Thymocytes, Light and Electron Microscopical Observations

Injection of 5×10^7 thymocytes in lethally irradiated recipients showed a diffuse localization of small lymphocytes throughout the PALS 24 hours after cell transfer (Fig. 3).

Under these experimental circumstances no lymphocytes were found in the follicular remnants at the periphery of the PALS. In contrast, at distinct areas of the PALS discrete accumulations of small lymphocytes (3.5–5.5 μ in diameter) were found. On close investigation, IDC were found in these areas and a close cell contact between small dark staining lymphocytes and cytoplasmic processes extending IDC was observed (Fig. 6).

Electron microscopic observation on IDC revealed a suprising low electron-density of the cytoplasm (Fig. 11). Only a few small mitochondria were found, the granular endoplasmic reticulum was rather poorly developed and the Golgi-complex consisted of small lamellae and vesicles. A characteristic feature was the presence of large empty vacuoles about 1 μ in diameter. Furthermore, complexes of small tubular vesicles were observed. Each vesicle was about 800 Å in diameter. In some occasions, these vesicles were found being pinched off by the rough

Fig. 6. PALS, 24 hours after X-irradiation and reconstitution with thymocytes. Note the high concentration of small dark staining lymphocytes, 3.5–5.5 μ in diameter. Arrows indicate IDC or their cytoplasmic processes (×920)

Fig. 7. PALS, 4 days after X-irradiation and reconstitution with thymocytes. IDC have extended their cytoplasmic web. Small lymphocytes (3.5–5.5 μ in diameter) and lymphoblasts (11–13 μ in diameter) (arrows) are frequently found to be in contact with IDC (×920)

Fig. 8. PALS, 6 days after X-irradiation and reconstitution with thymocytes, medium sized lymphocytes, 6–8 μ in diameter (*m*). The nucleus of the medium sized lymphocytes has more dispersed chromatin compared to small lymphocytes (*s*) (see also Fig. 5). (×920)

Fig. 9. PALS, 6 days after X-irradiation and reconstitution with thymocytes. The lymphatic vessel (arrows) contains medium sized T-lymphocytes. *a* splenic artery (×920)

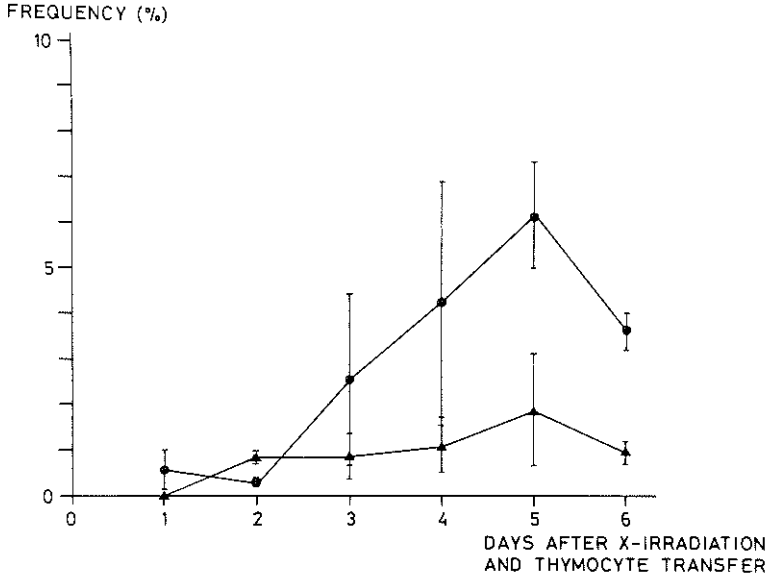


Fig. 10. Frequency of lymphoblasts (—●—) and mitotic lymphoid cells (—▲— in the PALS of lethally X-irradiated mice reconstituted with 5.10^7 thymocytes. Each point of the curves was obtained by counting 400 lymphoid cells in the PALS of each recipient mouse. The vertical bars represent the standard error

endoplasmic reticulum. In most of the observed IDC microtubules, 330 Å in diameter, were found, mostly in the neighbourhood of the nucleus (Fig. 11). Occasionally these structures were found in association with a cilium (Fig. 13). The number of phagosomes in IDC was rather low, especially when compared with macrophages. Therefore the IDC are thought not to be actively phagocytizing cells. The elongated nucleus showed a thin rim of condensed chromatin and usually one or two marginally located nucleoli were observed.

The cytoplasmic extensions of IDC interdigitated in a very complex way and small lymphocytes seemed to be trapped between these extensions (Fig. 12).

A remarkable demonstration of the intimate union between both cell types was the presence of fingerlike protrusions, extending from the lymphocyte and protruding into the cytoplasm of IDC (Fig. 12, arrows, Fig. 14). Electron dense material, often seen as cross bridges, about 120 Å in length, was apparent in the extra-cellular space of these contact regions (Fig. 14, arrows).

During the following days after X-irradiation and cell transfer, the cytoplasmic web of the IDC seemed to extend. Furthermore, lymphoblasts and mitotic lymphoid cells were found in the neighbourhood of IDC; most of the observed lymphoblasts were found to contact IDC (Fig. 7, arrows, Fig. 15). These blasts were devoid of endoplasmic reticulum (Fig. 15). and therefore thought to be T-lymphoblasts. As is demonstrated in Fig. 10 the percentage of lymphoblasts increased during the reconstitution period: on day 5 approximately 6 per cent of the lymphoid cells was transformed into blasts. However, on day 6 the number of blasts was significantly lower when compared to day 5.

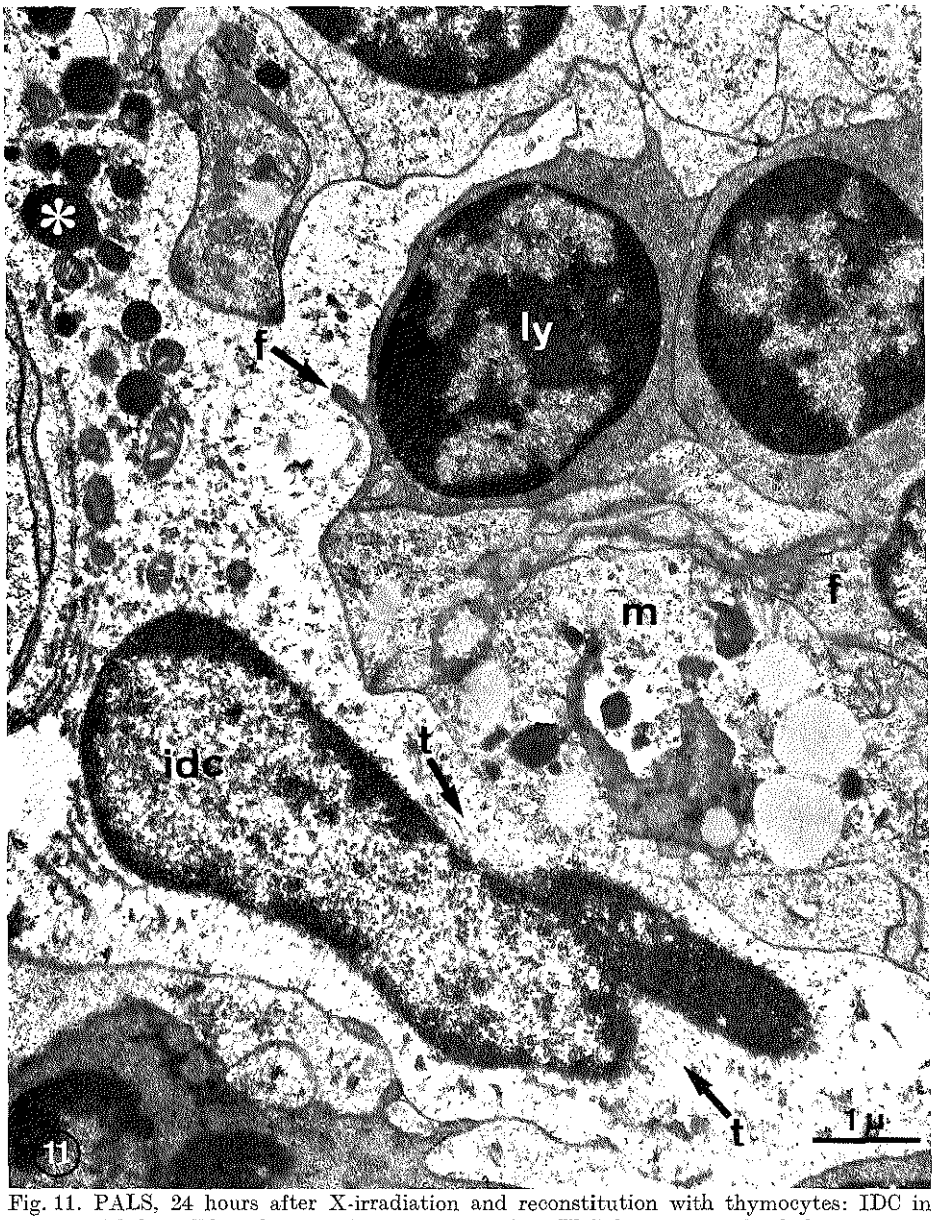
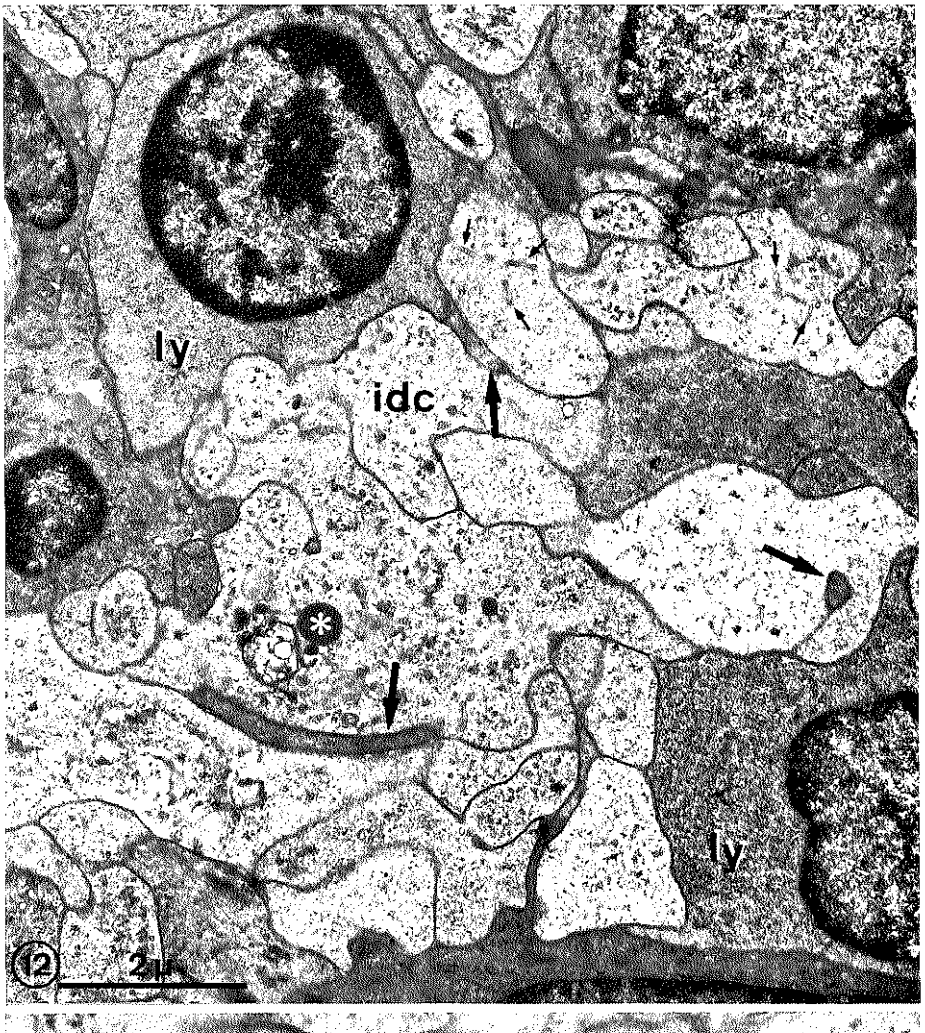


Fig. 11. PALS, 24 hours after X-irradiation and reconstitution with thymocytes: IDC in contact with 2 small lymphocytes. The cytoplasm of the IDC shows a remarkably low electron-density. * lysosome, *t* microtubules, *f* fingerlike cell contact between lymphocyte and IDC, *m* macrophage, *f* fibroblast. (×14000)

The mitotic index increased during the second day after X-irradiation and cell transfer and remained on a constant level during the following period. 5 Days after cell transfer the majority of the lymphoid cells was found to be medium sized (6–8 μ in diameter). These lymphocytes showed a lower affinity for toluidin blue in comparison with small lymphocytes.



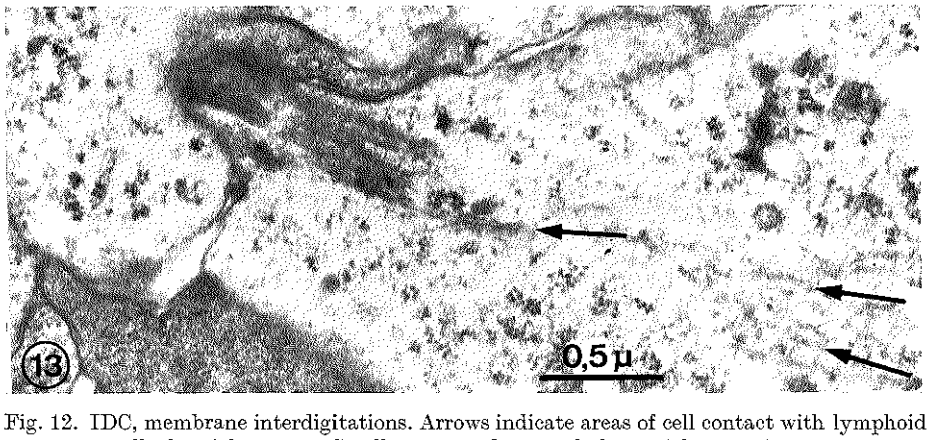


Fig. 12. IDC, membrane interdigitations. Arrows indicate areas of cell contact with lymphoid cells (ly). * lysosome. Small arrows indicate tubular vesicles. (×12500)

Fig. 13. IDC, demonstrating a cilium. Microtubules (arrows) are found to be in contact with the cilium. (×32000)

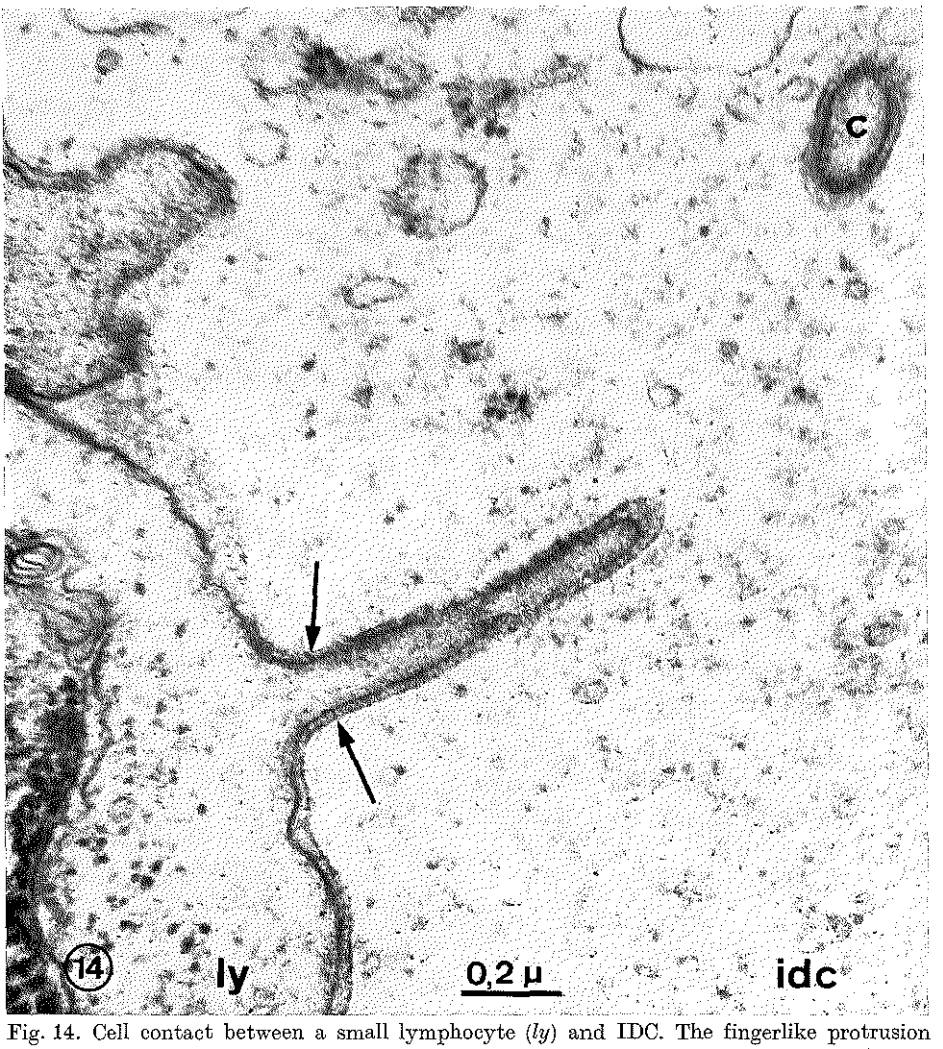


Fig. 14. Cell contact between a small lymphocyte (*ly*) and IDC. The fingerlike protrusion emerging from the lymphocyte is seen in longitudinal section and cross section (*c*). Arrows indicate extracellular cross bridges between both cell types. (×66000)

Electron microscopic observation of the medium sized lymphocyte showed a nucleus with more dispersed chromatin in comparison with small lymphocytes. The nucleus contained 2 or more nucleoli. Also the mitochondria had increased, both in number and size (Fig. 16). The latter were often concentrated opposite infoldings of the nuclear membrane. The cytoplasm of the medium sized lymphocyte showed a moderately low electron density and contained a fair number of monoribosomes. Polyribosomes consisting of 5 to 6 ribosomes were found less frequently (Fig. 17).

Coiled polyribosomes, consisting of more than 10 ribosomes, were found attached to the nuclear envelope and also to slender strands of endoplasmic

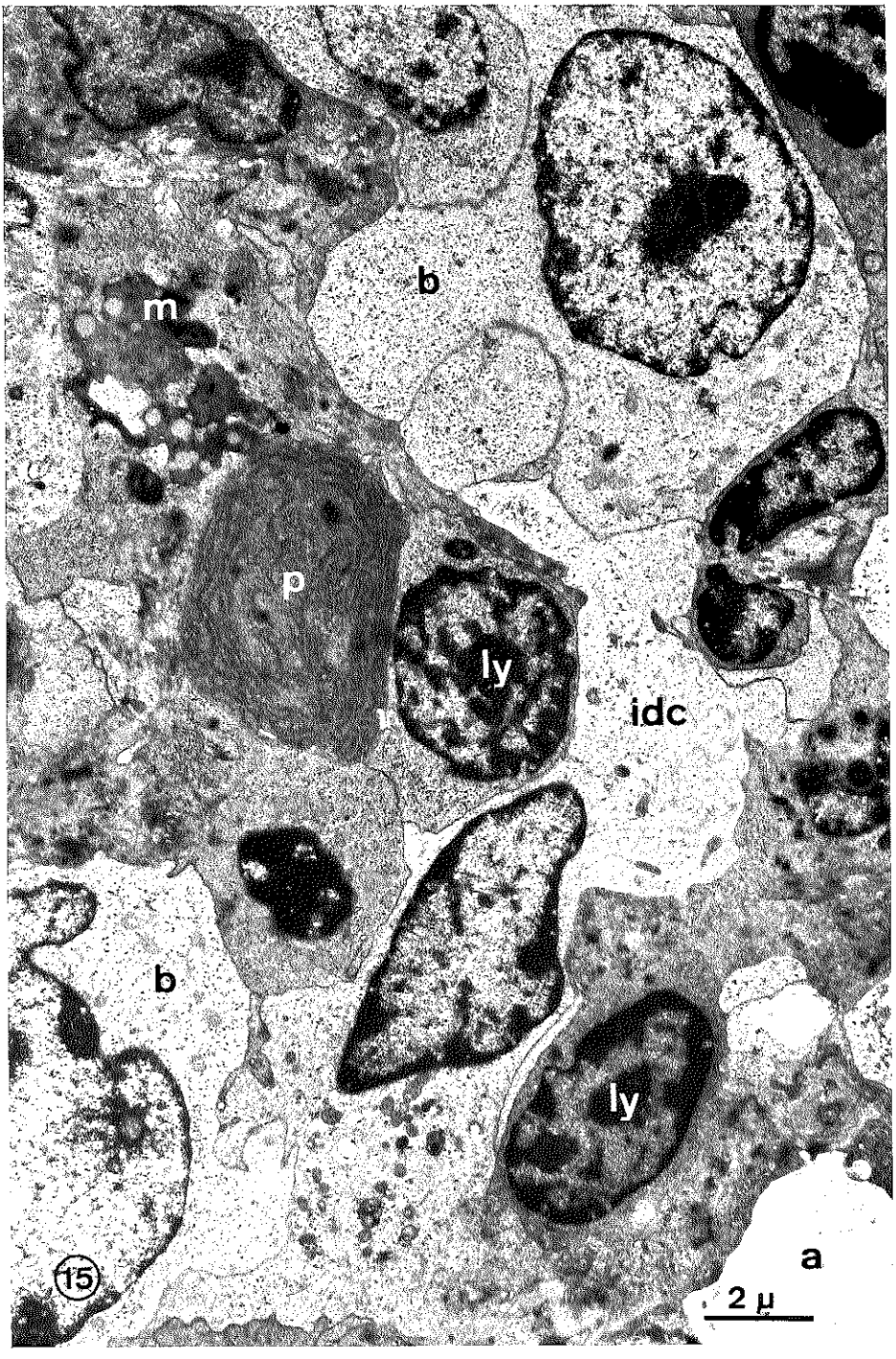


Fig. 15. PALS, 4 days after X-irradiation and reconstitution with thymocytes: IDC in contact with lymphoblasts (*b*), *ly* small lymphocyte, *p* plasma cell, *m* macrophage, *a* arteriole. (×7500)

reticulum (Fig. 17). A well developed Golgi region was present in the medium sized lymphocyte, furthermore multivesicular bodies and small vesicles about 800 Å in diameter were found throughout the cytoplasm (Fig. 16). It has to be noted that this type of lymphocyte was not restricted to the PALS only, they also occurred in the surrounding remnants of the marginal zone and in lymphatic vessels, which accompanied branches of the splenic artery (Fig. 9). Therefore these cells were thought to be part of the pool of recirculating T-cells.

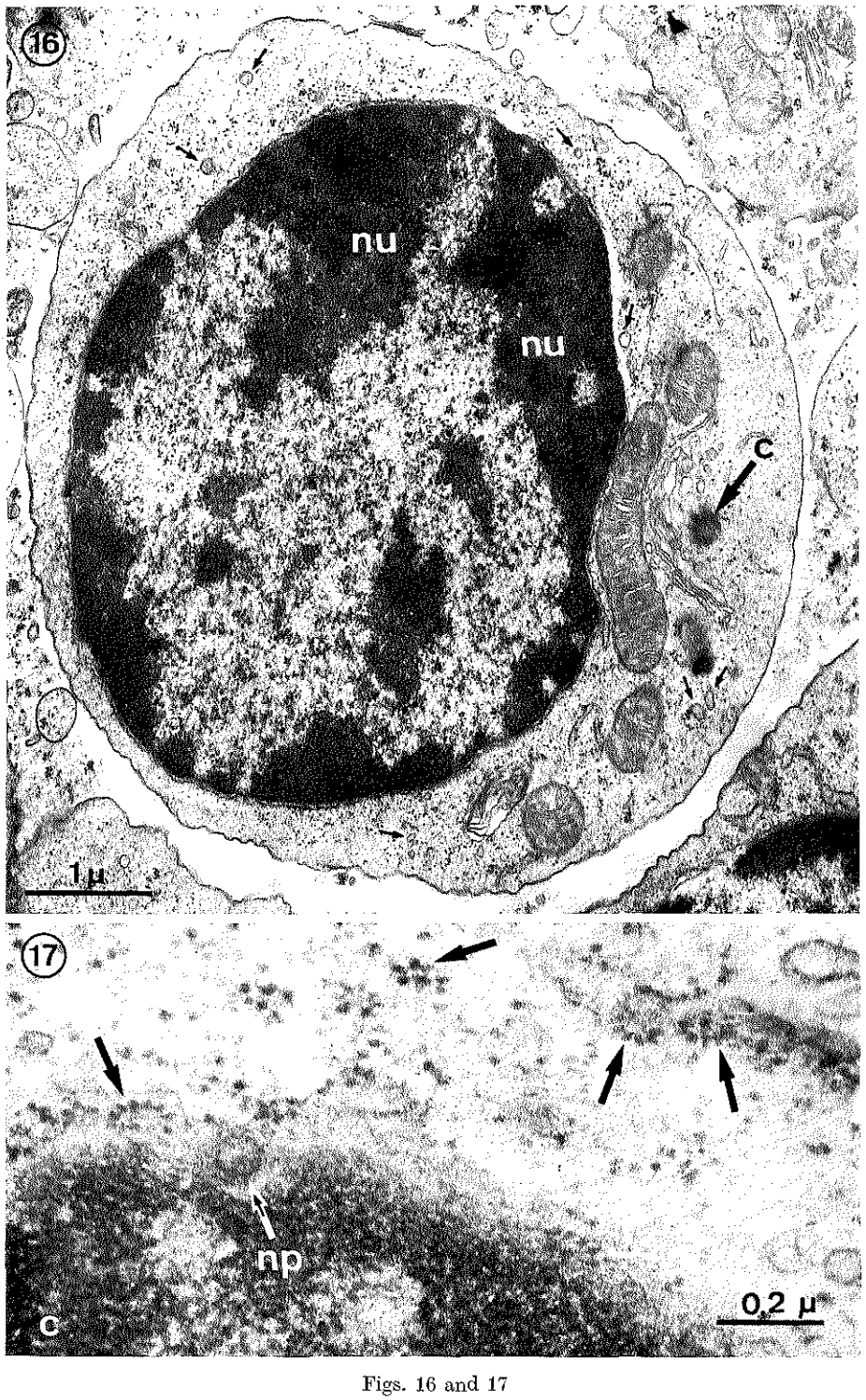
Discussion

The present report shows that intravenously injected syngeneic thymocytes or spleen cells from thymectomized, irradiated and bone marrow reconstituted mice localize in clearly defined compartments in the spleen of lethally irradiated mice, viz. the peri-arteriolar lymphatic sheath (PALS) and the follicular structures respectively. These observations are in agreement with those by Parrot *et al.* (1966), de Sousa *et al.* (1969), Parrot and de Sousa (1971), de Sousa (1971), Mitchell (1972), and Sprent (1973). Thymocytes were found to accumulate in the PALS around cytoplasmic processes of mononuclear phagocytes with a low phagocytic activity, so called Interdigitating Cells (IDC) (Veldman 1970). These cells resemble epithelioid cells (Sutton and Weiss 1966, Veerman, 1974, in press). IDC are thought to be characteristic for the thymus dependent areas in peripheral lymphoid organs.

A close interrelationship between lymphocytes and IDC has been demonstrated. A particularly striking form of this intimate cell contact is the presence of fingerlike protrusions extending from the lymphocyte and protruding into the cytoplasm of the IDC.

This type of cell-contact has also been described between lymphoid cells and mesenchymal reticular cells in the developing palatin tonsil of the rabbit (Leene 1971, 1972). Leene suggests that fingerlike protrusions retain lymphoid cells in a microenvironment which induces them to differentiate into antigen-reactive cells. McFarland (1969) has observed similar structures in electron microscopic studies of lymphocytes cultured *in vitro*. Motile lymphocytes show a cytoplasmic tail (uropod) which is used to attach the cell to solid structures in the immediate environment. The terminal end of the uropod shows numerous microspikes of about the same dimension as the fingerlike protrusions found on lymphocytes in our experiments. Studies on the interaction of lymphocytes and macrophages *in vitro* (Smith and Goldman 1970) demonstrate that particular areas of the lymphocyte membrane, on the posterior part of motile lymphocytes are used to contact macrophages. When this "interaction site" is occupied, immobilization of the lymphocyte is the result. Taken these data into account we suggest that thymocytes are able to contact IDC by means of specialized membranous structures, visible as fingerlike protrusions. This type of contact might be responsible for the prolonged presence of T-lymphocytes in the PALS. Possibly the extracellular material found in this contact-region represents specific receptors for the interaction of T-lymphocytes and IDC.

A close interrelationship between lymphocytes and mesenchymal reticular cells has also been described in the thymus (Metcalf and Ishidate 1962 and



Figs. 16 and 17

Hoshino 1962). These authors have demonstrated PAS-positive material in mesenchymal reticular cells, while surrounding lymphocytes showed a higher mitotic index than lymphocytes elsewhere in the thymus. This has been confirmed by Mandel (1969). Similar observations have been reported by Levaditi *et al.* (1973) who demonstrated that in tuberculous lesions T-lymphocytes surrounding epithelioid cells were frequently found to be in mitosis. In this scope the work of Hanifin and Cline (1970) is also of interest. These authors reported in vitro studies on T-lymphocytes and PPD-sensitized monocytes and macrophages. 36 Hours after onset of cell culture, close associations of lymphocytes and macrophages or monocytes were observed. In these immunological islands blastogenic transformation of lymphocytes occurred after 60–72 hrs of cell culture. Metcalf and Ishidate (1962) and also Clark (1968) and Gad and Clark (1968) assume that the PAS-positive material in the reticular cells in the thymus "triggers off" mitosis in primitive lymphoid cells. Veldman (1970), using the periodic-acid-silver-methenamine method, has demonstrated the occurrence of glycoproteins in complexes of small vesicles in IDC. Therefore, we would like to interpret these data to suggest that IDC produce a humoral factor, possibly a glycoprotein, which stimulates surrounding lymphocytes to transform into lymphoblasts.

This transformation is thought to be antigen dependent, since stimulation of lethally X-irradiated, thymocyte reconstituted mice with 4×10^8 sheep erythrocytes doubles the number of lymphoblasts on day 5 (van Ewijk, van der Kwast, unpublished observation). Possibly the fetal calf serum, present in BSS and the influx of antigens through the gastro-intestinal tract due to lethally X-irradiation account for the presence of lymphoblasts in the present experiments.

The medium sized T-lymphocytes observed in large amounts in the PALS, 6 days after irradiation and reconstitution with thymocytes, show a nucleus with less condensed chromatin, 2 or more nucleoli, well-developed mitochondria and a distinct Golgi region. Therefore, these cells are metabolically more active than the small lymphocytes observed on day 1. Morphologically, the medium sized lymphocytes are similar to cortisone resistant lymphocytes in the thymus medulla (Abe and Ito, 1970), which are known to be mature T-cells (Miller and Osoba 1967, Raff and Cantor 1971). The polysomes found in the cytoplasm and on the nuclear envelope are similar in structure to polysomes in plasma cells (van Ewijk, unpublished observation) and the assumption that T-cells can synthezise small amounts of immunoglobulin is therefore very temptive. Recent work of Marchelonis (1972) and Feldman *et al.* (1973) at least shows the presence of small amounts of monomeric IgM on the surface of T-cells.

Fig. 16. PALS, 6 days after X-irradiation and reconstitution with thymocytes: medium sized T-lymphocyte. Well developed mitochondria, a Golgi region with centrioles (*c*), pinocytotic vesicles (small arrows) and free ribosomes are found in the cytoplasm, *nu* nucleolus. (×18000)

Fig. 17. Detail of the cytoplasm of a medium sized lymphocyte. Arrows indicate coiled polysomes attached to the perinuclear envelope and to strands of the endoplasmic reticulum. Free rosette like polysomes consisting of 5 to 6 ribosomes are found throughout the cytoplasm, *np* nuclear pore, *c* condensed chromatin. (×75000)

It was mentioned before that in X-irradiated mice reconstituted with spleen cells, small lymphocytes predominantly localized in the follicles. However, a few lymphocytes also occurred in the thymus dependent area, 24 hours after cell transfer.

The following 3 possibilities which might account for the occurrence of lymphocytes in the PALS of these mice have to be considered:

a) the thymus is seeding T-lymphocytes before the 6th day after X-irradiation and spleen cell transfer,

b) X-irradiation with 850 rad does not kill all host lymphocytes,

c) part of the B-lymphocytes recirculates through the thymus dependent area towards efferent lymphatic vessels, localized next to branches of the splenic artery; the presence of these vessels was already demonstrated by Snook (1946).

The first possibility is at variance with experimental data from Gregory and Lajtha (1970) where T-cell recovery in lethally irradiated, bone marrow transplanted mice could not be demonstrated until two weeks after irradiation. The second possibility cannot be ruled out regarding our histological findings in lethally irradiated animals. In addition, Kettmann ad Dutton (1971), Anderson, Sprent and Miller (1972), Ito, Kino and Cudkowics (1973) and Nossal and Pike (1973) have demonstrated that at least the helper function of T-cells during antibody responses was retained in lethally X-irradiated mice. The third possibility has also to be considered; recent autoradiographic experiments with labeled B-cells have demonstrated the occasional presence of B-cells in the PALS of the mouse spleen (Mitchell 1973, van Ewijk, van der Kwast, to be published).

Obviously the follicles and the PALS are not strictly segregated areas for B- and T-cells. We have to consider the possibility that recirculating B-cells can traverse the thymus dependent areas, or that T-cells can travel through the thymus independent areas in the spleen. Indeed, T-cells have been demonstrated in follicle-center reactions (Gutman and Weissman 1972).

In conclusion we feel that the IDC is a characteristic cell type for the T-cell microenvironment in peripheral lymphoid organs. Circumstantial evidence suggests that IDC are involved in the transformation of small lymphocytes into medium sized immunocompetent T-lymphocytes. The latter cells are because of their ultrastructural feature thought to be inducer cells, which, in a humoral immune response, can stimulate B-lymphocytes to transform into immunoglobulin producing plasma-cells.

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Migration of B Lymphocytes in Lymphoid Organs of Lethally Irradiated, Thymocyte Reconstituted Mice

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Summary. The migration of radiolabeled intravenously injected B cells was studied in lymphoid organs of mice with clearly defined T cell microenvironments (B cell deprived mice or T mice). In the spleen, B cells were found to enter the peri-arteriolar lymphoid sheath (PALS) by two routes: a) by the marginal zone and b) by reticulin sheaths surrounding terminal arterioles. B cells migrated through the central and peripheral PALS and started the formation of primary follicles in the peripheral PALS from 6 hours after injection. Distinct primary follicles were noted at 18 hours after injection of the labeled B cells. After 24 hours small numbers of labeled cells were also noted in the efferent lymphatics of the spleen.

The reconstitution of B cell compartments in the mesenteric lymph node was delayed compared to the spleen. B cells entered the nodal stroma across the wall of postcapillary venules in the paracortex and by 6 hours were found throughout the paracortex. Isolated clusters of a few labeled cells were noted in the outer cortex at 18 hours after cell transfer. Defined primary nodules were seen only 24 hours after reconstitution. A minority of labeled cells was found at 24 hours in the cortico-medullary junctions and in medullary cords. B cells migrated through thymus dependent areas in both the spleen and the mesenteric lymph nodes, before localizing in B cell compartments. The immunological significance of this particular intralymphoidal migration route of B cells is discussed in view of cellular events during the humoral immune response.

Introduction

Lymphoid organs in rodents contain defined compartments for T and B lymphocytes (8, 14, 15, 16, 17). In the spleen, T cells populate the periarteriolar lymphoid sheath (PALS); in lymph nodes, T cells are found in the paracortex. B cells, on the other hand, localize in the spleen in follicles and non-follicular areas at the periphery of the PALS. In lymph nodes B cells occupy similar compartments in the outer cortex.

In a recent light- and electronmicroscopic study of the humoral immune response in the spleen (van Ewijk, Rozing, Brons and Klepper, submitted for publication), we found that upon intravenous (i.v.) stimulation of germfree mice with sheep red blood cells (SRBC) the first immunoglobulin producing plasmablasts arose in the thymus dependent area, i.e. the PALS. Ford and Gowans (4) have shown that in the isolated perfused spleen, the height of the immune response to SRBC depended on the number of nucleated cells added via the perfusate at the time of antigen addition. Thus, antibody forming cell precursors might be activated to differentiate into antibody forming plasma-cells upon entering the spleen. In view of our histological observations in SRBC stimulated germfree mice we assume that antibody forming cell precursors localize in the PALS. Due to this typical migration and localization pattern these cells may be stimulated by antigen retaining macrophages and activated T cells.

In the present paper we investigated the route of migration of radiolabeled B cells in lymphoid organs of mice with autoradiography, to test whether B cells indeed may localize in thymus dependent compartments. Since in normal mice the exact delineation of T and B cell compartments in lymphoid organs is

not clear (14), we decided to demarcate the thymus dependent compartments experimentally by means of X-irradiation and subsequent reconstitution with syngeneic thymocytes. "T mice" are totally devoid of B cells (3) and develop clearly defined T cell compartments within 6 days after reconstitution (2). The migration of [^{3}H]-leucine labeled B cells was followed in the spleen and mesenteric lymph node of T mice at intervals of 6, 18, 24, 42 and 66 hours after intravenous injection. The immunological significance of B cell migration within lymphoid organs of mice is discussed in view of the humoral immune response.

Materials and Methods

Animals. Male (DBA/2 × C57BL/ Rij) F1 mice, either 4-6 weeks or 15-20 weeks of age were used for the present experiments. They were purchased from the Medical Biological Laboratory, TNO, Rijswijk, The Netherlands.

X-irradiation. Recipient mice received 850 rad whole body X-irradiation from a Philips Möller MG 300 X-ray machine as reported in detail previously (2). Radiation control mice died in 9-16 days.

Preparation of T mice. Fifteen to twenty weeks old mice were lethally irradiated and injected intravenously (i.v.) with 5×10^7 syngeneic thymocytes from 6 weeks old donor mice, within 2 hours after irradiation. These mice were used for further experiments, 6 days after irradiation and inoculation of thymocytes. At that time the thymus dependent microenvironments in lymphoid organs were completely reconstituted and no B cells were detectable when tested with membrane fluorescence (3).

Preparation of B mice. Four to six weeks old mice were thymectomized according to the method reported by Miller (10). Two weeks after surgery they were lethally irradiated and injected i.v. with 5×10^6 syngeneic bone marrow cells, within two hours after irradiation. Before inoculation, the bone marrow cells were treated *in vitro* with anti Θ serum and complement to abolish contaminating T cells. Normal levels of B cells were detectable 4 weeks after reconstitution with bone marrow cells (14). The spleen of these mice was used as a B cell source and contained less than 3% T cells, when tested with membrane fluorescence (3).

B cell reconstituted T mice. B cell reconstituted T mice were prepared by i.v. injection of 5×10^7 spleen cells from B mice into the tail vein of T mice. B cell reconstituted T mice showed survival times similar to lethally irradiated bone marrow protected mice.

Radioactive labeling. Spleen cell suspensions of B mice (B cells) were prepared in phosphate buffered saline (PBS, pH = 7.2, 300 milliosmols). B cells were incubated with [^{3}H]-leucine (TRK. 170, Radiochemicals, Amersham) at a concentration of 10 μCi per 2×10^7 nucleated cells per ml. Incubation was allowed for 2 hours at 37°C, with continuous gentle shaking. The suspension was washed 3 times at room temperature with PBS supplemented with cold leucine. Cell viability was tested by dye exclusion. The final cell concentration was 10^8 nucleated cells per ml.

Preparation of tissues. Lymphoid tissues of T mice and B cell reconstituted T mice were fixed by total body perfusion of the mice with 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Details of the perfusion fixation method have been reported elsewhere (2). Perfusions were performed at intervals of 6, 18, 24, 42 and 66 hours after B cell transfer. The spleen and mesenteric lymph node of the perfused mice were excised, and 1 mm thick slices were cut with a razor blade. The tissues were osmificated, dehydrated and embedded in Epon 812 as described before (2). After polymerization of the Epon 1 μ thick sections were cut with a Reichert OM U III ultramicrotome. The sections were collected on thoroughly cleaned microscope slides.

Autoradiography. The sections were dipped in Ilford K5 emulsion and airdried for 24 hours. The film was exposed for 3 to 4 weeks at 4°C. After developing and fixing, the sections were stained through the emulsion with 1% toluidin blue in 0.1 M borax buffer (pH = 8.6), rinsed in tapwater and distilled water. The air-dried sections were covered with a coverslip using epoxy resin as embedding medium. Autoradiographs were studied both with brightfield and darkfield

illumination. In some micrographs, the darkfield image was superimposed on the brightfield image during photography. Micrographs were recorded with 25× and 40× planapo objectives on Ilford plus X Pan films (22° din).

Results

Reconstitution of the B cell compartment in the spleen of T mice

Details of the architecture of lymphoid organs of T mice have been reported elsewhere (2, 3) and will therefore not extensively be reported here. In short, these mice showed a depletion of the B cell compartments, characterized by a poor cellularity of follicular structures in the spleen and lymph nodes. Furthermore, in the spleen of T mice, the marginal zone was poorly structured, and the medullary cords of lymph nodes were depleted of lymphocytes and plasma cells.

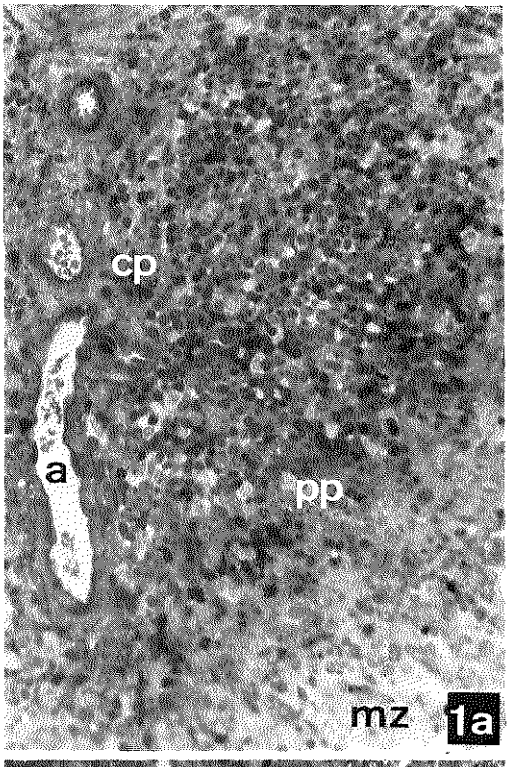
In our experiments we followed the localization pattern of radiolabeled B cells in the spleen and mesenteric node of T mice at intervals of 6, 18, 24, 42 and 66 hours after B cell transfer.

At 6 hours after reconstitution labeled cells were found in the red pulp, the marginal zone, the periarteriolar lymphoid sheath (PALS) and around the finest ramifications of terminal arterioles. In the PALS most of the label was confined to the peripheral part. A distinct minority of radiolabeled cells, however, also occurred in the central part of the PALS (Fig. 1a, b).

18 Hours after cell transfer, most of the labeled cells had left the marginal zone. Accumulations of labeled cells were noted around terminal arterioles, and distinct primary follicles were now observed at the peripheral site of the PALS (Fig. 2a, b). Follicular lymphocytes were small darkstaining lymphocytes (3.5-5.5 μm), whereas unlabeled lymphocytes in the PALS had a less condensed nucleus and were generally medium-sized (6-8 μm). Distinct marginal sinuses marked the outer border of these primary follicles. Next to the endothelial, adventitial and reticulum cells we noted the presence of a few mature plasma cells in the primary follicles. Cells which had a large clear nucleus with distinct small nucleoli were also noted in primary follicles. The morphology of these cells was similar to dendritic cells (DC), as observed in germinal centers of normal mice (14, 15). At 18 hours after injection of the B cells a definite number of labeled cells was still noted in the central part of the PALS.

At 24 hours after reconstitution the morphology of the white pulp was comparable to that at 18 hours after reconstitution, except that primary follicles had increased in size. The marginal zone was now largely depleted of labeled cells, however small accumulations of labeled cells were found around terminal arterioles (Fig. 3). Labeled cells were also observed in lymphatics close to branches of the central artery (Fig. 4 a, b).

During the next period, the number of labeled cells in the peripheral PALS further increased, resulting in a band of primary follicles along the peripheral part of the PALS. The number of labeled cells in the marginal zone had decreased when compared to that seen at 6 and 18 hours after reconstitution. Labeled cells were always found around terminal arterioles, also at the later intervals of 42 and 66 hours after injection of the B cells.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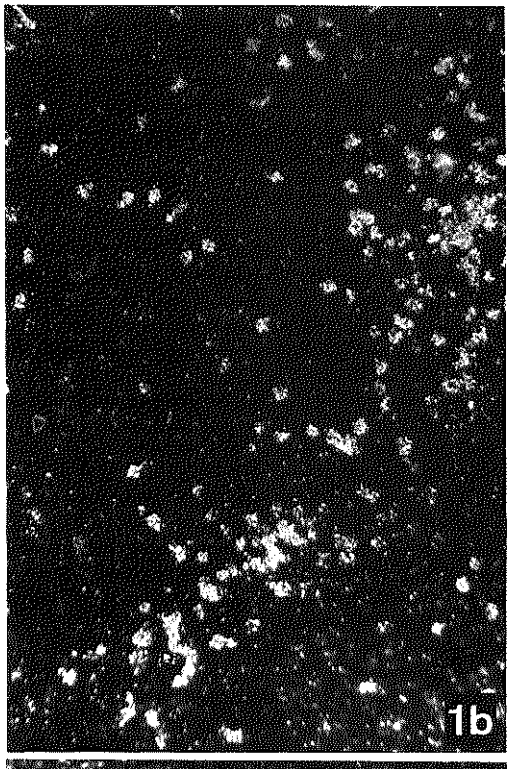
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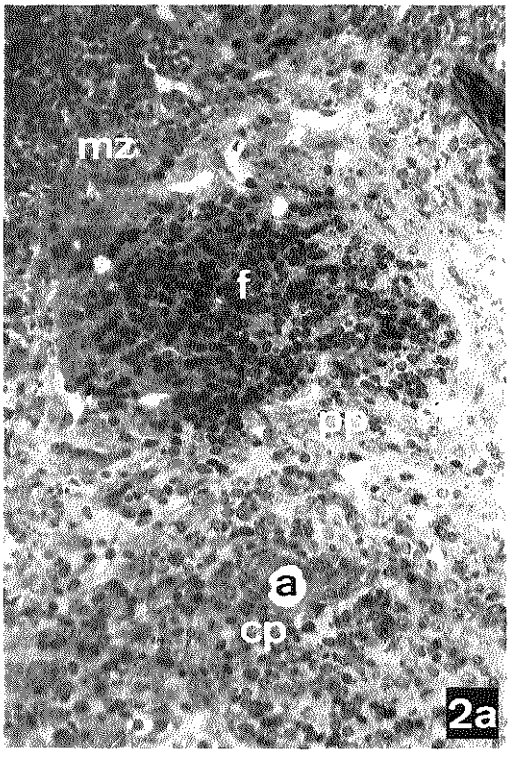
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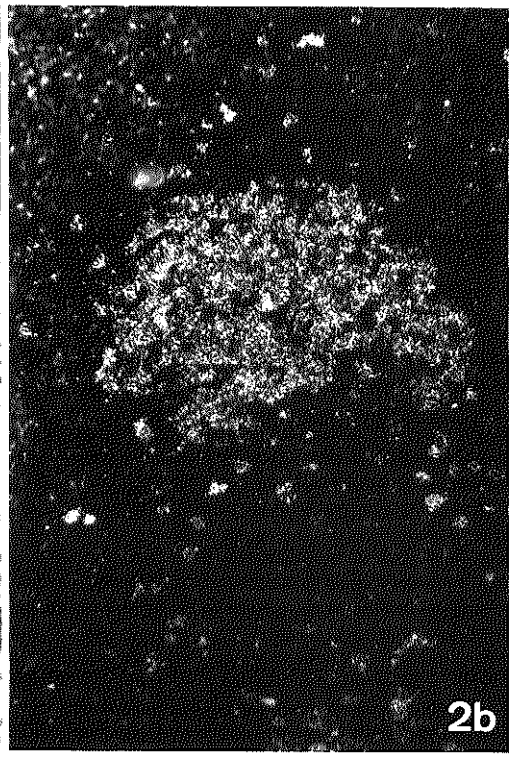
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Reconstitution of the B cell compartment in the mesenteric lymph node of T mice.

The reconstitution of the B cell compartment in lymph nodes was slower than in the spleen. By 6 hours after transfer of B cells, labeled cells were noted in the wall of postcapillary venules (PCV) and some labeled cells were scattered throughout the paracortex (Fig. 5 a, b). There was no preferential site of entry for B cells. They left the PCV all along the paracortex, and even in the medullary cords.

Initial formation of primary follicles was not seen before 18 hours after reconstitution. Isolated small clusters of labeled cells were found in the outer cortex under a layer of sinus-lining cells, adventitial cells and macrophages. As in the spleen we noted the nuclei of dendritic cells in this region. Sometimes mature (unlabeled) plasma cells were noted within primary follicles.

Distinct primary follicles were noted 24 hours after i.v. injection of the B cells (Fig. 6 a, b) and their size increased during the following period. Labeled cells were also noted at the cortico-medullary junction and in the medullary cords after 24 hours. Furthermore, they were noted in lymphatics in the superficial layer of the paracortex by this time. These lymphatics drain lymphocytes from the cortical region towards the medullary sinus (van Ewijk, unpublished observation). During the entire period of observation, labeled cells were noted in the wall of PCV as well as diffusely distributed throughout the paracortex.

Discussion

The purpose of the present investigation was to study the reconstitution of B cell compartments in lymphoid organs of mice with well defined T cell microenvironments and to follow the migration pathway of labeled B cells during reconstitution. In normal mice, small numbers of B cells have been

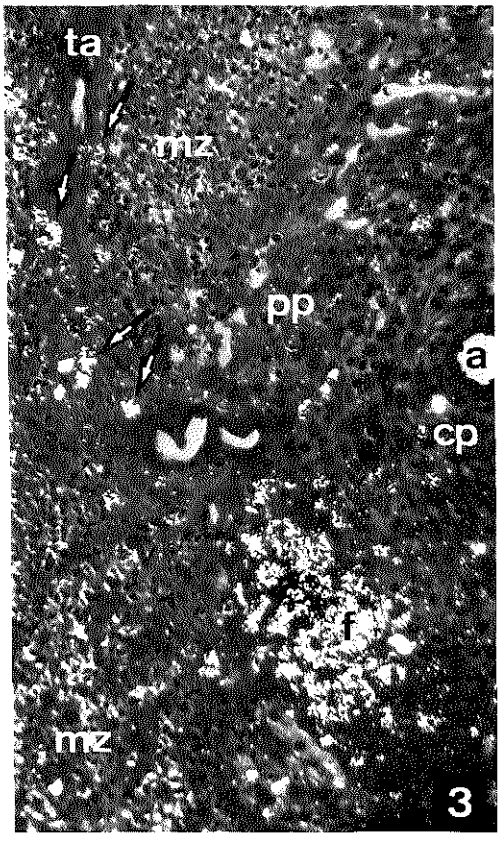
← Fig. 1a. Splenic white pulp of a T mouse, 6 hours after i.v. injection with (^{3}H)-leucine labeled B cells. *a* = central arteriole, *cp* = central PALS, *pp* = peripheral PALS, *mz* = marginal zone. Bright field, × 300.

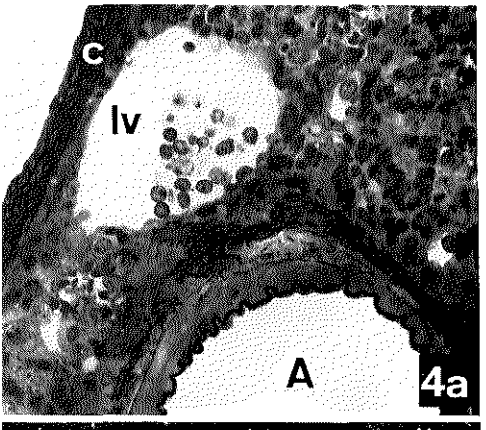
Fig. 1b. Dark field image of the same section as shown in Fig. 1a. The dark field image clearly shows the distribution of the autoradiographic grains. Labeled cells occur in the marginal zone, the peripheral and central PALS.

Fig. 2a. Splenic white pulp of a T mouse, 18 hours after reconstitution with (^{3}H)-leucine labeled B cells. A primary follicle (*f*) formed by small dark staining lymphocytes is located in the peripheral PALS (*pp*). The marginal zone (*mz*) is bordered from the follicle by distinct marginal sinuses. Lymphocytes in the central and peripheral PALS (*cp, pp*) show nuclei with generally a less condensed chromatin structure. *a* = central arteriole. Bright field, × 300.

Fig. 2b Dark field image of the same section as shown in Fig. 2a. Most of the labeled cells are localized in the primary follicle. A minority of labeled cells is scattered throughout the central and peripheral PALS. Very few labeled cells occur in the marginal zone at 18 hours after injection.

reported to be present in what is known as "thymus-dependent areas", like the peripheral part of the PALS in the spleen and the paracortex in lymph nodes (11). The role of this particular minority of B cells was uncertain until Nieuwenhuis and Ford (13) demonstrated that in rats these cells belonged to a population of migrating cells. Ford and Gowans (4) and Ford (5) have shown that in the isolated perfused spleen of rats the height of the immune response to SRBC was dependent of the number of lymphocytes which recently had migrated from the blood into the splenic white pulp. In a light-, fluorescence- and electronmiscroscopic study reported elsewhere (van Ewijk, Rozing, Brons and Klepper, submitted for publication) we followed the cellular events during





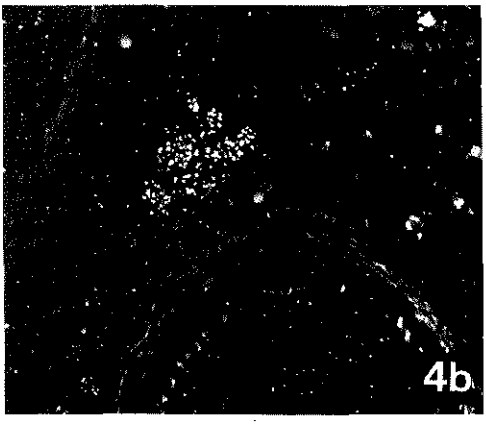


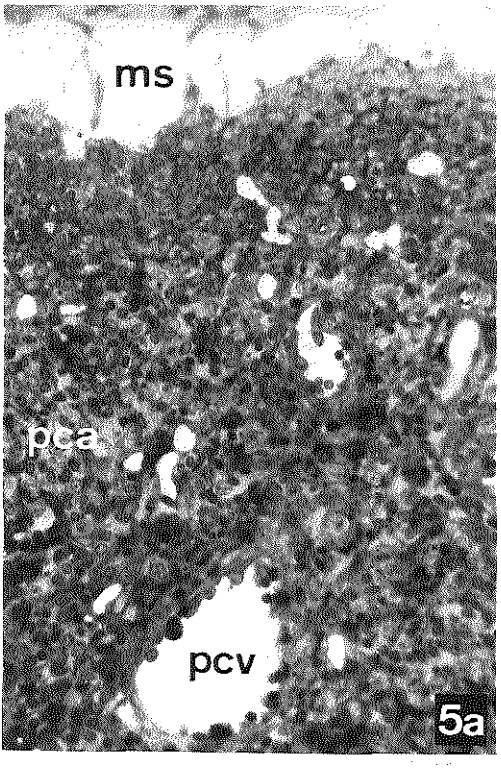
Fig. 3. Splenic white pulp, 24 hours after reconstitution with (^{3}H)-leucine labeled B cells. Labeled cells have left the marginal zone (*mz*). They occur around terminal arterioles (ta, see arrows) and in the peripheral and central PALS (*pp, cp*). A primary follicle (*f*) can be noted in the peripheral PALS. *a* = central arteriole. The dark field image is superimposed on the bright field image. × 300.

Fig. 4a. Splenic artery (*A*) and lymph vessel (*lv*) in the spleen of a T mouse, 24 hours after reconstitution with (^{3}H)-leucine labeled B cells. *c* = splenic capsule. × 300.

Fig. 4b. Dark field image of the same section as Fig. 4a. This micrograph shows the presence of labeled B cells in a lymph vessel, at 24 hours after injection.

the immune response to SRBC in the spleen of germfree mice and noted that the first plasmablasts originated in the peripheral PALS. This area is known to be thymus dependent (11, 14), but it always contained a few fluorescent B lymphocytes. From the above mentioned data one can argue that it is likely that also in mice a population of migrating B cells exists in the peripheral PALS, which contains antibody forming cell precursors. The peripheral PALS in the spleen appears to be an ideal area for the cellular cooperation during the immune responses between B cells, T cells and non-lymphoid cells since these cell types are all present in close proximity to each other. The present data support this hypothesis and indicate that in mice B cells in the PALS do belong to a migrating pool of lymphocytes.

B cells were found to enter the PALS via two routes: a) via the marginal zone, (cf. Ford, 5, Goldschneider and Mc Gregor, 6) and b) via reticulum sheaths surrounding terminal arterioles (cf. Nieuwenhuis and Ford, 13). Both entrance pathways were evident 6 hours after cell transfer, however, during the progress of the reconstitution period the first pathway seemed to become less important, since by 18 hours the marginal zone was nearly depleted of labeled cells. In contrast to the marginal zone, we found always a more or less constant number of labeled cells around terminal arterioles during the period of investigation. Since the number of i.v. injected labeled B cells in the peripheral blood stayed at a constant level during the first 18 hours (9), and since primary follicles increased in size during this period, it may be concluded that B cells continuously migrated into the PALS along the terminal arterioles during reconstitution. A number of labeled cells was always found in the central part of the PALS, which in normal mice and rats is known to be strictly thymus dependent. This finding contrasts earlier reported observations on B cell migration in normal rats, where B cells were only noted in the peripheral part of the PALS (13). In this context it may be noted that the white pulp architecture in rats and mice is different. In rats the reticulin fiber pattern in the peripheral part of the PALS is more conspicous than in mice, and in rats follicles are more clearly demarcated from the peripheral part of the PALS (14). One can assume that labeled cells located in the central PALS were contaminating T cells in the i.v. injected labeled spleen cell suspension of B mice. However, we have shown that the percentage of T cells in spleens of B mice was only 3 per cent (3), which is far too low to account for the number of centrally located labeled cells, especially those seen at 6 hours after reconstitution. The ratio of labeled cells in the central and peripheral part of the PALS was at this time roughly estimated at 1 : 2, (see also Fig. 1b). Probably B cells traverse the central PALS on their way to either the peripheral PALS, or to efferent lymphatics situated near the central artery (1, 14). Immunofluorescence studies on B cell reconstitution in lethally irradiated, fetal liver reconstituted mice (Rozing, Brons, van Ewijk and Benner, submitted for publication) indicated a similar migration pattern: Initially, 8 days after reconstitution, B cells were located within the central PALS, and formed small primary follicles at the periphery of the PALS at 11 days after injection of fetal liver cells. The presence of B cells in the central PALS probably also indicates an outflow of B cells from the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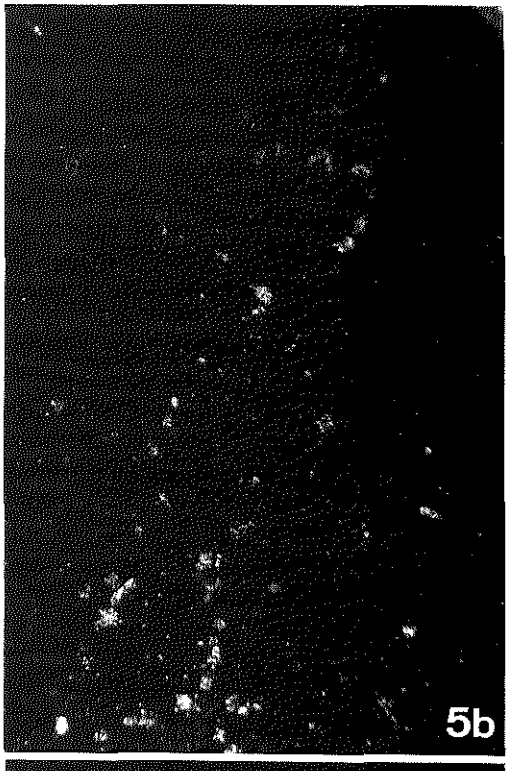
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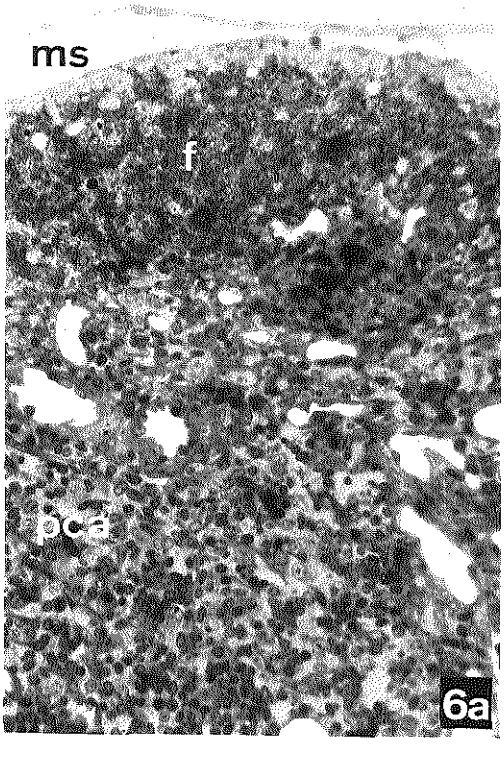
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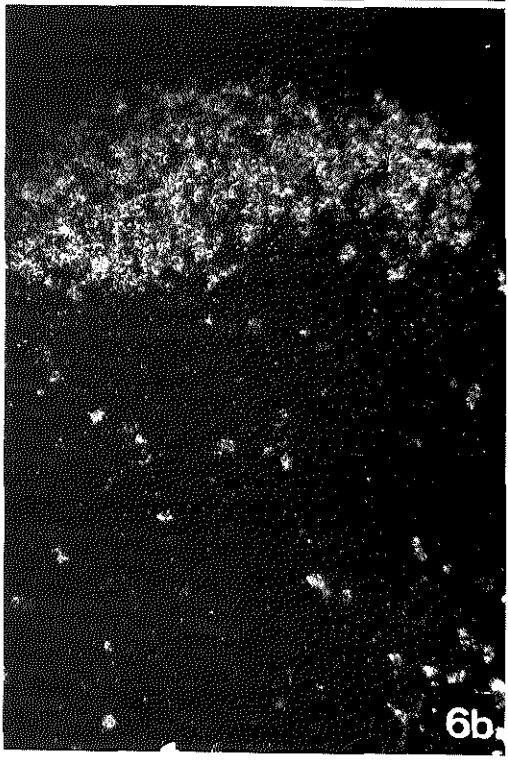
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PALS, since labeled cells were found in lymph vessels by 24 hours after injection (Fig. 4 a, b).

In lymph nodes of T mice, the reconstitution of primary nodules was delayed when compared to the spleen. In the spleen small foci of labeled cells were already seen by 6 hours, whereas in the mesenteric node these foci were not noticed until 18 hours after i.v. injection of B cells. A similar time difference in "B cell homing" has been described in the spleen and lymph nodes of normal mice (9) and rats (13).

B cells in the same way as T cells entered the lymph nodes across the endothelium of postcapillary venules, confirming our previously reported data on B cell migration in PCV of B mice (3) and those of other authors in normal mice (9) and rats (13). We could not determine a preferential site of entrance for B cells in the nodal stroma; labeled cells were found passing the endothelium of PCV in the paracortex and even in the medullary cords. As a result of this typical migration pattern, B cells were found scattered throughout the thymus dependent paracortex of the node.

The nature of the cell types which determine the ultimate homing of B cells in their specific compartments is at present not clearly known. Several cell types could play a role, such as reticulum cells, adventital cells, macrophages and dendritic cells. Dendritic cells (DC) are prominent in germinal centers and do not occur in T cell compartments (14, 15). They occur in close proximity to proliferating B cells and may be regarded as cells specific for B cell microenvironments. These DC can also occur in primary follicles, preliminary electronmicroscopic analysis of these areas, however, showed DC with a less extended cytoplasmic web as compared to DC in germinal centers.

We conclude from the present study that a) B cells entering the spleen migrate into the PALS via two routes: the marginal zone and the reticulin sheaths surrounding the terminal arterioles. b) The latter population of B cells may traverse T cell microenvironments, either on their way to B cell microenvironments, or on their way towards efferent lymphatics. c) B cells in the mesenteric node enter the lymphatic stroma along the total length of PCV, and migrate across the thymus dependent paracortex towards B cell microenvironments. d) B cell reconstitution in lymph nodes is delayed when compared with the spleen. e) The rate of B cell migration is independent of the presence

← Fig. 5a. Mesenteric lymph node of a T mouse, 6 hours after reconstitution with (^{3}H)-leucine labeled B cells. *ms* = marginal sinus, *pca* = paracortical area, *pcv* = post capillary venule. × 300.

Fig. 5b. Dark field image of the same section as shown in Fig. 5a. Labeled cells occur in the high endothelial wall of post capillary venules and are scattered throughout the paracortex.

Fig. 6a. Mesenteric lymph node of a T mouse, 24 hours after reconstitution with (^{3}H)-leucine labeled B cells. A primary follicle (*f*) is located in the superficial cortex. *ms* = marginal sinus, *pca* = paracortical area. × 300.

Fig. 6b. Dark field image of the same section as shown in Fig. 6a. Labeled cells have formed a primary follicle. They also occur in the wall of post capillary venules and are diffusely scattered throughout the paracortex.

of a resident population of B cells, since the transit times in T mice and in normal mice (9) and rats (13) are the same. f) The particular route of migration of B cells through T cell microenvironments may be of importance in immune responses which require the cooperation between B and T cells.

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Cellular Events during the Primary Immune Response in the Spleen

A Fluorescence-, light-, and electronmicroscopic Study in Germfree Mice

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Summary. The cellular events during the primary immune response in T and B cell compartments in the splenic white pulp were analysed in germfree mice immunized with sheep erythrocytes. Light-, fluorescence-, and electronmicroscopic studies revealed that initial formation of lymphoid blast cells occurred in the thymus-dependent area, i.e. the central periarteriolar lymphatic sheath (central PALS), 2 days after immunization. Lymphoblasts were found in close relation with erythrocyte containing macrophages and with interdigitating cells. With fluorescence microscopy these blast cells were Ig negative. Lymphoblasts in the central PALS showed many polyribosomes in the cytoplasm, but were virtually devoid of endoplasmic reticulum. The ultrastructure of lymphoblasts in the central PALS, and their relation with interdigitating cells, suggests that these cells are the progeny of antigen-activated T cells.

Cells with a positive cytoplasmic fluorescence, plasmablasts, appeared 3 days after immunization in the peripheral part of the PALS. During the progress of the immune response these cells accumulated around branches of the central arteriole, and moved along marginal zone bridging channels towards the red pulp. In the electronmicroscope plasmablasts showed many polyribosomes, short strands of rough endoplasmic reticulum near to mitochondria, and a few electron-dense bodies. The cell organelles of plasmablasts were frequently gathered in a so called "uropod", which is a morphological sign of active cell movement.

Germinal center formation started within primary follicles, 4 days after immunization. Blast cells in germinal centers did not show cytoplasmic fluorescence. During the course of the immune response, germinal centers extended in diameter, and fluorescent dendritic cells appeared at the peripheral site of the center.

From the present observations we concluded that: (1) the cellular cooperation between different lymphoid and non-lymphoid cell types during the immune response against SRBC resides in the PALS, (2) the cellular cooperation in the PALS results in the differentiation of B cells into immunoglobulin producing plasmablasts, (3) the cellular cooperation in the PALS preceeds the formation of germinal centers in primary follicles, hence germinal centers are not involved in early T-B cell cooperation.

Introduction

In rodents, the lymphoid part of the spleen, i.e. the white pulp, is organized around branches of the splenic artery, and can be divided in three areas: (a) the periarteriolar lymphatic sheath, (PALS), (b) follicles located at the periphery of the PALS and (c) a surrounding marginal zone (Weiss, 1972). T and B lymphocytes both enter the white pulp via the marginal zone (Ford, 1969; Nieuwenhuis and Ford, 1976) which consists of a meshwork of connective tissue cells to which the cells of the peripheral blood have free access via capillaries branching from the central artery. Upon entering the white pulp, cells of the lymphoid subclasses segregate each in distinct microenvironments: T cells home in the central part of the PALS (Waksman et al., 1962; Parrot et al., 1966), B cells occupy the peripheral PALS and the surrounding follicles (Sprent, 1973).

In two previous reported studies we have analysed at the ultrastructural level the T cell microenvironment in the white pulp of the spleen of irradiated,

reconstituted mice (Van Ewijk et al., 1974), and both the T and B cell microenvironment in the spleen of normal mice and rats (Veerman and van Ewijk, 1975). We found that similarly to lymph nodes (Veldman, 1970), the T cell microenvironment was characterized by the presence of reticular cells with a particularly low electrondensity, so called interdigitating cells (IDC), whereas the B cell microenvironment was characterized a.o. by very fine cytoplasmic ramifications of dendritic cells (DC). Both cell types showed prolonged cytoplasmic processes in close contact with surrounding lymphocytes.

The present paper reports the cellular events during antibody formation in the splenic white pulp of germfree mice in relation to the architecture of the T and B cell microenvironments, and attempts to define the morphological substrate of T-B cell interaction during the antibody response. We choosed for germfree mice since these mice show a very low background stimulation of their lymphoid tissues. We analysed the white pulp of mice immunized with sheep red blood cells (SRBC), using 1μ toluidin blue stained sections of Epon embedded tissue. This material guarantees a high resolution in the light microscope and provides the possibility for subsequent electronmicroscopic studies of prelocated areas of interest. To correlate these data with antibody production, we collected the serum and we examined splenic tissue of the same mice with the immunofluorescence technique of Gutman and Weissman (1972). This technique enables the immunological detection of T and B cells in frozen sections of lymphoid tissue, by virtue of their characteristic membrane antigens. Furthermore it provides insight in microenvironments where plasma cells originate during the immune response. From these studies we present evidence that, following intravenous stimulation of mice with a thymus dependent antigen, the microenvironment for the interaction between macrophages, T and B cells is located in the periarteriolar lymphatic sheath.

Materials and Methods

Mice. Germfree ND_2 male and female mice, 15-20 wk of age and weighing 25-35 g were purchased from the Radiobiological Institute, REPGO-TNO Rijswijk, The Netherlands. Before immunization, mice were transferred under sterile conditions from their respective germfree plastic isolators to laminar flow cabinets. During immunization and the following immune response period, mice were kept in the laminar flow cabinets with free access to sterile food and water.

Immunization. Mice were immunized intravenously (i.v.), with a sterile suspension of 4×10^8 sheep red blood cells (SRBC) in 0.5 ml phosphate buffered saline (PBS). As control, mice received 0.5 ml PBS i.v.

Preparation of tissues. Two experiments were carried out. In the first experiment, groups of 4 mice were sacrified at 12 hr intervals until 5 days after antigenic stimulation. Fixation of the spleen was carried out under Nembutal anaesthesia by total body perfusion of oxygenated PBS (pH = 7.2, 300 milliosmols), for 30 sec, followed by 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH = 7.2, 385 milliosmols) for 5 min. Details of this technique have been described elsewhere (Van Ewijk et al., 1974). The perfusion-fixed spleen was excised, slices of splenic tissue were post-fixed in 1% osmiumtetroxide in 0.1 M sodium cacodylate buffer, dehydrated in graded series of ethanol and embedded in Epon 812. After polymerization of the Epon, 1 μ sections were cut with a Reichert OM U III ultramicrotome. Sections were stained with 1% toluidin blue adjusted to pH = 8.6 with 0.1 M borax buffer. Areas of interest for ultrastructural analysis were selected in the last cut 1 μ section and relocated on the surface of the corresponding Epon block with a razor blade. In this way, cells observed in ultrathin sections could always be orientated in the complex white pulp architecture. Ultrathin sections were made with glass knives and collected on 300 mesh

copper grids or on 75 mesh copper grids covered with a formvar film. They were examined in a Philips EM 300 electron microscope, operated at 60 kV with a 20 μ objective aperture.

In the second experiment, groups of 4 mice were fixed at 24 hr intervals up to 4 days after stimulation. Also groups of mice were fixed 7, 12 and 15 days after antigenic stimulation. In this experiment we prepared material for light microscopy, electron microscopy and immunofluorescence. The serum of each mouse was collected for determination of haemagglutinin titers.

From each mouse 1 ml blood was obtained from the incised right atrium by adspiration with a capillary pipet at the start of the perfusion. Blood was stored overnight at 4°C. After centrifuging off the fibrin clot, serum was collected in plastic tubes and stored at −20°C. Haemagglutinintiters to SRBC were determined from each mouse using standard methods. Both mercapto-ethanol sensitive and mercapto-ethanol resistant titers were determined.

After perfusion with PBS for 2 min, the spleen was excised. Slices of about 1 mm thickness were cut with a razor blade and fixed in 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Further preparation of this material was carried out as described above.

For immunofluorescence, we employed the technique described by Gutman and Weissman (1972). Fresh slices of lymphoid tissues perfused with PBS were impregnated in Tissue-Tek II (Division Miles Laboratories, Naperville, Illinois, U.S.A.) and mounted in gelatin capsules (Eli Lilly and Company, Indianapolis, U.S.A. nr. 00). The capsules were then frozen on solid carbon dioxide and stored at −70°C. Prior to sectioning, the gelatin capsule was removed with a razor blade. The frozen specimen was mounted on a specimen stage with fresh Tissue-Tek and cut with a Bright 5030 rocking microtome (Bright Instrument Company Ltd., Huntingdon, England), at −25°C. Serial 5 μ thick sections were collected on thoroughly cleaned microscope slides. The sections were fixed immediately by dipping the slides less than one second in distilled acetone. The air dried sections were stored at 4°C.

to visualize B lymphocytes and plasma cells, an indirect immunofluorescence technique was used. Sections were incubated at room temperature with rabbit-anti-mouse-Ig antiserum (RAM-Ig) (Nordic Laboratories, Tilburg, The Netherlands) for 20 min. as a first step, and washed three times with PBS (pH = 7.8) for another 20 min. Next, the sections were incubated with goat-anti-rabbit-Ig conjugated to rhodamine (TRITC-GAR-Ig) (Nordic Laboratories, Tilburg, The Netherlands) for 20 min., and again washed three times with PBS. The incubated sections were covered with buffered glycerol (9 parts glycerol and 1 part PBS, pH = 7.8), a coverslip was applied and the edges were sealed with paraffin. The slides were examined with a Zeiss fluorescence microscope equipped with a vertical illuminator IV/F and an Osram HBO-50 mercury lamp as a light source. Black and white photographs were taken on Kodak Plus X Pan Films (22 DIN) with exposure times ranging from 30 to 60 seconds.

Results

Serum titers

To correlate the cellular events in the spleen with the presence of circulating antibodies during the immune response to SRBC, we collected the serum of each mouse at the immediate start of the perfusion. As shown in Fig. 1, the IgM response started between the second and third day after intravenous stimulation of mice with SRBC. The peak IgM serum titer probably occurred between the 4th and 7th day. After the 7th day a decrease in the IgM titer was observed, followed by an increase in the period from day 12 onwards. IgG serum hemagglutinin to SRBC was detectable in a very low concentration on day 4. The IgG titer increased during the next days, decreased slightly between day 7 and day 12 and subsequently surpassed the 7th day level on day 15.

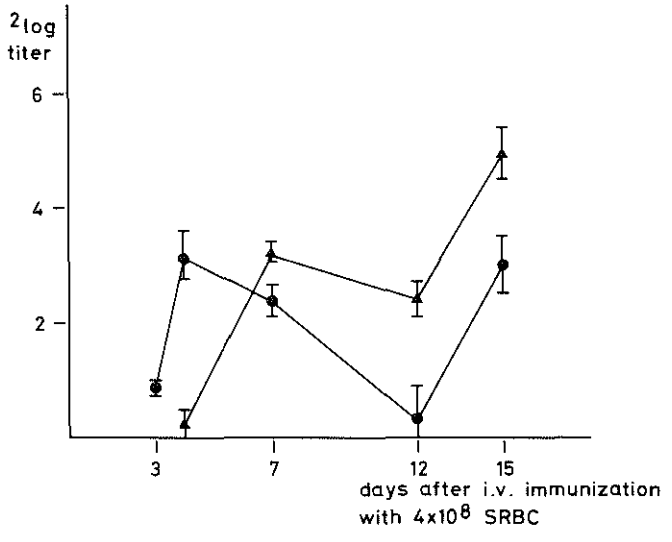


Fig. 1. IgM haemagglutinin titer ⊢●⊣ and IgG haemagglutinin titer ⊢▲⊣ in germfree mice, stimulated with 4×10^8 SRBC i.v. The vertical bars represent the standard error of the mean.

Light microscopy

1 μ Toluidin blue stained Epon sections of the spleen of unstimulated germfree mice showed the lymphoid tissue organized around branches of the central arterioles. Details of the architecture of the white pulp have been dealt with before (Veerman and van Ewijk, 1975), therefore we will not extensively consider this subject here. There were two main differences with respect to normal conventional mice: (a) germinal centers were not observed and (b) lymphoid blast cells occurred in very low numbers in the PALS of germfree mice. The quiescent state of the lymphoid tissue of germfree mice permits therefore an accurate localization of the first appearing lymphoid blast cells during the immune response upon antigenic stimulation.

12 Hours after immunization, many macrophages with phagocytized erythrocytes were observed in the red pulp and the marginal zone. Macrophages, located in the margin of follicles and the peripheral PALS were sometimes seen trapping erythrocytes in the lumen of vascular sinuses of the marginal zone (Fig. 2).

The PALS and follicles were initially devoid of erythrocyte containing macrophages. However, it was noted that from 36 hr onwards erythrocyte containing macrophages appeared in the PALS. Blast transformation of lymphoid cells was first noted 48 hr after stimulation. Lymphoblasts measuring about 12 μ appeared in the central PALS always in association with macrophages (Fig. 3) and with processes of interdigitating cells (IDC) (Fig. 4). By this time also mitotic figures were found in the central PALS.

Between two and three days after stimulation, very large blast cells (about 15 μ), presumably plasmablasts, were noted in the PALS (Fig. 5, 16, 19). These

blast cells were scattered through the peripheral PALS and occurred frequently in close proximity with macrophages. With the light microscope short profiles of rough endoplasmic reticulum could be observed in the cytoplasm, and the pale staining nucleus contained a prominent nucleolus. Groups of plasmablasts were also situated in between reticular sheaths around terminal arterioles, crossing the marginal zone. Here many cells in mitosis were found (Fig. 6). These reticular sheaths formed a direct pathway for plasmablasts from the PALS to the red pulp. Mitchell (1973) called these routes "marginal zone bridging channels".

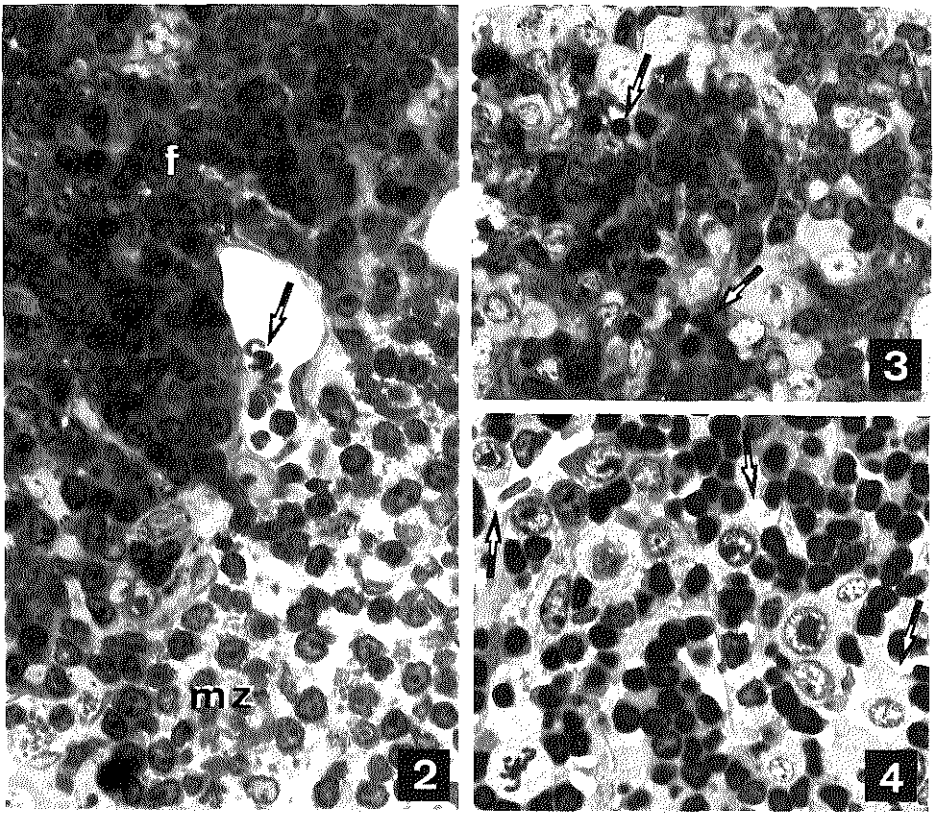


Fig. 2. Trapping of erythrocytes by a macrophage located in the margin of a follicle (arrow), 12 hr after immunization. *f* = follicle, *mz* = marginal zone (×850).

Fig. 3. Central PALS, 48 hr after immunization. Lymphoblasts occur in the neighbourhood of erythrocyte loaded macrophages. The latter cells are indicated by arrows (×850).

Fig. 4. Central PALS, 48 hr after immunization. Lymphoblasts measuring about 12 μ are present in close juxtaposition with cytoplasmic processes of interdigitating cells (arrows). Note the mitotic lymphoblast in the lower left corner of the micrograph (×850).

4 Days after stimulation, the number of plasmablasts was increased both in the PALS and in marginal zone bridging channels. By this time initial germinal center formation was noted: small clusters of blast cells and mitotic cells occurred within the primary follicles of some of the mice (Fig. 7). These clusters of blasts were surrounded by small, darkly staining lymphocytes, and frequently the nucleus of a dendritic cell was observed (Fig. 9). The clusters were outlined by cytoplasmic processes of reticulum cells.

On day 7, the majority of the plasmablasts had disappeared from the PALS, only a few mature plasma cells were found near large branches of the central artery. Most of the plasma cells were now located in the red pulp. Germinal

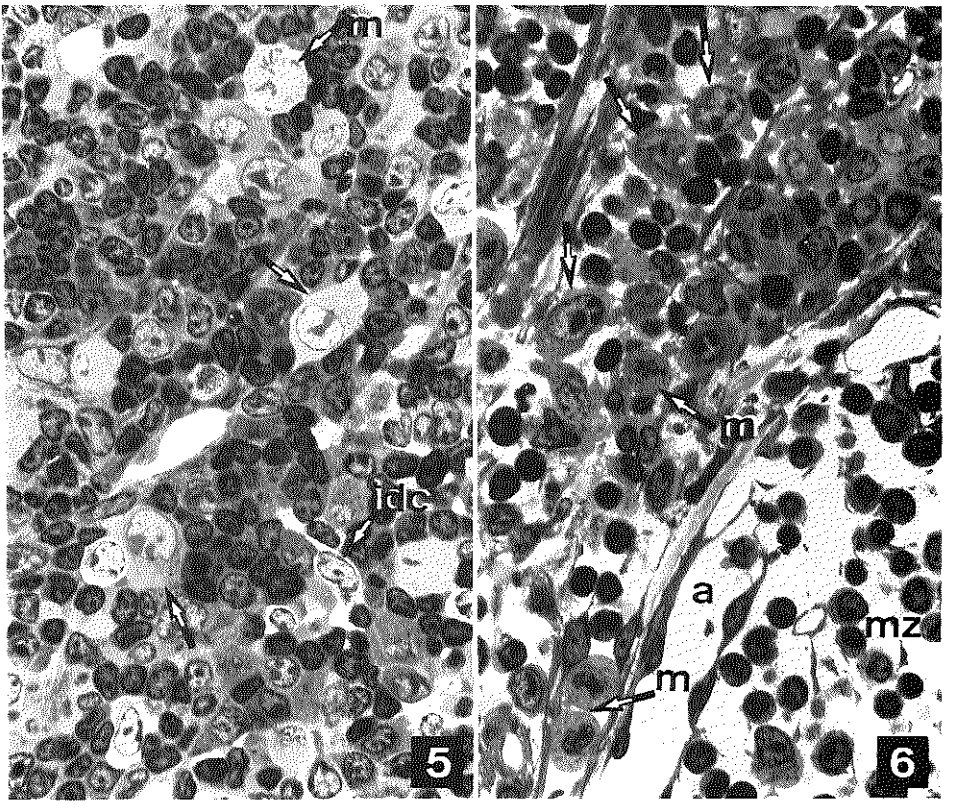


Fig. 5. PALS, 72 hr after immunization. Large lymphoid blast cells, about 14 μ in diameter (arrows), presumably plasmablasts, are found in between reticular sheaths of the peripheral PALS. Note the large nucleolus and the strands of rough endoplasmic reticulum in the plasmablasts. The interdigitating cell (*idc*) is situated in the central PALS. *m* = mitotic plasmablast. (×850).

Fig. 6. Marginal zone bridging channel, 72 hr after immunization. Plasmablasts (arrows) occur in between the fine reticular sheaths accompanying a terminal arteriole (*a*). Note the mitotic plasmablasts (*m*), *mz* = marginal zone (× 850).

centers occurred in follicles of each observed mouse and contained dendritic cells as well as tingible body macrophages. At 15 days after stimulation, the germinal centers were very prominent (Fig. 8), they measured about 200 μ, and contained many cells in mitosis, large tingible body macrophages and dendritic cells (Fig. 10). The number of plasma cells in the PALS was further decreased to values comparable with control mice.

Immunofluorescence

Frozen, dried sections of the spleen of control germfree mice, incubated with RAM-Ig and TRITC-GAR-Ig showed positively stained primary follicles and marginal zones (Fig. 11). The PALS did not stain with this method, which indicated that the labeling was specific for Ig-determinants. All B cells in the follicles showed a membrane fluorescence of more or less equal intensity, however, cells in the marginal zone were more strongly fluorescent than cells in follicles (Fig. 11, 12). This phenomenon was noticed in all mice, whether immunized or not. B cells were also noted in low numbers in the peripheral PALS (Fig. 12). Consequently, the peripheral PALS is not exclusively a thymus dependent area. Plasma cells, showing a bright cytoplasmic fluorescence, occurred in the red pulp. Only very few of them were found in the white pulp. If present, they were located close to the large branches of the central artery.

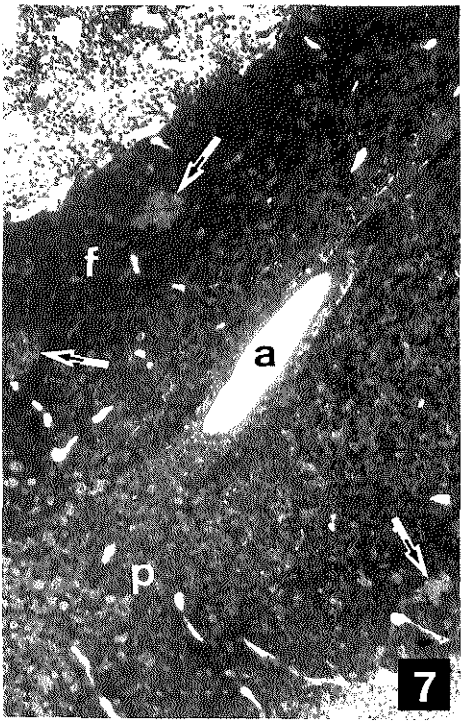
The first increase in the number of cells with cytoplasmic fluorescence in the white pulp was noted 3 days after stimulation. These cells initially occurred in the peripheral part of the PALS, and were frequently seen in the neighbourhood of particle containing macrophages, as judged by phase contrast studies of the same section. The appearance of cells with a cytoplasmic fluorescence was correlated with the presence of the large lymphoid blast cells in the PALS as observed in 1 μ Epon sections, and with the appearance of IgM antibodies in the peripheral blood. It seems therefore likely to assume that these cells are IgM producing plasmablasts.

Four days after stimulation, the number of these plasmablasts was further increased and now most cells were situated around branches of the central artery (Fig. 13) and in marginal zone bridging channels. It must be noted that not all parts of the splenic white pulp responded synchronously with the formation of plasmablasts; the white pulp of stimulated mice also showed regions devoid of plasmablasts and as such comparable to the splenic white pulp of control mice, even at 4 days after antigen administration.

As in the lightmicroscopic sections, we noted in some mice on day 4 the start of germinal center reactions: small foci of non-fluorescent blast cells were observed within positive staining follicles (Fig. 14).

7 Days after stimulation, the number of plasma cells in the PALS had returned to normal values, while brightly fluorescent cells were now observed mostly in the red pulp. Apparently the plasma cells had moved out of the PALS towards the red pulp during the progress of the immune response. At this time, germinal centers were observed in spleen sections of each mouse.

Germinal centers were prominent at 12 and 15 days after stimulation and showed always a cuff of dendritic cells at the peripheral site of the germinal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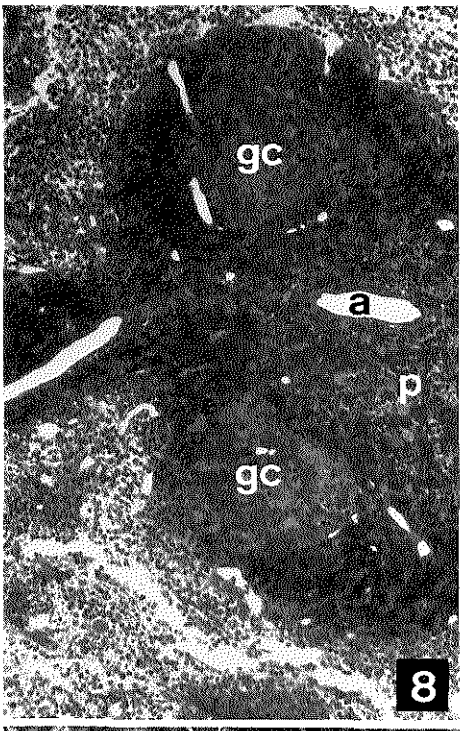
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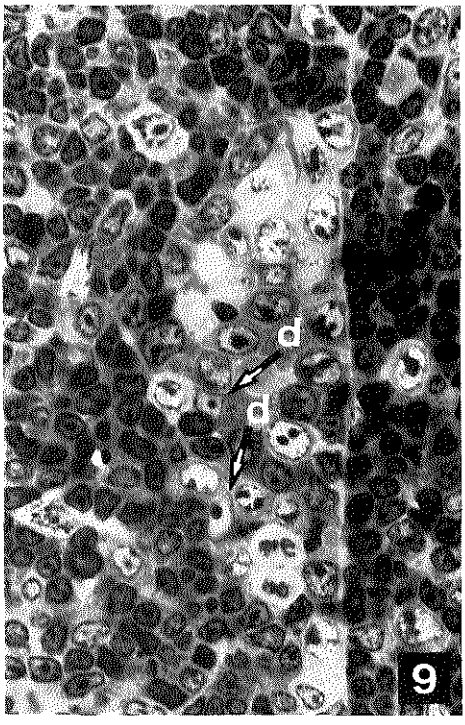
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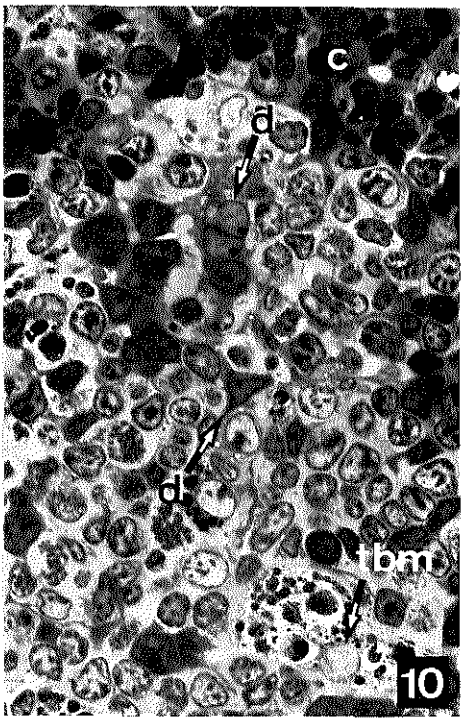
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center (Fig. 15). A few plasma cells in the white pulp were present around central arterioles. They did not occur in follicles, nor in germinal centers or in the marginal zone.

Electron microscopy

Since blast transformation initially started in the PALS, we decided to study this area on closer inspection with the electron microscope, attempting to analyse the ultrastructure of cell types involved in the immune response to SRBC. Submicroscopic analysis of the PALS, 3 days after immunization revealed the following non-lymphoid cell types: (a) reticulum cells, forming the basic frame work of the white pulp, (b) interdigitating cells (IDC), occurring only in the central PALS, they were characterized by their translucent cytoplasmic extensions (Fig. 17, 18) and (c) macrophages, containing phagosomes with degradation products of erythrocytes (Fig. 18). 4 Types of lymphoid cells stayed within the meshwork of non-lymphoid cells: (a) small, electrondense lymphocytes (mean diameter = 4 μ), (b) medium-sized, moderate electrondense lymphocytes (mean diameter = 6 μ), (c) lymphoid blast cells and (d) mitotic cells (Fig. 17, 18). Since the ultrastructural features of most cell types have been previously reported (van Ewijk et al., 1974; Veerman and van Ewijk, 1975), we will mainly focus on lymphoid blast cells.

Blast cells were scattered through the central and the peripheral PALS. Blast cells in the central PALS measured about 12 μ and were frequently found in contact with IDC (Fig. 17, 18). They showed abundant polyribosomes but very few strands of rough endoplasmic reticulum (RER) and resembled closely the T lymphoblasts described before (van Ewijk et al., 1974). More peripherally, blast cells were found either free (Fig. 19) or in association with macrophages. These blasts were generally larger than the blasts in the central PALS, they measured about 14 μ. They contained a few dense bodies and strands of rough endoplasmic reticulum, nearly always located around mitochondria. Most of these cell organelles were located near nuclear invaginations. The nucleus contained a very large, marginally located

Fig. 7. Splenic white pulp, 4 days after immunization. Blast cells are diffusely spread throughout the PALS (*p*). Germinal centers occur in follicles (*f*) as isolated groups of lymphoid blast cells (arrows). *a* = central arteriole (*a* = 135).

Fig. 8. Splenic white pulp, 15 days after immunization. Large germinal centers (*gc*) accur at the periphery of the PALS (*p*). *a* = central arteriole (× 135).

Fig. 9. Germinal center, 4 days after immunization. Note the lymphoid blast cells, mitotic blasts and the nuclei of dendritic cells (*d*). The germinal center is bordered by cytoplasmic processes of reticulum cells (×850).

Fig. 10. Germinal center, 15 days after immunization. Many blast cells and mitotic cells occur in between processes of dendritic cells (*d*). Note the typical tingible body macrophages (*tbm*). *c* = lymphocytes, present in the surrounding lymphocyte corona.

nucleolus. The latter lymphoid blast cells resemble closely plasmablasts in rabbits (de Petris and Karlsbad, 1965), in rats (Kuhlman and Avrameas, 1972) and B lymphoblasts present in lymphocyte cultures stimulated with endotoxin (Janossy et al., 1972) or lipopolysaccharide (Shohat et al., 1973).

Throughout the PALS plasmablasts were observed showing morphological signs of active migration (Fig. 20). Moving cells have their cell organelles gathered in a so called "uropod" and move in a direction opposite to the uropod (Bessis, 1973). Microfilaments and microtubules, known as cell organelles involved in locomotion were found in the uropod and were

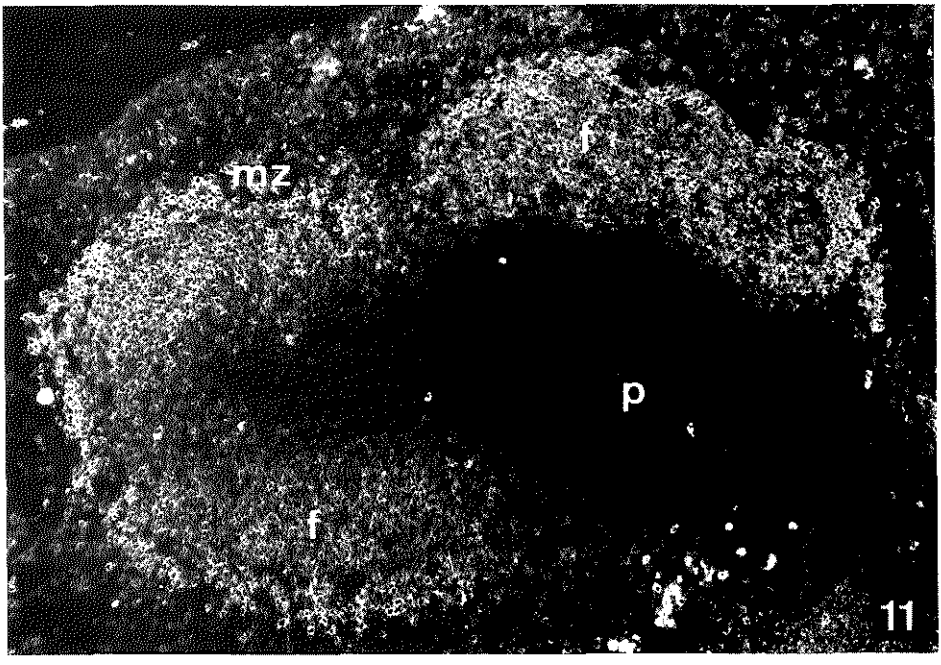


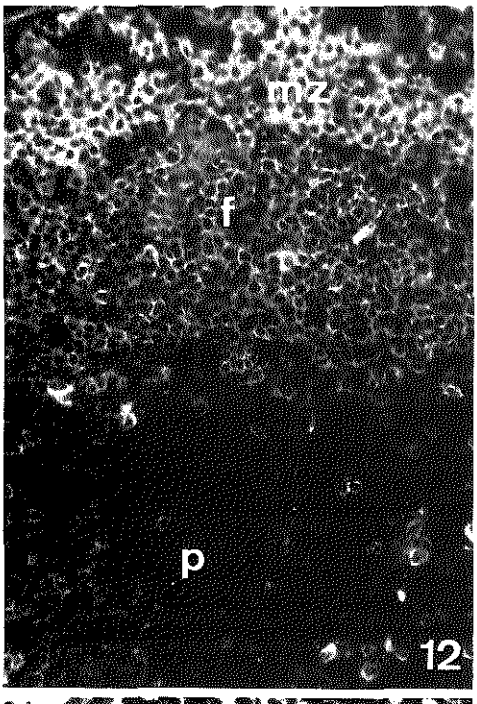
Fig. 11. Frozen dried section of the splenic white pulp of a control germfree mouse. The section was incubated with RAM-Ig followed by TRITC-GAR-Ig. Primary follicles (*f*) and marginal zone (*mz*) are positively stained, whereas the PALS (*p*) is negative (×165).

Fig. 12. White pulp of a control germfree mouse. Cells in the marginal zone (*mz*) show a more strongly membrane fluorescence than cells in the follicles (*f*). A few membrane Ig positive cells occur in the peripheral PALS (*p*) (×280).

Fig. 13. White pulp of a germfree mouse, 4 days after immunization. Cells with a strong cytoplasmic fluorescence occur in the PALS, most of these cells are located near the central arteriole (*a*). *f* = follicle, *mz* = marginal zone, *rp* = red pulp (×150).

Fig. 14. Start of a germinal center (between arrows) in a primary follicle. The blast cells are larger in diameter than the surrounding lymphocytes, and show a weak membrane fluorescence. No cytoplasmic fluorescence was observed (×280).

Fig. 15. White pulp of a germfree mouse, 15 days after immunization. A few cells with strong cytoplasmic fluorescence occur in the PALS (*p*). Note the "dendritic staining" of the peripheral part of the germinal center. The central part of the germinal center (*gc*) is Ig negative. *c* = lymphocyte corona (×150).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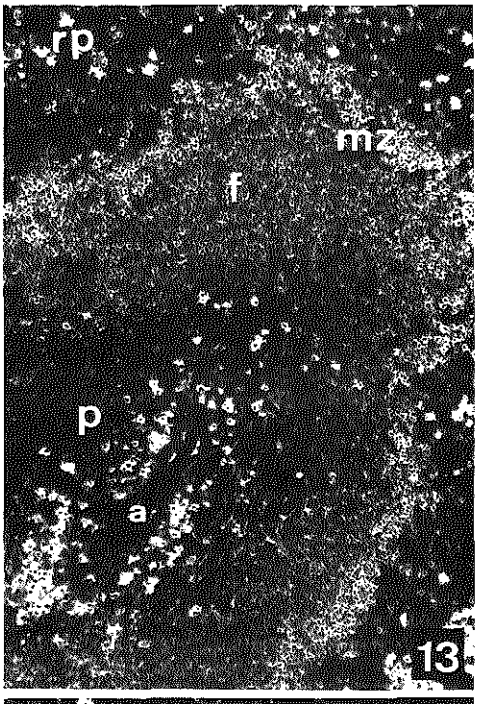
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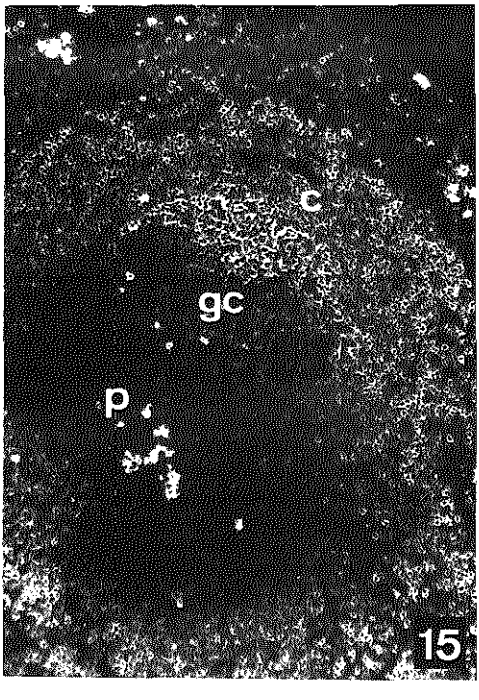
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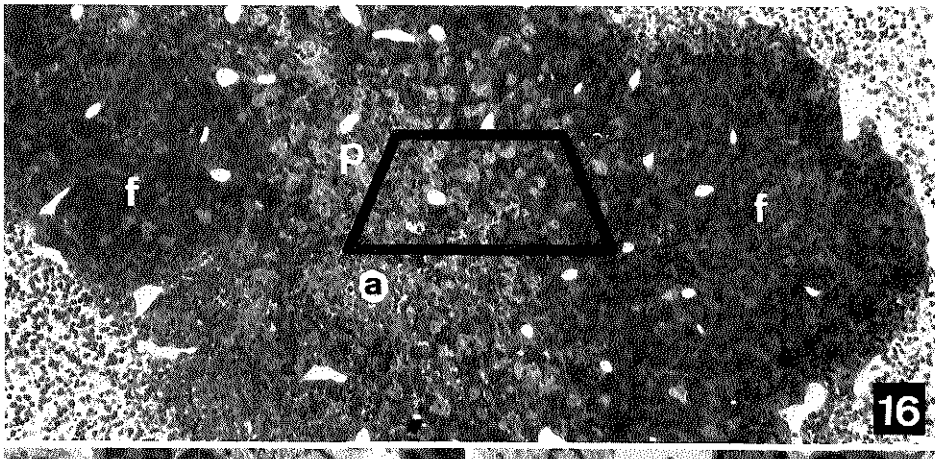
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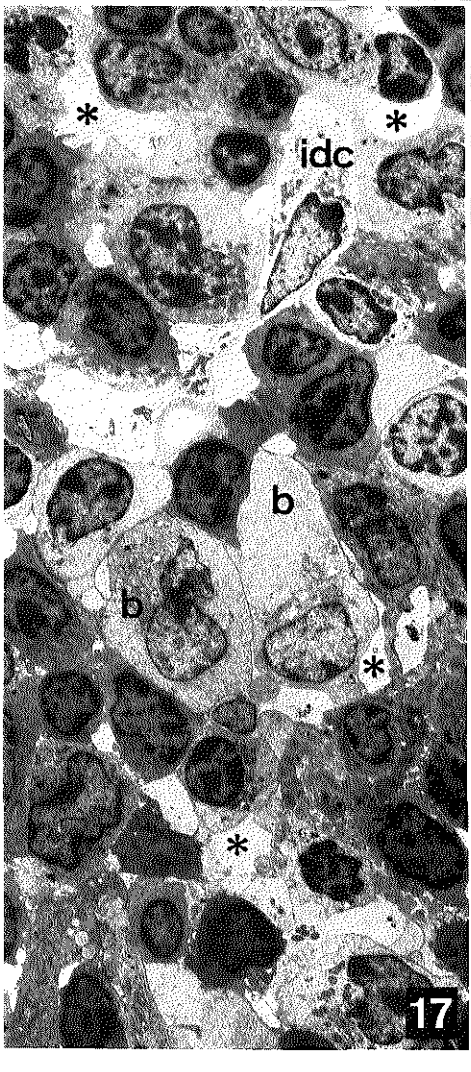
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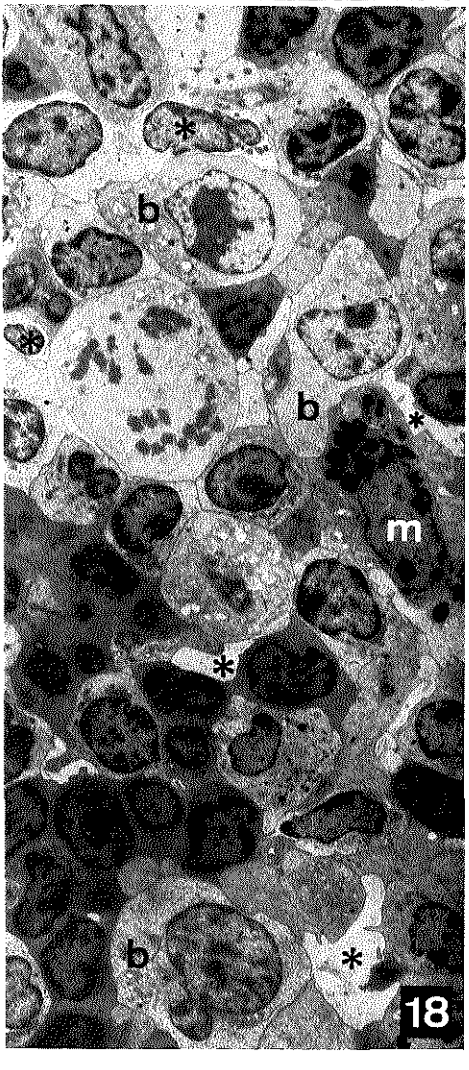
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orientated in the long axis of the cell. Moving plasmablasts always contained a high concentration of pentameric and helical polyribosomes evenly distributed throughout the cytoplasm. Mitochondria, short profiles of RER, Golgi lamelles and dense bodies were concentrated in the uropod. Membrane invaginations were sometimes observed at the terminal site of the cell.

Discussion

The goals of the present investigation were: a) to delineate T and B cell compartments in the splenic white pulp of unstimulated mice, b) to follow the cellular events in T and B cell compartments during a primary immune response, c) to localize the area where interaction between T and B cells may occur upon immunization with a thymus-dependent antigen. Since conventional mice nearly always show morphological signs of immune reactivity probably due to environmental antigens, we preferred to follow the course of the immune response in germfree mice.

The immunofluorescence studies on frozen section of the splenic white pulp in unstimulated germfree mice have shown a clear distinction between thymus dependent and thymus independent areas. Membrane fluorescence of cells stained with RAM-Ig followed by TRITC-GAR-Ig was positive in the follicles and marginal zone and negative in the PALS. These results are similar to immunofluorescence studies on T and B cell microenvironments in lymph nodes reported by Gutman and Weissman (1972). We found, surprisingly, that cells in the marginal zone were more strongly fluorescent than cells in follicles. The question arises whether this population of B cells is a distinct subpopulation of B cells with a specific localization pattern, preferentially in the marginal zone. Marginal zone cells in the spleen of rabbits have been shown to contribute to the pool of antibody forming cell precursors (Nieuwenhuis and Keuning, 1974). Also, marginal zone cells may enter the white pulp and belong therefore to a population of recirculating cells (Ford, 1969). Recirculating B cells in the thoracic duct of nude mice have been shown to posses a higher concentration of Ig determinants on the cell membrane than B cells either in

← Fig. 16. White pulp of a germfree mouse, 3 days after immunization. Lymphoid blast cells are scattered through the PALS (*p*). The trapezium indicates the area selected for subsequent ultrastructural studies. *f* = follicle, *a* = central arteriole (×150).

Fig. 17. Central part of the PALS, 3 days after immunization. Interdigitating cells (*idc*) show their typical translucent cytoplasm. Lymphoid blast cells (*b*) contain numerous polyribosomes but very few strands of rough endoplasmic reticulum. Blast cells contact processes of interdigitating cells (asterisks) (×2100).

Fig. 18. Central part of the PALS, 3 days after immunization. A lymphoid blast cell (*b*) contacts a macrophage (*m*), loaded with degradation products of erythrocytes. The asterisks indicate interdigitating cells or their cytoplasmic processes. Lymphocytes indent these cytoplasmic processes by means of fingerlike protrusions, as shown in the lower right corner of the micrograph. Note the large mitotic lymphoid blast cell (×2100).

spleen or in bone marrow (Osmond and Nossal, 1974). Taken together, these data provide evidence that the more strongly fluorescent B cells in the marginal zone represent a distinct population of B cells, possibly antibody forming cell precursors and/or B memory cells.

When the PALS was inspected at higher magnification, we found a small percentage of membrane fluorescence positive cells throughout the PALS, mostly located at the periphery. Apparently, B cells can localize in the PALS, and this area is therefore not exclusively thymus dependent. Recently published autoradiographic studies by Nieuwenhuis and Ford (1976) provide

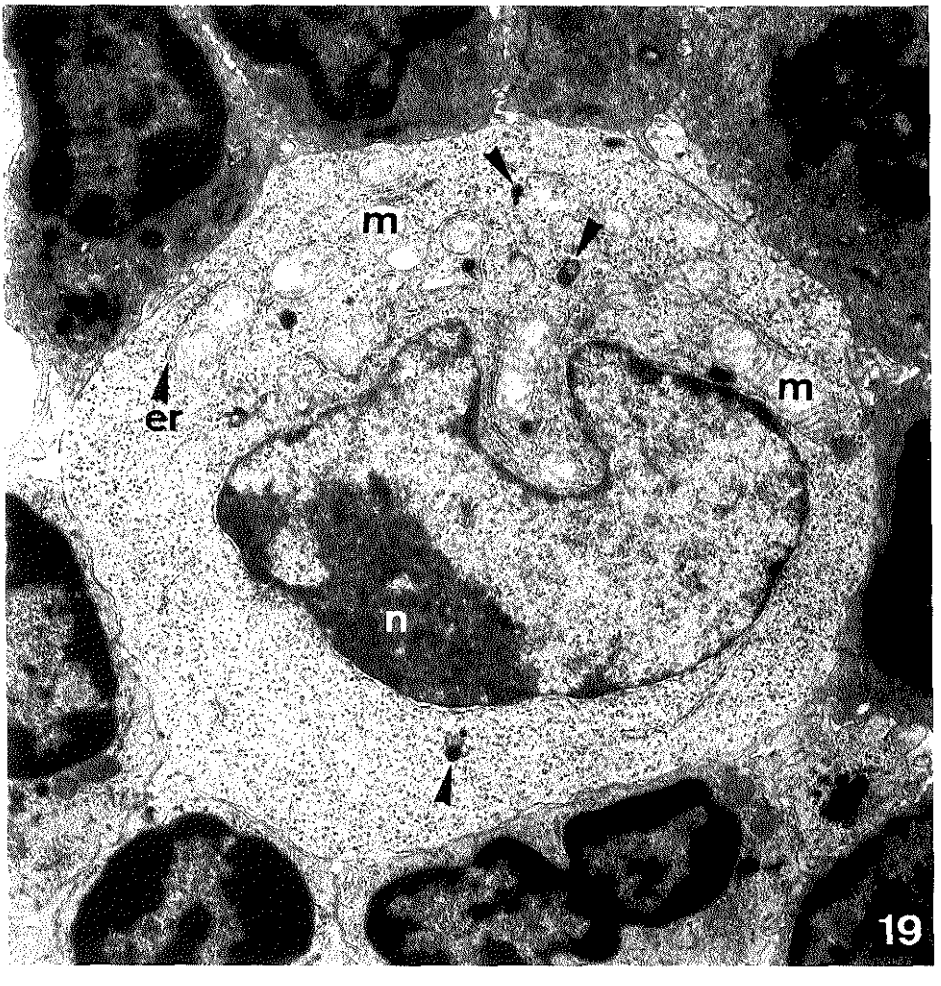

Fig. 19. Large plasmablast located in the peripheral PALS. Many polyribosomes, either pentameric or helical are found throughout the cytoplasm. Short profiles of rough endoplasmic reticulum (*er*) occur near to mitochondria. Dense bodies (*arrows*) are scattered through the cytoplasm. Most of the cell organelles are concentrated near to nuclear invaginations. The nucleus contains a prominent nucleolus (*n*) (×6500).

evidence that in rats B cells in the peripheral PALS belong to a pool of migrating B lymphocytes. They showed that B lymphocytes entered the spleen via the marginal zone and subsequently migrated along the reticulin sheaths surrounding terminal arterioles towards the PALS. From here, the cells migrated along the peripheral PALS to the lymphocyte corona surrounding germinal centers. The local transit time from marginal zone towards corona was approximately 24 hr. Autoradiographic experiments carried out in our laboratory on the migration of B cells in T mice (adult lethally irradiated mice, i.v. injected with syngeneic thymocytes) showed a similar migration pattern: B cells entered the thymus dependent area and subsequently formed primary follicles, in 18 - 24 hr (van Ewijk and van der Kwast, submitted). Together these data indicate that B cells on their way to specific "homing" areas, can migrate through thymus dependent areas. This particular traffic route is probably conductive to an efficient collaboration between T and B cells.

When germfree mice were immunized i.v. with SRBC, we noted that initial blast formation occurred in the PALS. This observation is in agreement with the results of other reported histological studies on the immune response in the spleen of rodents (Langevoort (1963), for a review on this subject see also Fitch and Wissler (1971). These studies concerned conventional animals and were generally based on methylgreen pyronin stained paraffin sections. As a consequence, distinction between T and B cells and between T and B microenvironments was not possible.

In the present study, the first blast cells were noted in the thymus dependent central PALS, 2 days after immunization. At that time, however, our immunofluorescence studies failed to demonstrate cells with cytoplasmic fluorescence, i.e. plasmablasts. Plasmablasts were first noted 3 days after immunization, and they were mostly located in the peripheral part of the PALS. Electronmicroscopic observation of the central PALS revealed that blast cells in this area contained many free ribosomes but only very few strands of rough endoplasmic reticulum. They were found in close contact with processes of IDC and erythrocyte containing macrophages. In earlier electronmicroscopic studies on the T cell microenvironment in lethally irradiated, thymocyte transferred mice we reported identical relationships between T lymphoblasts and IDC (van Ewijk et al., 1974). In contrast, early appearing blast cells in the peripheral PALS were not found in contact with IDC. These cells were generally larger than blast cells in the central PALS and always contained dense bodies and strands of rough endoplasmic reticulum. Based on these observations we suggest that the early lymphoblasts in the central PALS are the progeny of antigen-reactive T cells responding to the antigenic stimulus provided by macrophages, whereas the later on appearing large blasts in the peripheral PALS belong to the B cell lineage. Thus, T cell activation is a necessary prerequisite for B cell activation during the immune response against SRBC. These results agree with T6T6 chromosome marker studies of the immune response against SRBC in mice reported by Davies et al. (1969), in which it was noticed that T cell proliferation starts before B cell proliferation. This phenomenon was also recently observed with thymidine suicide experiments (Bachvaroff and Rapaport 1975).

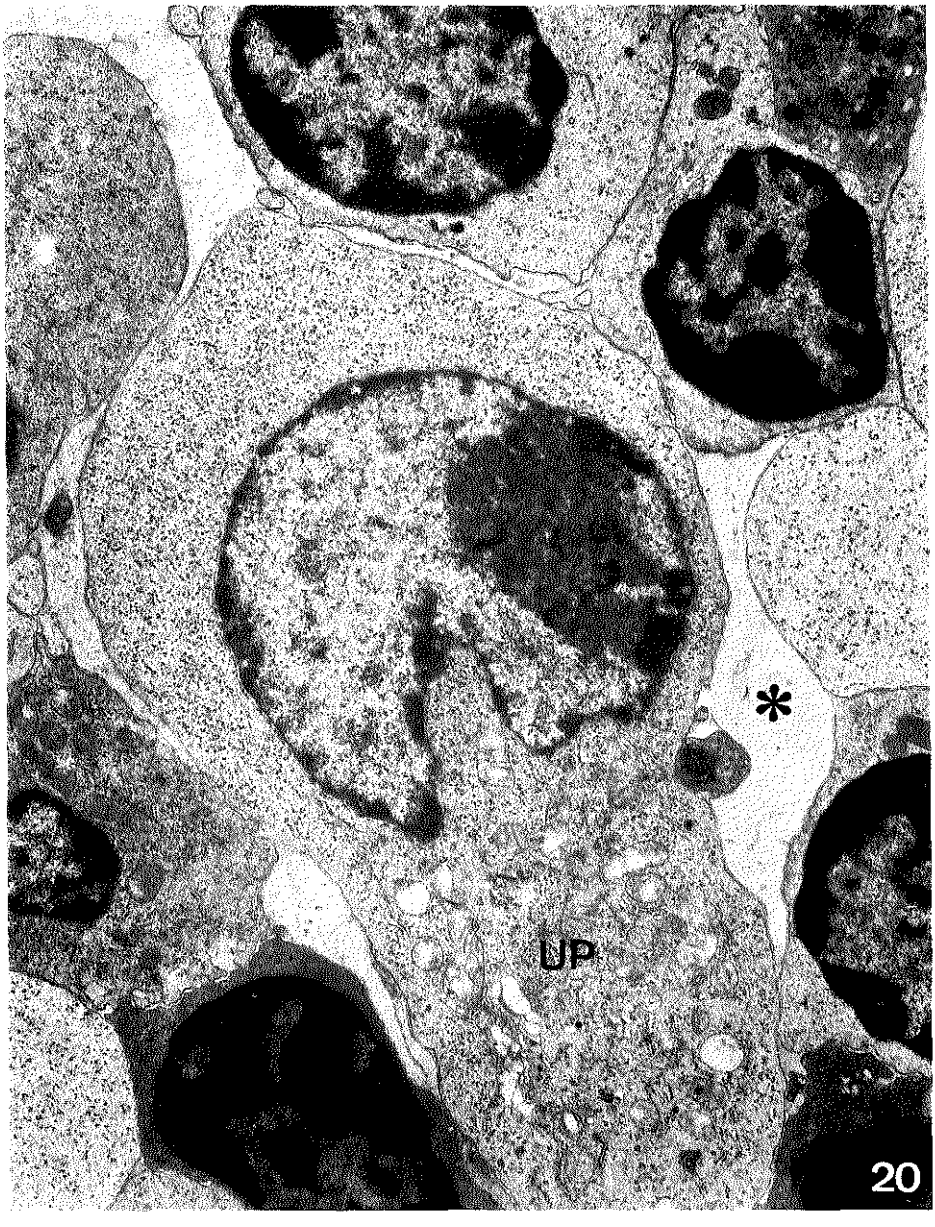

Fig. 20. Large plasmablast showing an uropod (*UP*) in which all cell organelles are gathered. Uropod formation is an indication for cell movement. The plasmablast migrates through the central part of the PALS in a direction opposite to the uropod. Note the cytoplasmic processes of interdigitating cells, indicated by an asterisk (×6500).

The precise function of IDC during the immune response is at this moment unknown. However, two functions of IDC may be of importance: a) IDC are capable of retaining T lymphocytes within their cytoplasmic web by means of specialized membrane structures (Veerman and van Ewijk, 1975). Thus, IDC are involved in the homing of T cells in thymus dependent areas. b) IDC have been shown to produce glycoproteins (Veldman, 1970; Veerman and van Ewijk, 1975; Friesz, 1976), which are probably involved in T cell stimulation. This assumption is supported by the observation that T lymphoblasts have a close relation with processes of IDC (van Ewijk et al., 1974).

The early events in the spleen of SRBC stimulated germfree mice can be integrated to the following hypothesis on cellular cooperation: a) Macrophages pick up antigen from the red pulp and/or marginal zone and migrate into the PALS. b) Antigen-reactive T cells, migrating through the PALS are activated upon contact with macrophages (for review see Basten and Mitchell, 1976). c) These T cells contact IDC for a prolonged period. Possibly the IDC stimulates further proliferation and differentiation of antigen-activated T cells. d) Antigen-reactive B cells streaming through the PALS on their way to follicles move through microenvironments where T cell activation and proliferation occurs. e) Either by cell-cell contact with antigen-activated T cells and macrophages or by factors secreted by antigen-activated T cells and macrophages, the antigen-reactive B cells are stimulated and transform into plasmablasts. f) These plasmablasts in turn migrate towards the central PALS, follow the reticulum sheaths surrounding the central arterioles and terminal arterioles and migrate finally via marginal zone bridging channels into the red pulp. We postulate therefore that the PALS is the appropriate microenvironment in which the interaction between macrophages, interdigitating cells, T cells and B cells occur upon stimulation of mice with a thymus dependent antigen. This cellular cooperation leads to the differentiation of B cells to immunoglobulin producing plasmablasts. These plasmablasts synthesize most likely antibodies of the IgM-class, since in the early phase of the immune response to SRBC only IgM antibodies to SRBC could be detected in the serum (Fig. 1). We found no evidence that this particular differentiation occurred in the marginal zone, as has been suggested by Mitchell (1972) or in follicles or germinal centers as has been suggested by Gutman and Weissman (1972).

The next phase of the immune response in the spleen of germfree mice was characterized by the appearance of germinal centers. Germinal centers were not found in follicles of unstimulated germfree mice, they appeared from the fourth day after immunization. Initially, germinal center formation started in the follicles as isolated clusters of lymphoblasts. With fluorescence microscopy these clusters were Ig negative, especially with regard to cytoplasmic fluorescence. This observation supports the view that germinal centers are not directly involved in the production of Ig producing plasmablasts in the early period of the immune response (Galj-Peczalska et al., 1969). We cannot rule out the possibility that in the following response period germinal centers contribute to antibody formation. However, the fluorescence staining of germinal centers on day 7, 12 and 15 was of a "dendritic" type and as such clearly different from the cytoplasmic fluorescence of plasma cells in the red

pulp. It seems therefore more likely that Ig in germinal centers is cytophilically bound to the cytoplasmic processes of dendritic cells, which are known to occur at the peripheral site of the center (Nossal and Ada, 1971; Abe and Ito, 1973). This cytophilic Ig is part of immune complexes (van Rooyen, 1973) and probably transported towards dendritic cells by antigen-antibody binding lymphocytes (Veerman and van Rooyen, 1975). The exact mechanism of germinal center reactions remains to be established, however, Wakefield and Thorbecke (1968) relate germinal center reactions to the formation of memory B lymhocytes, whereas Nieuwenhuis and Keuning (1974) consider germinal centers as an amplification system in B cell formation.

The present investigation indicates that at least the thymus dependent IgM response to SRBC starts in the PALS, and that the cooperation between the different lymphoid and non-lymphoid cell types resides in this same area. The site of the development of IgG plasmablasts is at present unknown. The localization of these cells with specific anti IgG antisera will be subject of further studies on the cellular events during the immune response.

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Scanning Electron Microscopy of Homing and Recirculating Lymphocyte Populations

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The surface structure of T and B lymphocytes *in vivo* was investigated using scanning electron microscopy. For these studies the spleen and mesenteric lymph node of mice enriched for B lymphocytes (adult thymectomized, lethally irradiated, bone marrow reconstituted mice, B mice) and of mice enriched for T lymphocytes (adult, lethally irradiated, thymocyte transferred mice, T mice) were examined. Both types of lymphocytes demonstrated a smooth cell surface when they were situated in their respective microenvironment, whereas recirculating T and B cells exhibited numerous microvilli on the cell surface. In postcapillary venules, known to be the major sites of entry of lymphocytes in lymph nodes, lymphocytes were in contact with the endothelial wall by means of these microvilli. While passing the endothelial lining, lymphocytes withdrew their microvilli and appeared smooth upon arrival in the lymphatic stroma. It is suggested that microvilli on the surface of lymphocytes play a role in cellular recognition mechanisms.

The two major classes of lymphocytes B cells and T cells home in different compartments in peripheral lymphoid organs. In the lymph node, B cells predominantly occur in the outer cortex, while T cells occupy the paracortical areas (1–4). In the lymphatic part of the spleen, the white pulp, B cells are localized in follicles and in the peripheral part of the periarteriolar lymphatic sheath (PALS) (1). T cells on the other hand occupy the central area of the PALS (5).

With the transmission electron microscope, B and T cells are difficult to distinguish with morphological criteria. However, there is some evidence that T and B lymphocytes differ in two aspects: B cells generally show more polyribosomes in their cytoplasm than T cells (6) and B cells show a nuclear sfaeridium (7). In addition, scanning electronmicroscopic studies of Polliack *et al.* (8) and Lin *et al.* (9) have provided evidence that the surface morphology of T and B cells differs *in vitro*: T cells generally have a smooth cell surface, while B cells exhibit a surface with many microvillous projections.

It is well known that T cells, B cells, and macrophages cooperate during the humoral immune response against various types of antigens, resulting in the differentiation of antigen reactive B cells into antibody producing plasma cells (10). During this cooperation process there is evidence that in addition to humoral factors (11, 12), cellular contacts between T cells, B cells, and macrophages play also an important role in B cell activation (13, 14).

In view of the data by Polliack *et al.* (8) and Lin *et al.* (9), scanning electron microscopy seems to be an appropriate tool to investigate T–B cell cooperation at

the ultrastructural level. To initiate these studies, we have investigated the scanning electron microscopic aspects of T and B areas in spleen and lymph nodes of adult thymectomized, lethally irradiated mice, reconstituted with bone marrow cells (mice depleted of T cells, B mice). In addition, peripheral lymphoid organs of adult, lethally irradiated mice reconstituted with thymocytes (mice depleted of B cells, T mice) were examined. Evidence is presented that the surface structure of lymphocytes, as observed *in vivo*, reflects the state of physiological activity of the lymphocyte and is not a morphological criterium for the distinction of the two classes of lymphocytes.

MATERIALS AND METHODS

Mice. Male (DBA/2 × C57BL/Rij)F_1 mice, either 4–6 wk or 15–20 wk of age were used for the present experiments. They were purchased from the Medical Biological Laboratory TNO, Rijswijk, The Netherlands.

X-irradiation. Recipient mice received 850 rad whole body x-irradiation from a Philips Möller MG 300 X-ray machine, as reported earlier (15). Radiation control mice died in 9–16 days.

Preparation of B mice. Four- to six-week-old mice were thymectomized according to the method reported by Miller (16). Two weeks after surgery they were irradiated and injected intravenously with 5×10^6 syngeneic bone marrow cells, within 3 hr after irradiation. The bone marrow cells were treated with anti θ serum and complement *in vitro* before inoculation, in order to abolish contaminating T cells. The lymphoid tissues of these mice were examined 4 wk after reconstitution.

Preparation of T mice. Fifteen- to twenty-week-old mice were lethally irradiated and injected intravenously with 5×10^7 syngeneic thymocytes from 6-wk-old donor mice within 3 hr after irradiation. The lymphoid tissues of these mice were examined 6 days after cell transfer.

Immunofluorescence staining of B and T cells. Cell suspensions of the spleen and mesenteric lymph node of normal mice, T mice, and B mice were prepared in a balanced salt solution containing 5% newborn calf serum as described in detail by Benner, Meima, Van der Meulen, and Van Muiswinkel (17). Before reacting with antisera, the cells were washed three times in a solution consisting of 5% bovine albumin in phosphate-buffered saline (5% BA-PBS) (18). For the detection of B lymphocytes, 25 μl aliquots of 10^6 cells were added to 25 μl of a fluorescein-labeled swine anti-mouse immunoglobulin serum (FITC-SwAM-Ig, Nordic, Tilburg, The Netherlands) and incubated at 4°C for 30 min with gentle shaking every 10 min. Subsequently, the cells were washed three times with 1% BA-PBS and resuspended in approximately 30 μl. A drop of the cell suspension was mounted on a slide in buffered glycerol (9 parts glycerol, 1 part PBS), covered with a cover slip, and the edges were sealed with paraffin. The FITC-SwAM-Ig reacted with all classes of mouse Ig as well as with Ig kappa and Ig lambda light chains.

To detect T lymphocytes, an indirect immunofluorescence technique was used. Portions of 10^6 cells, in about 25 μl, were added to 25 μl of a rabbit anti-mouse

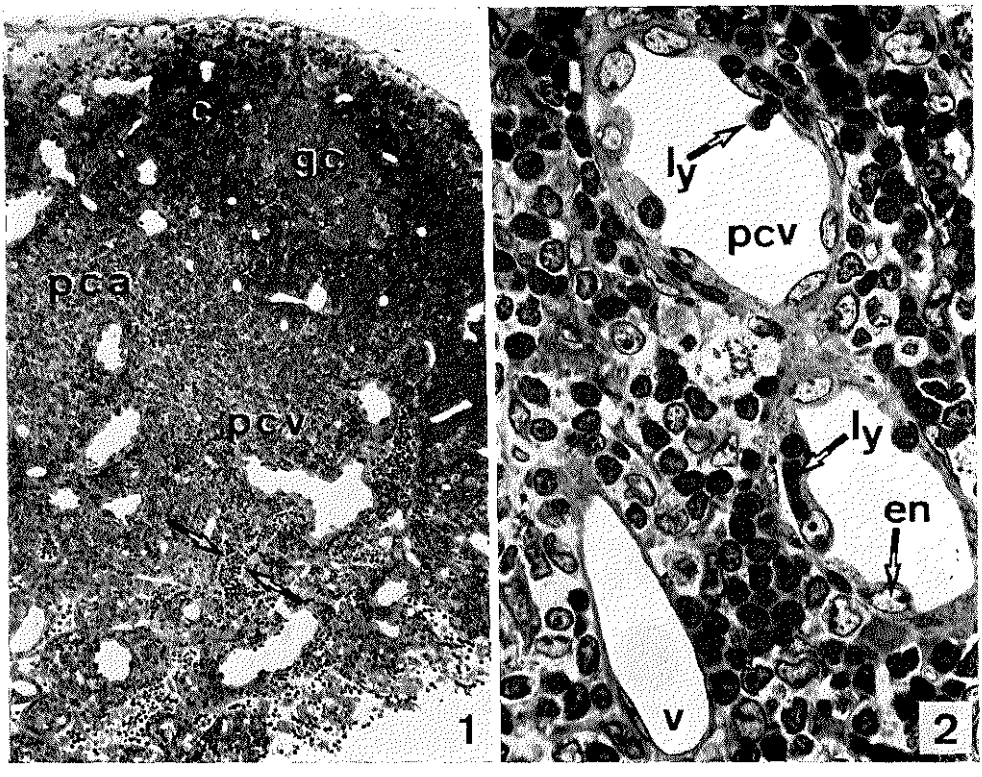


FIG. 1. Survey of the outer cortex (c) and paracortex (pca) of the mesenteric lymph node of a B mouse. In the cortex a germinal center (gc) is surrounded by a corona of small dark staining lymphocytes. The depleted paracortex shows large postcapillary venules (pcv) and lymph vessels (arrows). (×110).

FIG. 2. Postcapillary venule (pcv) in the paracortex of the mesenteric lymph node of a B mouse. Lymphocytes (ly) migrate between the high endothelial cells (en). The venule (v) is lined by flat endothelial cells. In these vessels diapedesis of lymphoid cells was not observed. (×920).

thymocytes serum (ATS) and this mixture was then incubated at 4°C for 30 min with gentle shaking every 10 min. The ATS was obtained as described by Veldkamp, De Reuver, and Willers (19). The cells were washed three times in 1% BA-PBS, resuspended in a final volume of 25 μl, and then mixed with 25 μl of a fluorescein labeled horse anti-rabbit immunoglobulin serum (FITC-HAR-Ig, Bloedtransfusiedienst, Amsterdam, The Netherlands). After incubating for another 30 min at 4°C with gentle shaking every 10 min, the cells were washed three times with 1% BA-PBS and mounted on a glass slide as described above. As a control, cells were first incubated with normal rabbit serum or with 5% BA-PBS (instead of with the ATS) and subsequently with FITC-HAR-Ig. The slides were examined with a Zeiss ultraviolet microscope equipped with a vertical illuminator IV/F and an Osram HBO50 mercury lamp as a light source.

Preparation of lymphoid tissue for light microscopic and scanning electron microscopic observations. To examine B and T areas of the spleen and mesenteric lymph node with the light and electron microscope, 8 B mice as well as 8 T mice

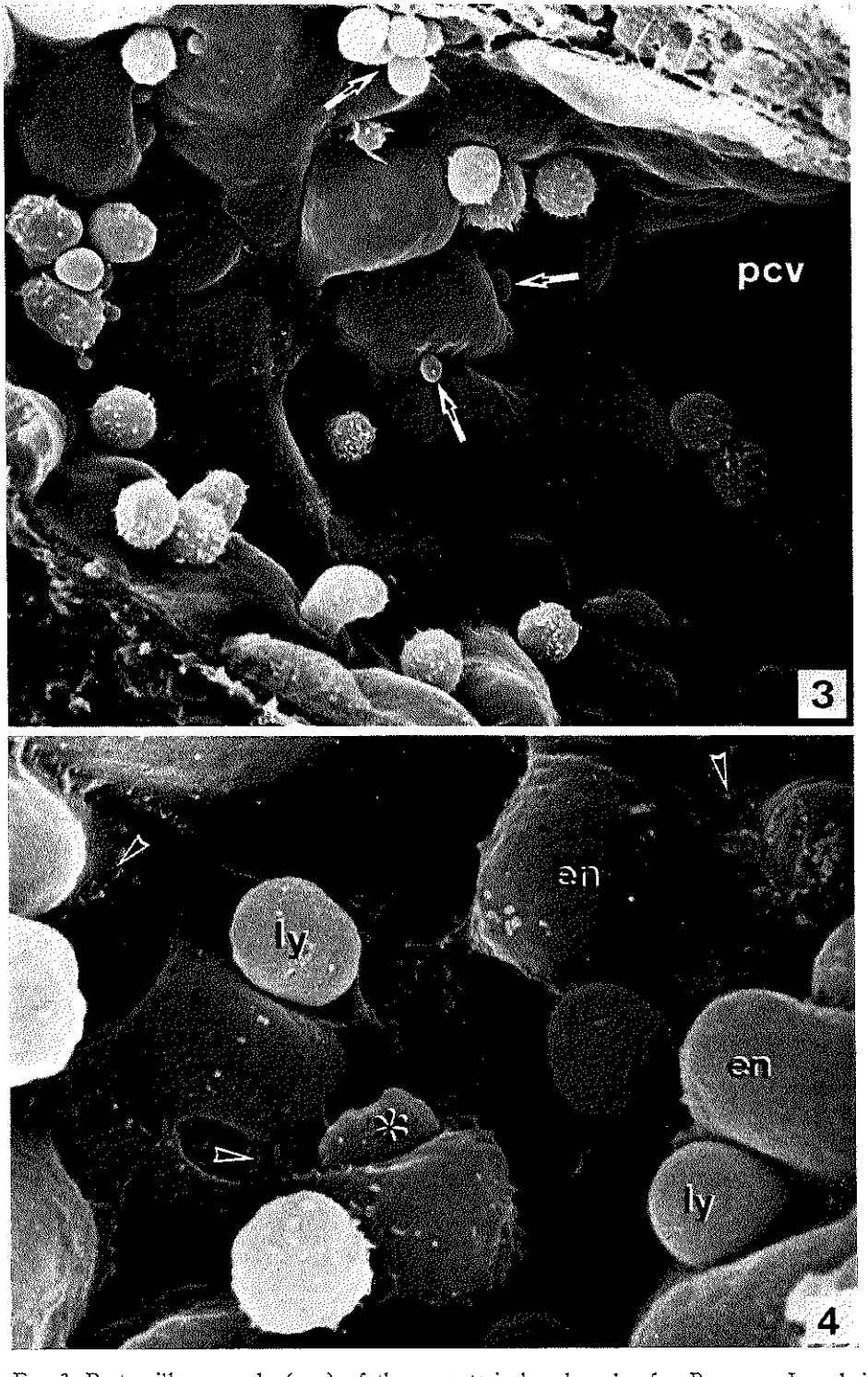


FIG. 3. Postcapillary venule (pcv) of the mesenteric lymph node of a B mouse. Lymphoid cells have attached to the endothelial wall. Blood is cleared from the lumen of the PCV due

TABLE 1

IMMUNOFLUORESCENCE OF LYMPHOCYTES IN SPLEEN AND MESENTERIC LYMPH NODE OF B MICE, T MICE, AND NORMAL CONTROL MICE

Cells tested from	Spleen		Mesenteric lymph node	
	B cells	T cells	B cells	T cells
	(%)[c]	(%)	(%)	(%)
B mice[a]	93.0 ± 1.8[d]	2.8 ± 1.4	81.8 ± 1.2	11.0 ± 1.0
T mice[b]	0.0	97.6 ± 7.3	0.0	98.7 ± 5.8
Normal control mice	56.6 ± 1.9	35.4 ± 1.8	28.9 ± 1.1	62.8 ± 1.2

[a] B mice were adult thymectomized, lethally irradiated, bone marrow reconstituted mice

[b] T mice were adult, lethally irradiated, thymocyte reconstituted mice.

[c] Percentage of all lymphocytes.

[d] Average ± 1 SEM. At least five individual mice were used for one determination.

[e] As a control for the immunofluorescent procedure to detect T lymphocytes, cells were first incubated with normal rabbit serum or with 5% BA-PBS and subsequently with FITC-HAR-Ig. In all cases these controls turned out to be completely negative.

were killed by total body perfusion fixation using a solution of 1.5% glutaraldehyde in 0.075 *M* sodium-cacodylate buffer. This fixation procedure has been reported in detail previously (15). At autopsy, the mediastinum of B mice was carefully inspected for thymic remnants using a dissecting microscope. Only animals free from thymic tissue were used in the present study.

For light microscopic studies, slices of the fixed tissue were osmificated, dehydrated in graded series of ethanol, immersed in epoxy-propane, and embedded in Epon 812. Polymerization was carried out at 37°C for 16 hr, at 45°C for 8 hr, and at 60°C for another 48 hr. Serial 1 μm thick sections were cut with a Reichert OM U2 microtome and were stained with an aqueous solution of 1% toluidine blue adjusted to pH 8.6 with 0.1 *M* borax buffer.

For scanning electron microscopic studies, the glutaraldehyde fixed specimens were dehydrated and immersed in amyl-acetate. The specimens were then transferred to a Critical Point Drying apparatus (Polaron, type E 3000). The amyl-acetate was exchanged for liquid carbon dioxide, then the temperature inside the apparatus was raised slowly to 36°C. When the pressure inside the chamber reached to 1400 lb/in^2, the gaseous carbon dioxide was ventilated. The dried tissue was mounted on specimen stages with colloidal silver and covered with gold using a sputter coater (Polaron, type E 5000). The specimens were examined in a Cambridge MK IIA scanning electron microscope, operated at an accelerating voltage of 20 kV, with 200 μm diam. illumating apertures.

to the perfusion fixation. The smooth sphaerical bodies (arrows) are probably lymphocytes which migrate across the endothelium. Compare with Fig. 2. (×2300).

FIG. 4. Endothelial lining of a PCV. Ridges occur between the endothelial cells (en). Smooth lymphocytes (ly) are situated in these ridges. They leave the circulation between the endothelial cells (asterisk). The endothelial cells contact each other by means of small cytoplasmic protrusions (arrows). (B mouse). (×5800).

RESULTS

The Mesenteric Lymph Node of B Mice

T cells were virtually absent in adult thymectomized, lethally irradiated, bone marrow reconstituted mice (B mice) examined 4 wk after bone marrow transplantation (Table 1). In histological sections of the mesenteric lymph node the absence of T cells was demonstrated by a marked depletion of the paracortical area (PCA). In contrast, the outer cortex was densely populated with lymphocytes and germinal centers were observed (Fig. 1). The medullary cords contained large numbers of plasma cells. Postcapillary venules (PCV) were present and showed the characteristic high endothelial cells (Fig. 2). Migration of lymphocytes through the wall of the PCV was frequently observed: single lymphocytes or groups of two or three lymphocytes were apparent between the high endothelial cells (Fig. 2). Migration of lymphocytes through the endothelial wall was not restricted to the cortical part of the PCV, lymphocytes crossing the endothelium in the (thymus-dependent) paracortex were also observed. Apparently these cells then migrated from the paracortex to the cortex, where they accumulated in follicular structures.

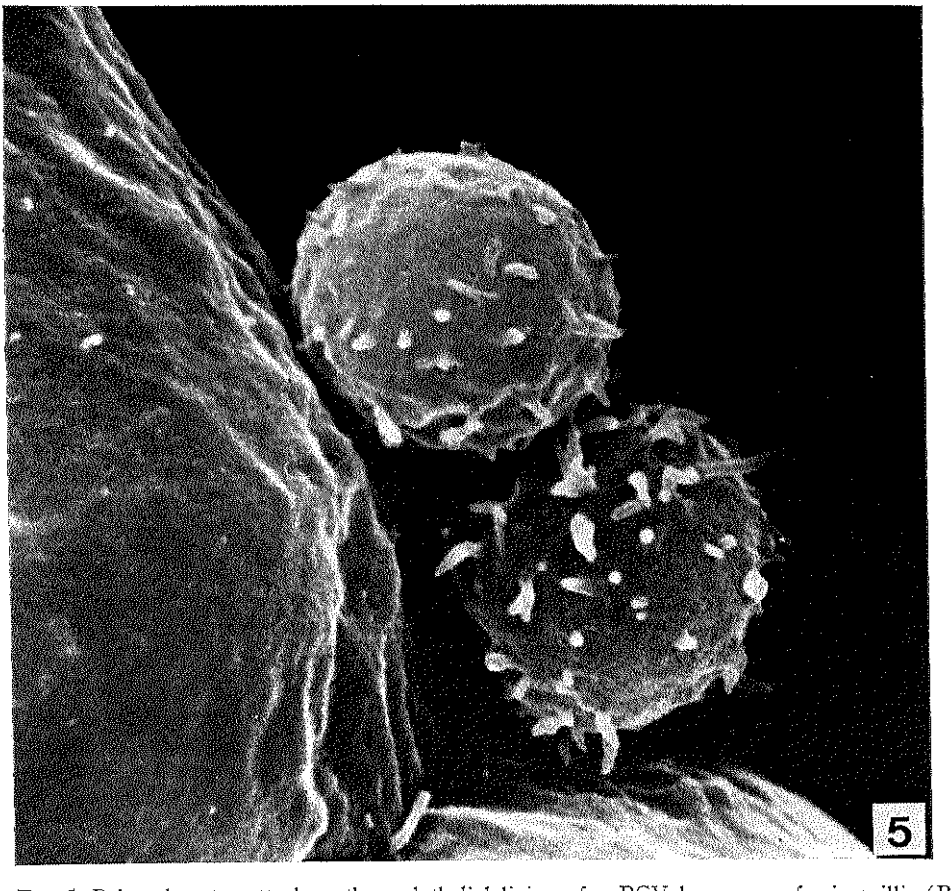


FIG. 5. B lymphocytes attach to the endothelial lining of a PCV by means of microvilli. (B mouse). (×11,500).

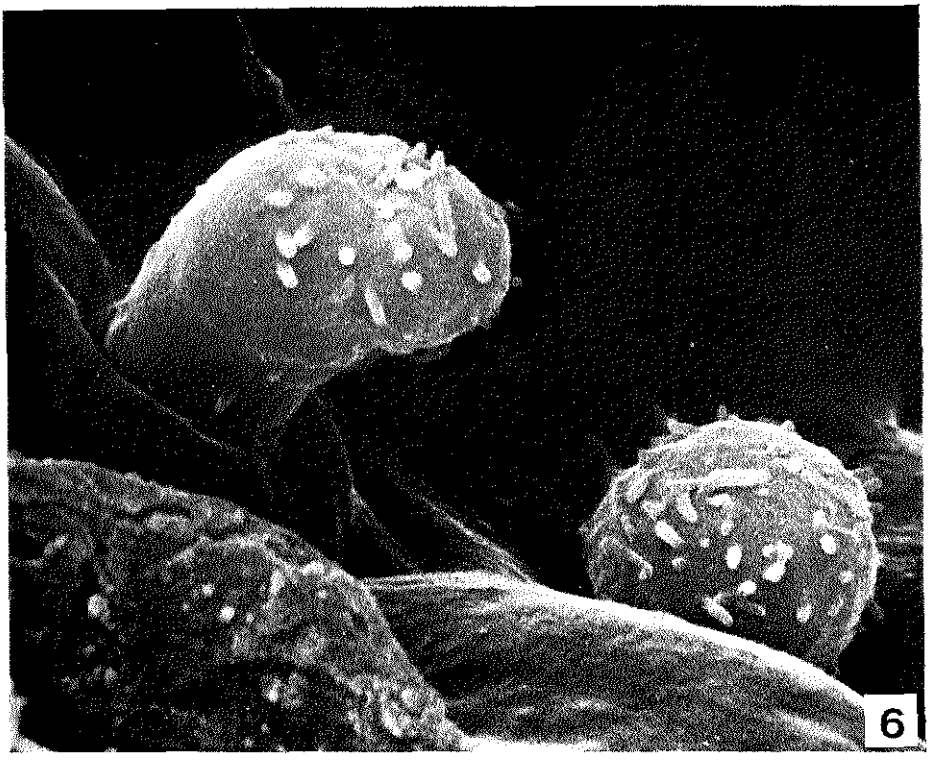


FIG. 6. Diapedesis of a B lymphocyte in a PCV of the mesenteric lymph node. The surface of the passing lymphocyte is smooth at the site of the endothelial wall. (B mouse). (×10,500). Compare with Fig. 2.

Scanning electron microscopic observations on PCV of the mesenteric lymph node demonstrated the hilly appearance of the endothelial wall (Fig. 3). Intercellular ridges were found between the endothelial cells, in these regions the endothelial cells contacted each other by means of protrusions (Fig. 4). Lymphocytes in the lumen of PCV were found to attach to the endothelial wall with microvilli (Fig. 5). Frequently small groups consisting of two to four lymphocytes were observed between the bulges of the endothelial cells. Lymphocytes actually leaving the PCV were also seen (Fig. 6, compare with Fig. 2). Passing lymphocytes showed only microvilli on the luminal side of the lymphocyte cell surface (Fig. 6). Much smaller smooth spherical bodies were found at other places between endothelial cells (Fig. 3, arrows). These structures probably represent lymphocytes arrested during their transit from the recirculating blood to the lymphatic compartment.

Lymphoid cells in the outer cortex of the lymph node (homing area of B cells) did not show microvilli (Fig. 7, Table 2). Their cell surface was usually smooth, but sometimes a few stubby structures occurred.

The Spleen of B Mice

The white pulp of the spleen of the B mice showed a depleted central area of the PALS (Fig. 8). Follicles and follicle centres on the other hand showed a

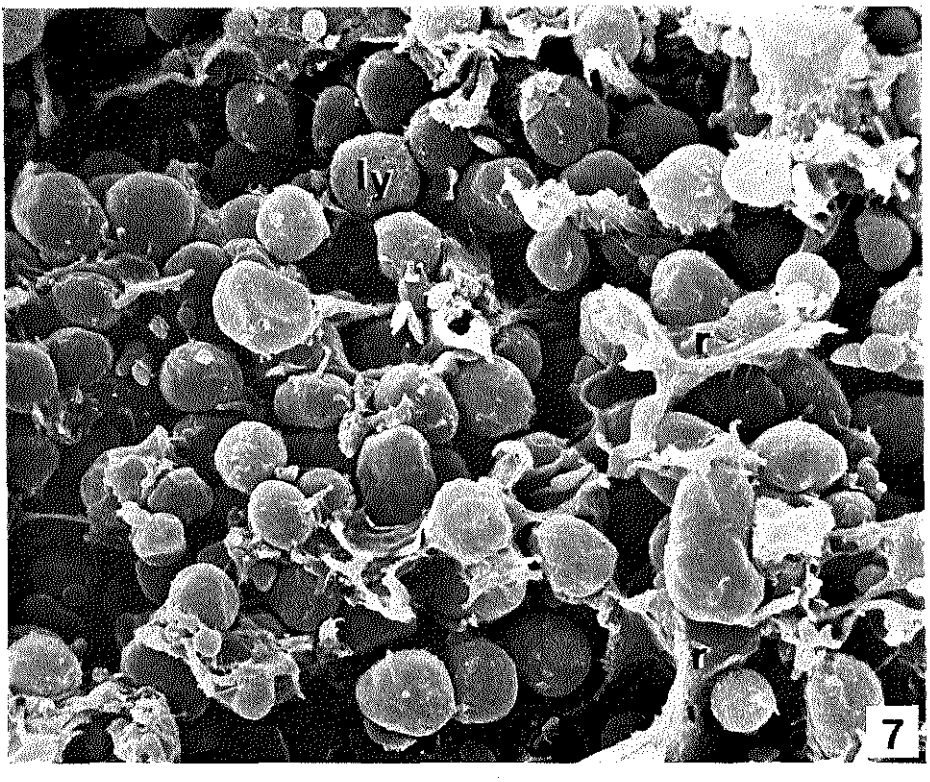


FIG. 7. B lymphocytes (ly) in the cortex of the mesenteric lymph node of a B mouse. In contrast to lymphocytes in postcapillary venules, these lymphocytes display a smooth cell surface. r = reticulum cell. (×2300).

normal architecture. A marginal zone was also present, occupied by lymphocytes, macrophages, reticulum cells, and plasma cells.

Scanning electron microscopy of follicles in the spleen revealed the presence of lymphocytes between slender processes of reticulum cells. These lymphocytes showed a smooth cell surface like follicular lymphocytes in lymph nodes. In contrast, lymphocytes in the marginal zone clearly showed the presence of microvilli. (Table 2). They were frequently found to contact each other by means of these structures (Fig. 9).

The Mesenteric Lymph Node of T Mice

Lethally irradiated and thymocyte reconstituted mice were sacrificed 6 days after cell transfer. Immunofluorescent studies demonstrated that in cell suspensions of the spleen and the mesenteric lymph node of these mice B lymphocytes were no longer present (Table 1). Light microscopic observations of the mesenteric lymph node revealed the absence of follicular structures in the cortex (Fig. 10).

Medullary cords were less prominent when compared to those in lymph nodes of B mice. Medullary sinuses were occupied with large macrophages, characterized by a distinct ruffling membrane (Fig. 11). Macrophages were frequently found to be associated with reticulum cells. The paracortical area was populated with lymphoid cells. However, when this area was compared to PCA of normal mice

it appeared that the PCA of T mice contained less lymphocytes. Postcapillary venules running through the paracortex showed their typical high endothelial cells. Lymphocyte passage was found all along the venules, always in those places were high endothelial cells were evident.

Scanning electron microscopic observations of the mesenteric lymph node of T mice revealed the presence of well preserved macrophages showing an extensive ruffling membrane. They were settled on reticulum cells; the latter cells showed a rather flat appearance (Fig. 12). T lymphocytes varied in surface architecture. Most T cells were round, showing distinct microvilli at their cell surface, while others showed rather broad cytoplasmic extensions (Fig. 13, arrows). These membrane structures are probably involved in active amoeboid locomotion of the lymphocyte (20). The distribution of the microvilli on T cells differed from cell to cell. Most T cells showed a random distribution of microvilli, however, on other T cells the microvilli were aggregated on one pole of the cell (Fig. 13, asterisk). Their size varied from stublike structures (0.3 μm in length) to slender fingerlike protrusions (1 μm in length).

In contrast to the recirculating lymphocytes in the sinuses, lymphoid cells present in the stroma of the paracortical area (homing area of T cells) displayed a smooth surface with only a few stublike structures (Fig. 14, Table 2).

The Spleen of T Mice

The white pulp of the spleen of T mice was reduced to a narrow peripheral lymphatic sheath which closely surrounded the central artery (Fig. 15). Follicles

TABLE 2

FREQUENCY OF SMOOTH AND VILLOUS CELLS PER LOCATION AS EXAMINED IN THE SCANNING ELECTRON MICROSCOPE.[a]

		Spleen		Mesenteric lymph node		
		Specific homing area[b]	Marginal zone	Specific homing area[b]	Post-capillary venule	Medullary sinus
		(%)	(%)	(%)	(%)	(%)
B mice[c]	Smooth cells[e]	98	19	97	38	2
	Villous cells	2	81	3	62	98
T mice[d]	Smooth cells[e]	92	3	95	34	7
	Villous cells	8	97	5	66	93

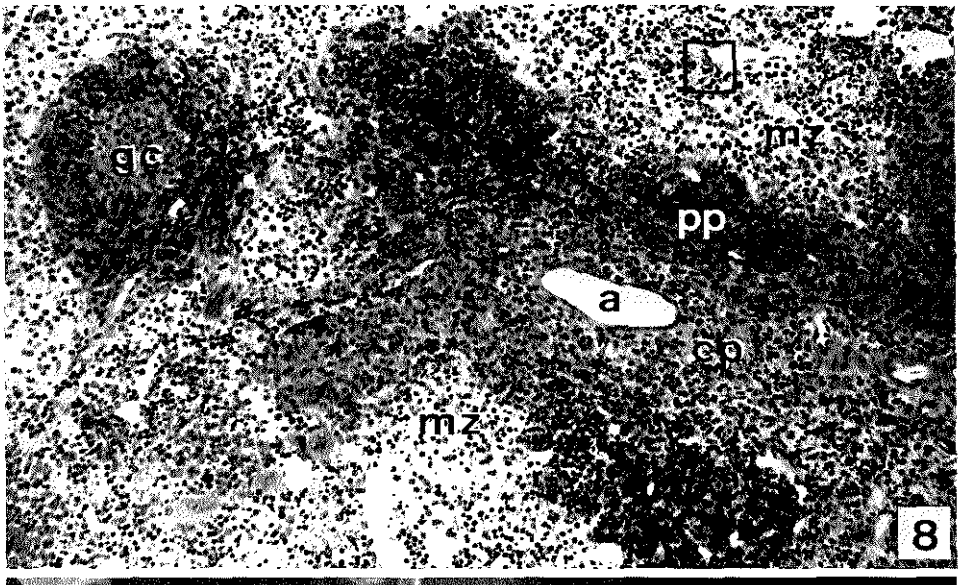
[a] From each mouse a minimum of two specimens per organ was examined. Only undamaged, totally visible round cells were counted.

[b] The specific homing area represents for B cells the follicles in the spleen and mesenteric lymph node. For T cells it represents the periarterolar lymphatic sheath in the spleen and the paracortical area in the mesenteric lymph node.

[c] Per location 125–660 cells were counted.

[d] Per location 50–810 cells were counted.

[e] Cells were qualified as smooth when cell surface projections were smaller than 0.2 μm.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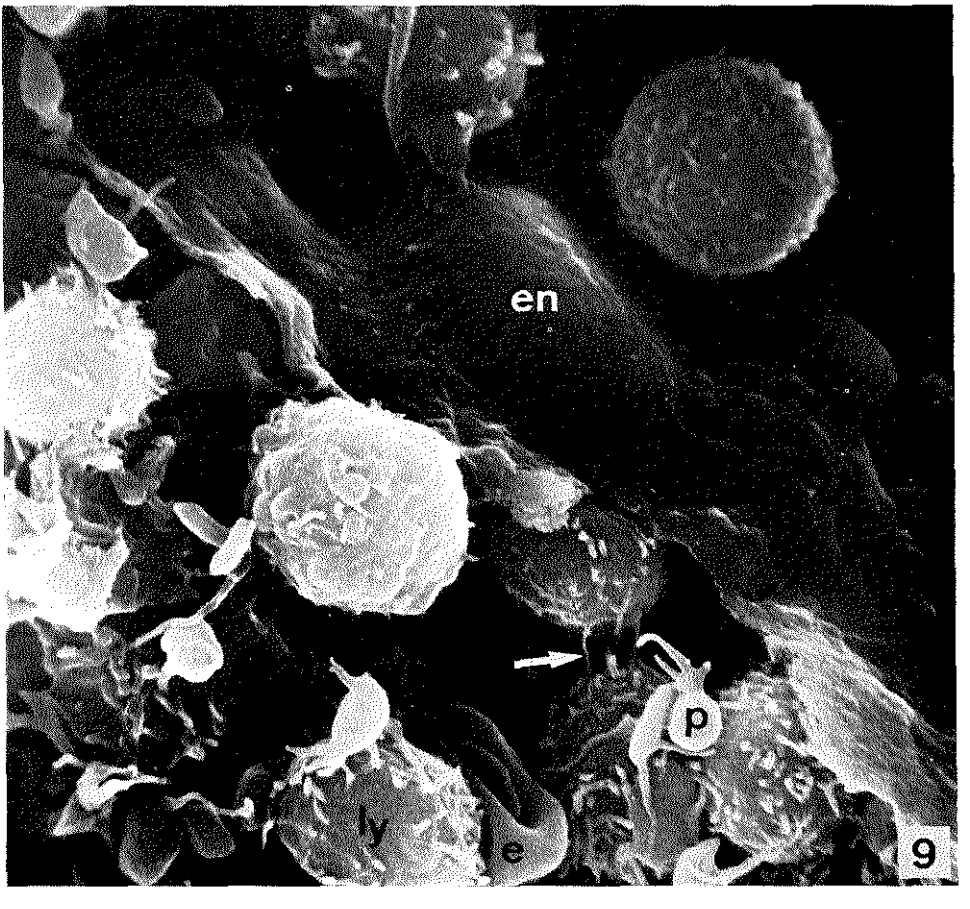
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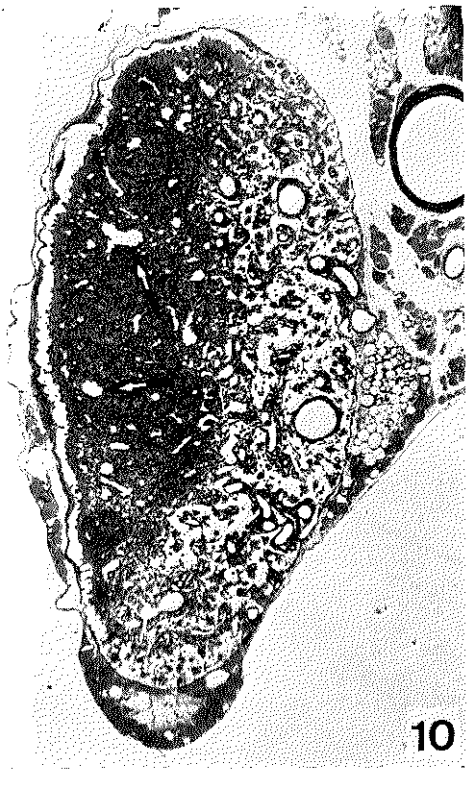


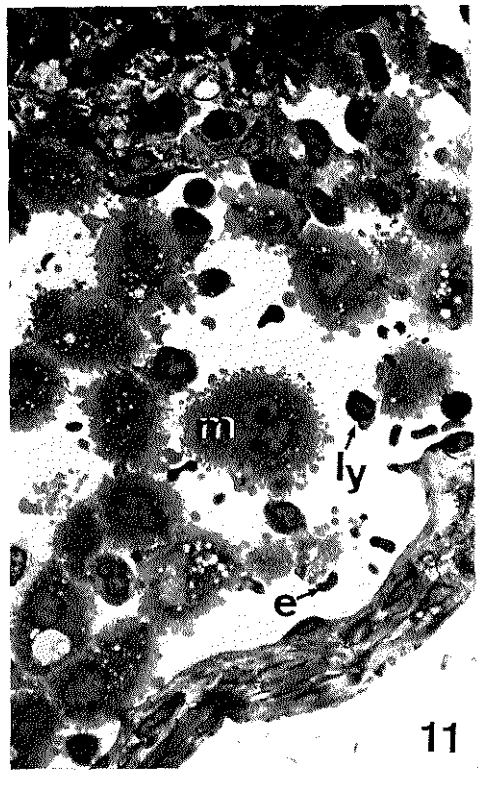


FIG. 10. Low magnification of a semithin section of the mesenteric lymph node of a T mouse. Follicles are absent, medullary cords are reduced in diameter. Postcapillary venules (arrows) are present throughout the paracortex. (×110).

FIG. 11. Medullary sinus of the same lymph node. Large macrophages (m) show an extensive ruffling membrane. Several lymphocytes (ly) are in contact with macrophages. A few erythrocytes (e) are present in the lymph node sinuses. (×920).

were absent and a marginal zone was not clearly expressed. Many macrophages were present in the red pulp and in the remnants of the marginal zone. In the peripheral part of the white pulp mature plasma cells were also found. Their number was reduced compared with the splenic white pulp of B mice and normal mice.

In the scanning electron microscope the central area of the PALS was found to contain reticulum cells and lymphocytes. The lymphocytes again showed a smooth surface. In contrast, the lymphocytes in the most peripheral part of the PALS as well as in the remnants of the marginal zone did show fair numbers of microvilli (Fig. 16, Table 2). They were frequently found to be in contact with

FIG. 8. Survey of the splenic white pulp of a B mouse. Lymphocytes occur in the peripheral part of the PALS (pp). The central PALS (cp) remains depleted of lymphocytes. The boxed area refers to Fig. 9. gc = germinal center, mz = marginal zone, a = central arteriole (×130).

FIG. 9. Marginal zone (left) and red pulp sinus (right) in the spleen of a B mouse. Compare with the boxed area in Fig. 8. B lymphocytes (ly) in the marginal zone contact each other by means of microvilli (arrow). en = endothelial lining of the red pulp sinus, p = platelet, e = erythrocyte (×5800).

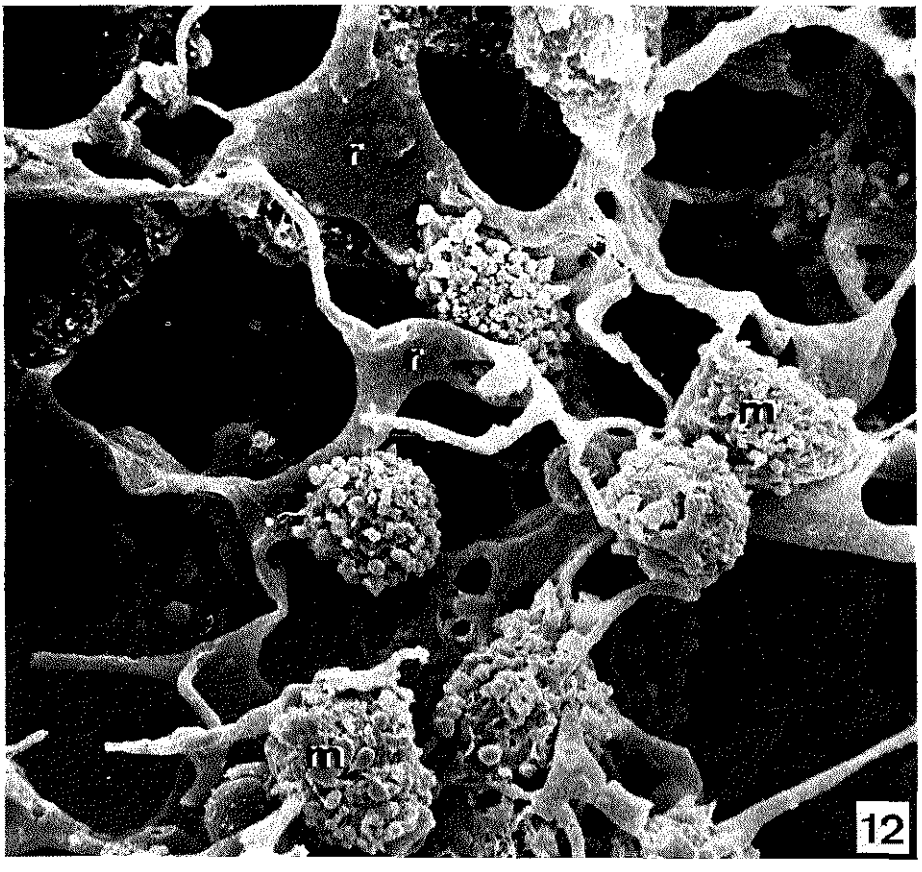


FIG. 12. Macrophages (m) and reticulum cells (r) in the medullary sinus of the mesenteric lymph node of a T mouse. The macrophages exhibit a prominent ruffling membrane which is well preserved due to the critical point drying of the tissue. (×2300).

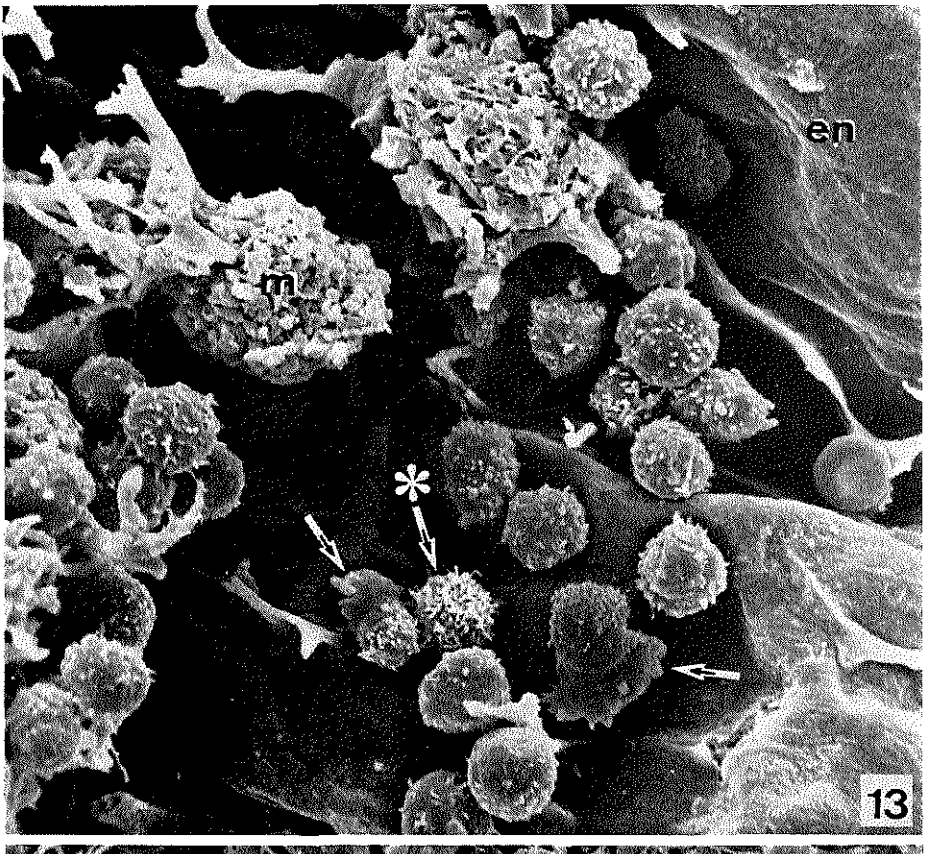
adventitial cells of red pulp sinuses and also with reticulum cells in the marginal zone (Fig. 16, ly'). The latter cells formed a three-dimensional meshwork at this site of the white pulp. In the marginal zone oval cells were found as well, which were approximately twice as large as the round lymphocytes. They also exhibited microvilli (Fig. 16, p). Possibly these cells are lymphoblasts or mature plasma cells.

DISCUSSION

The lymphatic tissues of T and B mice have been observed with the scanning electron microscope while focusing the attention mainly on two types of areas.

FIG. 13. Various types of T lymphocytes in the medullary sinus of the mesenteric lymph node. Most cells show microvilli, some of them display a broad cytoplasmic extension (arrows). This structure is probably involved in the locomotion of the cell over the endothelium. The lymphocyte indicated by an asterisk shows aggregation of microvilli at one pole of the cell. en = endothelial lining of the medullary sinus, m = macrophage (T-mouse) (×2300).

FIG. 14. T lymphocytes (ly) in the stroma of the paracortical area of the mesenteric lymph node. The lymphocytes are smooth surfaced. They are situated between slender processes of reticulum cells (r). (T mouse) (×2300).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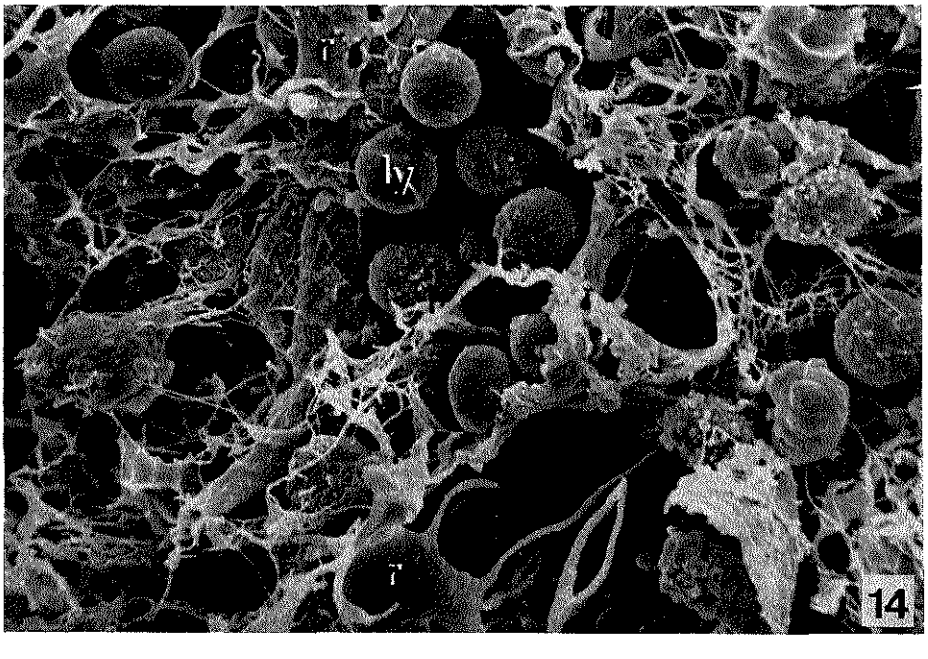
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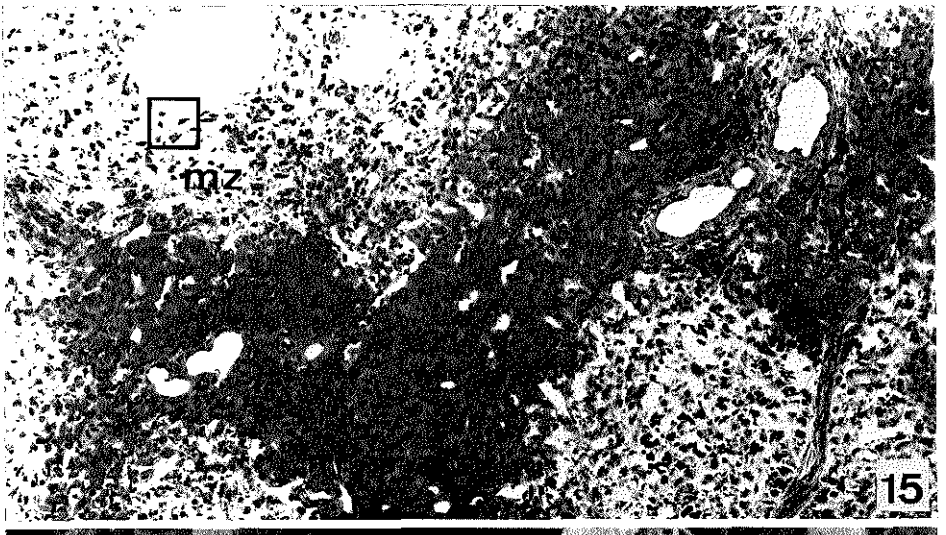
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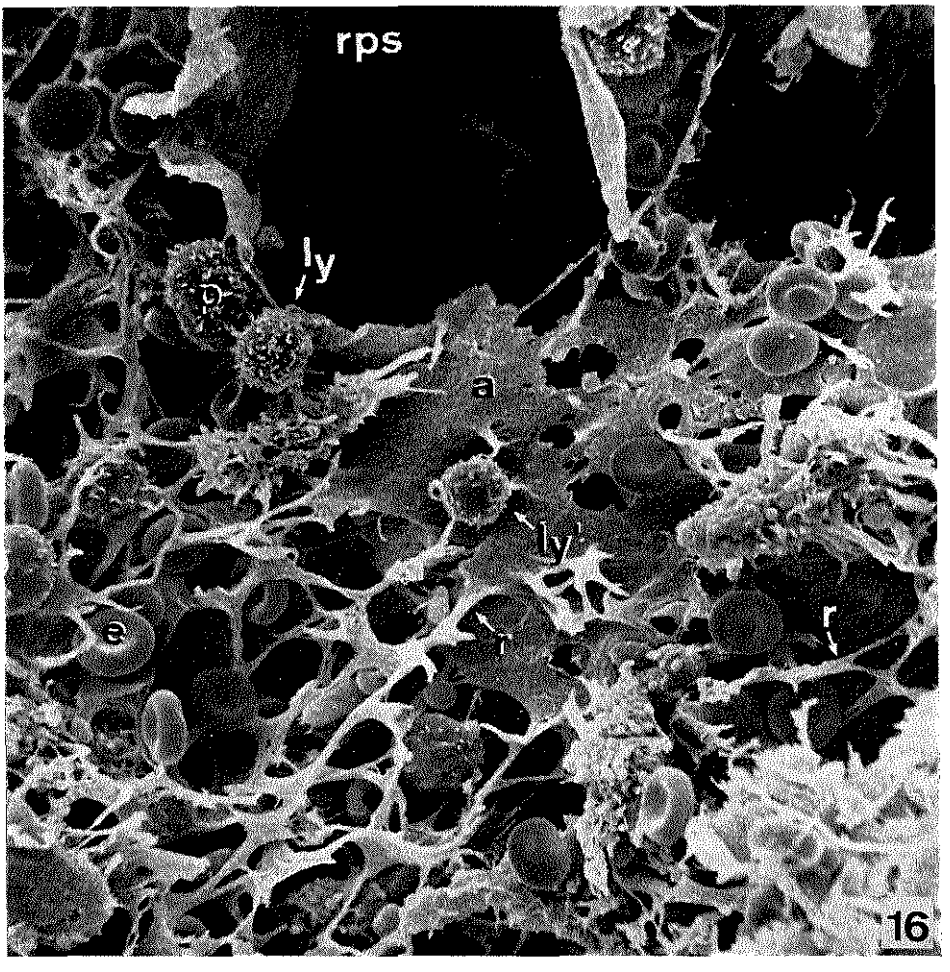
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First, areas were studied which are known to be specific microenvironments for sessile T and B lymphocytes. Second, we have investigated areas where lymphocyte recirculation is known to occur. Sessile T cells occur in the paracortical area of lymph nodes (3), in the spleen they populate the central area of the periarteriolar lymphatic sheath (5). Sessile B cells populate the follicles in the outer cortex of the lymph nodes and the spleen (21). Recirculating lymphocytes are demonstrable in postcapillary venules and efferent lymphatic vessels of lymph nodes (22). In the spleen they occur in the marginal zone (23).

From the present study it is obvious that at least *in vivo* both classes of lymphocytes in mice cannot be distinguished with the scanning electron microscope. Both T and B cells demonstrated microvilli when they were present in postcapillary venules, lymph vessels, sinuses, and marginal zone. Both T and B cells were smooth surfaced when they had reached their respective microenvironments (Table 2). Similar results were obtained in studies of the spleen and mesenteric lymph node of nude mice and of mice, lethally irradiated and reconstituted with 5×10^6 dexamethason-resistant thymocytes (van Ewijk, to be published).

In contrast to our observations, data reported by Polliack and co-workers (8) did suggest a difference in surface architecture of T and B cells. These authors reported that in human peripheral blood 20% of the lymphocytes demonstrated microvilli, while 80% appeared smooth. This finding correlated well with immunological estimates of the percentage of B and T cells in peripheral blood. However, in a subsequent paper (24) on spontaneous rosette forming cells and rosette forming cells of complement receptor lymphocytes, which are known to be specific markers for human T cells and B cells respectively, they found that 15% of the spontaneous rosette forming cells were indistinguishable from complement receptor rosette forming cells, since both were villous. On the other hand, 10% of the B cells turned out to have a smooth cell surface. In this same area the experiments reported by Lin and Wallach (25) are of interest. They studied rosette formation of lymphoid cells in a clone of human leukemic lymphocytes (Molt-4 cells). These cells have T cell properties: they have the same surface structure as thymocytes when viewed by the scanning electron microscope, they lack detectable surface immunoglobulin and form spontaneous rosettes with sheep erythrocytes. However, when rosetting was followed with the scanning electron microscope, it was found that Molt-4 rosette forming cells displayed a time dependent modification of their surface topography: during the rosetting process the smooth surface developed abundant long microvilli. Comparable results have been obtained by Scott, Linthicum, and Sell (26) in scanning electron microscopic studies of rabbit peripheral blood lymphocytes. They noted that cells fixed in suspension had a smooth surface, while cells which were fixed after settling on a glass covers slip

FIG. 15. Survey of the splenic white pulp of a T mouse. The PALS is reduced in diameter and follicles are absent. The marginal zone (mz) is depleted of lymphoid cells. The boxed area refers to Fig. 16. (×130).

FIG. 16. Marginal zone and red pulp sinus (rps) in the spleen of a T mouse. Compare with the boxed area in Fig. 15. T lymphocytes (ly) display microvilli on their surface. Reticulum cells (r) are continuous with the adventitial cells (a) of the sinus. The lymphocyte in the centre of the micrograph (ly') is in contact with a reticulum cell and a cytoplasmic process of an adventitial cell. p = plasma cell, e = erythrocyte (×2300).

displayed progressive formation of microvilli. Also, Lin, Wallach, and Tsai (27) have demonstrated that the number and size of microvilli on cells of the bovine NBC-6 lymphocyte line varied depending on the temperature of the medium. At 25°C the cell surface of these cells was largely obscured by numerous microvilli. Chilling to 4°C reduced their number and increased their diameter.

The last three studies give substantial evidence for the influence of physiological conditions on the presence or absence of microvilli on the surface of lymphocytes.

The question arises what the function of these organelles might be. Scanning electron microscopic studies on various types of cells in tissue culture suggest that these structures are involved in the initiation of intercellular contact during cell aggregation (28). Recently, we have reported fingerlike protrusions emerging from T lymphocytes which are in contact with interdigitating cells in the central area of the PALS (15). In these studies we could demonstrate intercellular bridges (receptors?) between both cell types. It is therefore tempting to speculate on the function of microvilli on lymphocytes in terms of a functional exposure of specific receptor sites on the cell surface to the surrounding cells. In postcapillary venules this mechanism would account for the arrest of lymphocytes on the surface of the high endothelial cells.

Our observations support the view that both T and B cells recirculate via PCV (29). We could not establish whether T and B cells left the venule in different areas. Lymphocytes left the PCV always at places where high endothelial cells were present. In B mice, lymphoid cells were found leaving the vessel throughout the (thymus dependent) paracortical area. It appears that after passing the endothelial lining, the lymphocytes actively migrate through the stroma of the lymph node towards their specific microenvironment. Which factors influence this particular migration pathway of lymphocytes in the stroma is not yet understood.

There have been several reports published in the literature on the route of transit of lymphocytes from the lumen of the postcapillary venules into the surrounding lymphatic tissue. Marchesi and Gowans (30) and Sainte Marie (31) suggested a pathway which led directly through the cytoplasma of endothelial cells. However, Mikata and Niki (32) and Schoefl and Miles (33) studying serial sections of PCV in the electron microscope presented evidence for an intercellular pathway. Our observations are in favour of the intercellular migration pathway: most lymphocytes were found in the intercellular ridges in between the endothelial cells. In addition, it appears from the present observations that the surface structure of lymphocytes passing the endothelial border changes during transition. The lymphocyte on the left of Fig. 6 is probably in the initial stage of transit. It may be noted that the number and size of the microvilli are reduced toward the endothelial wall. Figure 3 shows several smooth spherical bodies which are smaller in size than the lymphocytes present in the lumen of the venule. These observations strongly suggest that the lymphocyte retracts its microvilli while migrating across the wall of the PCV.

In conclusion, the present results suggest that a smooth cell surface represents a quiescent state of the lymphocyte when it is situated in its specific microenvironment. The exposure of microvilli, on the other hand, might indicate an activated state of recirculating lymphocytes. Microvilli on the surface of lymphoid cells are considered to be cell organelles involved in cellular recognition mechanisms.

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Cell surface labelling of mononuclear cells with antisera associated to turnip yellow mosaic virus or alphalpha mosaic virus particles. A freeze-etch study

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Synopsis. Turnip yellow mosaic virus (TYMV), and alphalpha mosaic virus (AMV) were used as immuno-electron microscopical markers to detect cell surface receptors on mononuclear cells in freeze-etch replicas. TYMV particles were conjugated with vacuum-distilled glutaraldehyde to rabbit IgG anti-mouse immunoglobulins (TYMV–RAMIg conjugate) or to rabbit IgG anti-mouse θ antigen (TYMV–RAMTh conjugate). B-lymphocytes incubated with TYMV–RAMIg conjugate showed either randomly distributed particles or patches of virus particles on the etched surface of the cell membrane. Mouse thymocytes incubated with TYMV–RAMTh conjugate, however, showed only a random distribution of the virus particles. Human mononuclear cells incubated with rabbit IgG anti-AMV and AMV for the demonstration of the receptors for the Fc fragment of IgG showed the oblong shape of the AMV particles on the etched cell membrane. Fc receptors were either randomly distributed or aggregrated into patches. It is concluded that both types of virus particles are useful markers for the demonstration of membrane receptors in freeze-etch replicas of labelled cells.

Introduction

Differentiation between lymphoid subpopulations based on pure morphological criteria using transmission electron microscopy (TEM) is hampered by the heterogeneity in cell size, the shape of the nucleus, and the number and distribution of cell organelles (Zucker-Franklin, 1969; Veerman & van Ewijk, 1975). According to Polliack *et al.* (1973), Lin *et al.* (1973) and others, the surface structure of the two major lymphocyte subclasses, i.e., T- and B-cells, can be distinguished by scanning electron microsocopy (SEM). These studies on lymphocyte suspensions *in vitro* revealed that T-lymphocytes had a smooth cell surface, whereas B-lymphocytes showed many microvilli. Recently, Alexander *et al.* (1976), criticized these observations and argued that the procedures used for the preparation of cells for SEM could influence the topography. SEM investigations on lymphoid subpopulations *in vivo* (van Ewijk *et al.*,

1975) also failed to reveal characteristic differences in the cell surface of T- and B-cells, but showed that, regardless of their origin, lymphocytes in recirculation pathways possessed microvilli, whereas those present in their respective micro-environments showed a smooth cell surface.

Adequate submicroscopical characterization of lymphoid cells seems only possible if specific cell surface markers are being used. Several marking techniques using different electron-dense markers have been reported for TEM studies, e.g. ferritin (Singer, 1959) and horse-radish peroxidase (Nakane & Pierce, 1966; Avrameas, 1969).

The present paper describes a method for the demonstration of cell surface determinants on lymphoid subclasses by the use of small virus particles as electron microscopical markers. The freeze-etch technique has been used to investigate the labelled cells since this method allows high resolution studies of relatively large membrane areas in the transmission electron microscope (Moor & Mühletahler, 1963; Pinto da Silva & Branton, 1970).

Materials and methods

Viruses

Turnip yellow mosaic virus (TYMV) and alphalpha mosaic virus (AMV) used as immuno-electronmicroscopical markers were generously provided by Dr E.M.J. Jaspars of the Department of Biochemistry, University of Leiden, The Netherlands. Virus suspensions were stored in a concentration of 42 mg/ml in a solution of 0.01 M sodium acetate (pH 6.4) supplemented with 0.001 M sodium azide.

Antisera

Lyophilized rabbit IgG anti-mouse immunoglobulin (RAMIg) and rabbit IgG anti-human immunoglobulin (RAHuIg) was obtained commercially (Nordic Immunological Laboratories, Tilburg, The Netherlands). Rabbit anti-mouse θ serum (RAMTh) was prepared by Dr R. Benner and Dr W. B. van Muiswinkel (Department of Cell Biology and Genetics, Erasmus University, Rotterdam) according to Jooste *et al.* (1968). Rabbit anti-AMV was prepared by repeated intravenous injections of rabbits with AMV in concentration series from 0.5 to 4 mg AMV. Rabbits were immunized at 3-day intervals over a period of 25 days. Tested in the Ouchterlony assay, a precipitation line was found at a serum dilution of 1:27.

Coupling of viruses to antisera

As coupling reagent, glutaraldehyde (Fluka AG, Buchs S. G., Switzerland) was used. In order to remove glutaric acid and polymerized glutaraldehyde, the glutaraldehyde solution was vacuum distilled according to Fahimi & Drochmans (1965). Having a final concentration of about 45%, spectrophotometric analysis of the purified glutaraldehyde showed only one peak, at 280 nm. The stock solution was stored at pH 5.0 under gaseous nitrogen in the dark at 4°C in 1 ml aliquots. Before use, the glutaraldehyde was diluted with phosphate-buffered saline (PBS), pH 7.2, to a final concentration of 1.5%.

The coupling of virus to immunoglobulins was carried out in two steps analogous to the method for peroxidase conjugation described by Avrameas & Ternynck (1971).

(1) 2.1 mg TYMV was added to 0.1 ml 1.5% glutaraldehyde to a final volume of 0.5 ml PBS. After stirring at room temperature for 30 min, the mixture was dialysed for 48 h against PBS, pH 7.2, at 4°C to remove excess glutaraldehyde.

(2) The diffusate was then transferred to a glass tube and 0.3 ml dissolved RAMIg or 0.3 ml RAMTh was added to the glutaraldehyde-activated virus suspension after which the mixture was stirred for 30 min at room temperature.

The crude conjugates of TYMV coupled to RAMIg, (TYMV–RAMIg conjugate), or RAMTh (TYMV–RAMTh conjugate) were purified by gel filtration on a Sephadex G200 column (Pharmacia, Uppsala, Sweden) equilibrated with PBS, pH 7.2. The virus-containing fraction, as judged by electron microscopical analysis of negatively-stained samples, was collected and concentrated to a final volume of 2 ml by vacuum dialysis using collodion bags (SM 13200; Sartorius-Membranefilter GmbH, Göttingen, Germany). A sample of the conjugate, containing approximately 0.5% protein, was evaluated with respect to homogeneity in an analytical ultracentrifuge (Spinco, model E).

The virus suspension and the purified conjugate were investigated with the transmission electron microscope according to the negative-staining method described by Horne & Pasquali-Ronchetti (1974). We used 2% sodium molybdate (pH 5.3) as the initial stain and 1% uranyl acetate (pH 5.4) as a secondary stain.

Cells

Lymphoid cell suspensions enriched for B-lymphocytes were obtained from the mesenteric lymph node of nude mice (*nu/nu*, the outcome of the fourth backcross matings to B 10 LP/JP h mice, obtained from the Central Institute for the Breeding of Laboratory Animals, Bilthoven, The Netherlands). The cell suspensions were prepared by pressing the node gently through a nylon gauze with a pore size of 100 μm.. Cells were collected in PBS and washed three times before incubation with the conjugate. Suspensions of T-cells were similarly obtained from the thymus of 4 to 6-week-old (DBA/2xC57BL/Rij) F1 male mice.. Human mononuclear cells were obtained from peripheral blood by Ficoll/Isopague density gradient centrifugation as described elsewhere (De Vries *et al.*, 1976).

Labelling experiments

In order to demonstrate surface immunoglobulins on B-lymphocytes, 0.1 ml TYMV–RAMIg conjugate was added to 3×10^7 nucleated cells from the mesenteric lymph node of nude mice. The cells were incubated at 4°C for 30 min and then washed three times with PBS to remove unbound conjugate. As controls, 3×10^7 B-lymphocytes were incubated, as described above, with RAMIg before incubation with TYMV–RAMIg conjugate; or with TYMV, pre-incubated with 1.5% glutaraldehyde and dialysed for 48 h against PBS (glutaraldehyde-activated virus); or with 2.1 mg untreated TYMV.

In order to determine the specificity of the antiserum component of the conjugate, 3×10^7 T-lymphocytes were incubated with 0.1 ml TYMV–RAMIg conjugate.

For the demonstration of θ-antigen on the T-cell membrane, 3×10^7 T-cells were incubated with 0.1 ml TYMV–RAMTh conjugate and subsequently processed as described for Ig-receptors. As control, thymocytes were incubated with RAMTh

serum before incubation with the TYMV–RAMTh conjugate. After three washes with PBS, the cells were processed for freeze-etching.

For the demonstration of receptors for the Fc fragment of IgG, we used a procedure analogous to the method of EA rosettes, described by Zeylemaker *et al.*, (1974). For this preparation, 3×10^7 cells were incubated with 0.3 ml dissolved, heat-inactivated rabbit IgG anti-AMV and with 0.2 mg AMV, in a final volume of 1.0 ml PBS. After incubation at 37°C for 15 min, the cells were spun down and incubated at room temperature for another 20 min. As the control, 3×10^7 mononuclear cells were incubated with 0.3 ml RAHuIg before incubation with 2.0 mg AMV. After three washes with PBS, the cells were processed for freeze-etching.

Freeze-etching of labelled cells

For freeze-etch studies, the labelled cells were fixed briefly in 1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer (pH 7.2, 370 mosm), washed twice with 0.14 M sodium cacodylate buffer and concentrated to a volume of about 0.1 ml. Aliquots of

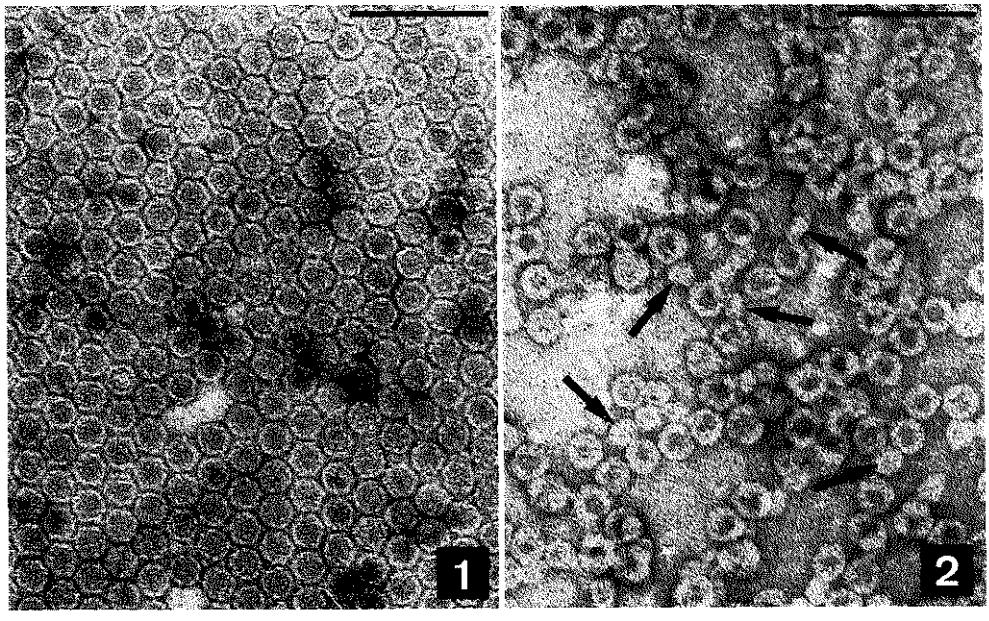

Figure 1. TYMV particles, negative–positive staining (Horne & Pasquali-Ronchetti; 1974). Note the regular hexagonal pattern of the assembled virus particles. Bar = 1000 Å.

Figure 2. TYMV conjugated with RAMIg, negative–positive staining. The regular hexagonal pattern is no longer present. Smaller ellipsoid particles (150–200 Å) are visible adjacent to virus particles (arrows). Bar = 1000 Å.

about 2 µl were transferred to silver specimen holders and quickly frozen in solid nitrogen (for details, see Ververgaert, 1973). In the present study, no cryoprotective agent was used. The frozen specimen holders were mounted on the pre-cooled specimen stage (–150°C) of a Polaron freeze-etch module or a Balzers B.A. 360 M freeze-etch unit. The frozen specimens were fractured at –100°C under a vacuum

below 2×10^{-6} Torr, and etching was allowed to proceed for periods as stated in the legends to the figures. During etching, the anti-contamination shield in the freeze-etch device was kept at $-192°C$. Shadowing was carried out with a platinum–carbon mixture evaporated under an angle of 45° and with carbon evaporated under an angle of 90°. The specimen holders were then transferred to atmospheric pressure and room temperature, and the replicas stripped off in distilled water and cleaned in 15% sodium hypochlorite. After being washed in distilled water, the replicas were collected on copper grids (400 mesh) and examined in a Philips EM200 or a Philips EM300 electron microscope, operated with an accelerating voltage of 60 kV and a 40 μm objective aperture.

Results

TYMV and TYMV–RAMIg conjugate

TYMV particles prepared for negative staining aggregated in a hexagonal pattern during air-drying of the specimens (Fig. 1). The virus particles showed a uniform diameter of 280 Å. Due to the staining with uranyl acetate, the core of the virus particles was positively contrasted. In preparations of TYMV–RAMIg conjugate, the virus particles did not show this typical configuration, but were distributed randomly (Fig. 2). Adjacent to approximately 50% of the virus particles, smaller negatively-stained particles measuring 150–200 Å were present.

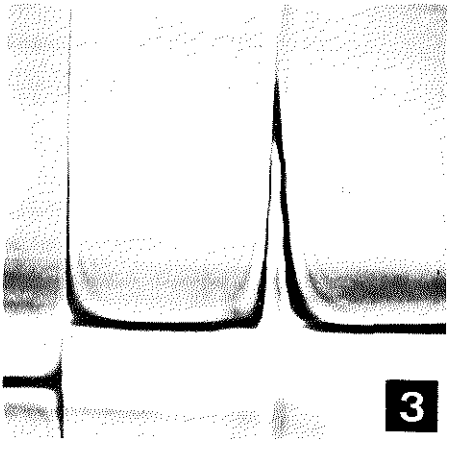

Figure 3. Analytical ultracentrifuge diagram of TYMV–RAMIg conjugate run at 32 000 rev/min at 21°C, 15 min after start.

The TYMV–RAMIg conjugate was studied for homogeneity in an ultracentrifuge. As can be seen from Fig. 3, the conjugate showed only one peak, suggesting the presence of only one molecular species. No distinction could be made between conjugated and non-conjugated virus particles, because of the large difference in molecular weight of the virus particles (mol. wt. 5.4×10^6) and IgG molecules (mol. wt. 1.6×10^5).

Freeze-etched cells

Replicas of freeze-etched lymphocytes showed the hydrophobic fracture plane and the outer surface of the cell membrane, as illustrated in Fig. 4. The fracture face showed a homogeneous distribution of intramembranous particles of about 80 Å in diameter. Pits measuring about 100 Å in diameter were also observed.

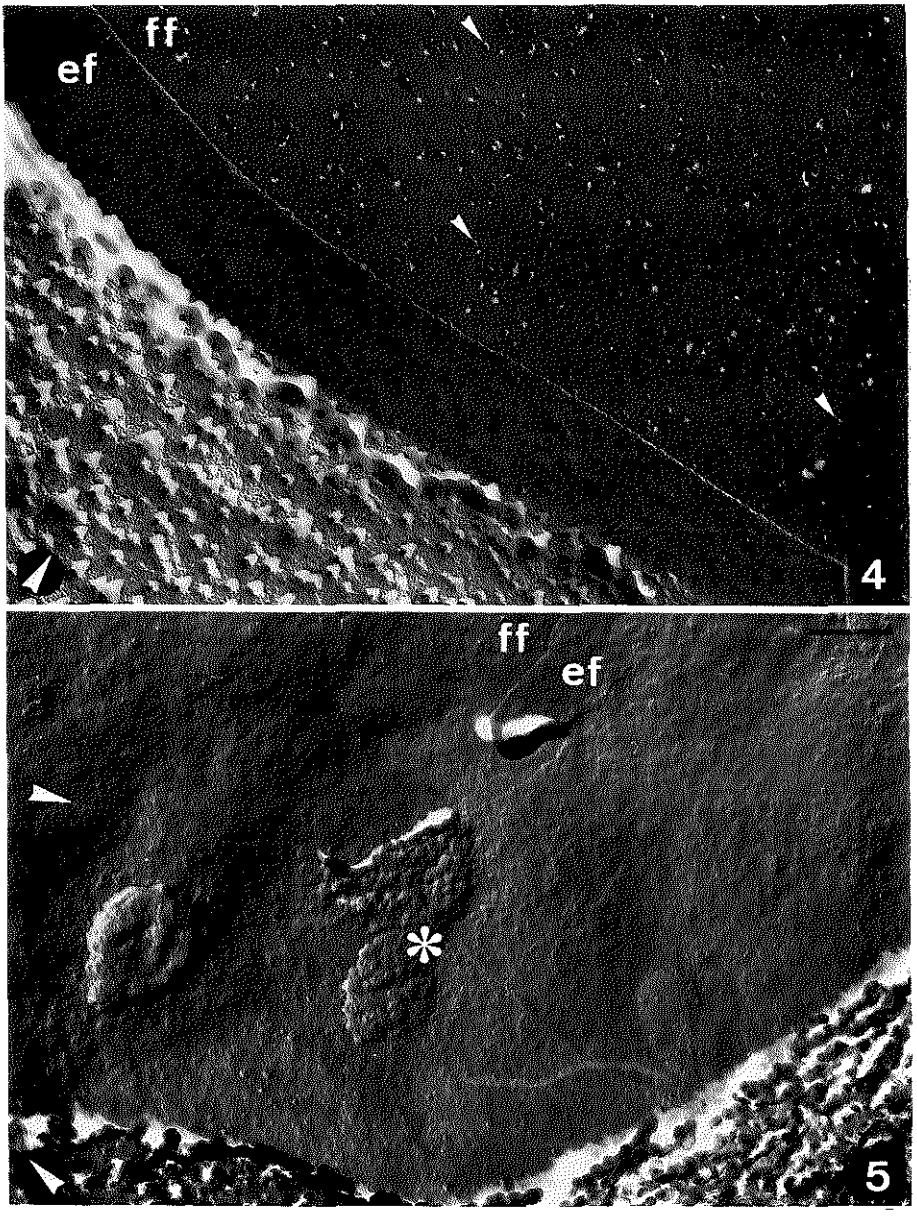

Figure 4. Freeze-etch replica of a mouse B-lymphocyte. The fracture face (ff) shows particles (white arrows) and pits (black arrows). The etched face (ef) is smooth, intramembranous particles can be seen protruding the etched face of the membrane. The cell was frozen without cryoprotectant and etched for 3 min at −100°C. The encircled arrow indicates the platinum evaporation direction. Bar = 1000 Å.

The etched surface of the cells showed neither particles nor pits. However, under a low angle of platinum shadowing the intramembranous particles appeared to protrude the cell surface. We were not able to discriminate between T- and B-cells on the basis of cell surface topography.

Mouse B-lymphocytes incubated with TYMV–RAMIg conjugate frequently showed aggregation of the marker into patches (Fig. 5), as well as a random distribution of the virus particles. When the cells were incubated successively with RAMIg and TYMV–RAMIg conjugate, no virus particles could be detected on the etched surface.

When mouse thymocytes were incubated with TYMV–RAMTh conjugate, only a random distribution of the virus particles on the etched surfaces was observed (Fig. 6). Incubation of thymocytes with RAMTh serum before incubation with TYMV–RAMTh conjugate resulted in a total absence of virus particles on the etched surfaces, and the same occurred after incubation of thymocytes with TYMV–RAMIg conjugate.

In order to determine whether non-specific binding of TYMV or glutaraldehyde-activated TYMV to the cell membrane could mask the specific labelling with the conjugates, lymphoid cells were incubated with TYMV and glutaraldehyde-activated TYMV suspensions. Non-specific binding by B-or T-cells did not occur in either case.

Receptors for the Fc fragment of IgG on human mononuclear cells were detected after incubation of these cells with rabbit IgG anti-AMV and AMV (Fig. 7). Both patches and random distribution of the characteristic 250–500 Å particles were seen. After incubation with RAHuIg antiserum and AMV, no virus particles were observed, thus indicating the specificity of the labelling with rabbit IgG anti-AMV and AMV for Fc receptors.

On the etched surfaces of labelled cells, the virus particles seemed to be impressed into the cell membrane (Fig. 4). The fracture plane of labelled thymocytes showed pits with a diameter corresponding to that of virus particles. In control experiments, such pits were not observed.

Discussion

In freeze-etch replicas of lymphocytes from thymus and lymph node suspensions, we could not differentiate between cell membranes of the two lymphoid subclasses on the basis of morphological criteria. Both cell types showed particles and pits on the fracture plane. With respect to the intramembranous particles, some evidence has been obtained that in erythrocytes these structures are related to antigenic structures on the cell surface (Tillack *et al.*, 1972). In mouse thymocytes, however, Matter & Bonnet (1974) were unable to demonstrate a relationship between intramembranous particles and the θ-antigen.

← *Figure* 5. Freeze-etch replica of a mouse B-lymphocyte after incubation with TYMV–RAMIg conjugate. On the etched face (ef) of the cell membrane aggregates of the virus particles are seen (asterisk), adjacent to areas free of label. Etching was allowed for 5 min at -100°C. The white arrow indicates the separation line between fracture face (ff) and etched face. The encircled arrow indicates the platinum evaporation direction. Bar = 2000 Å.

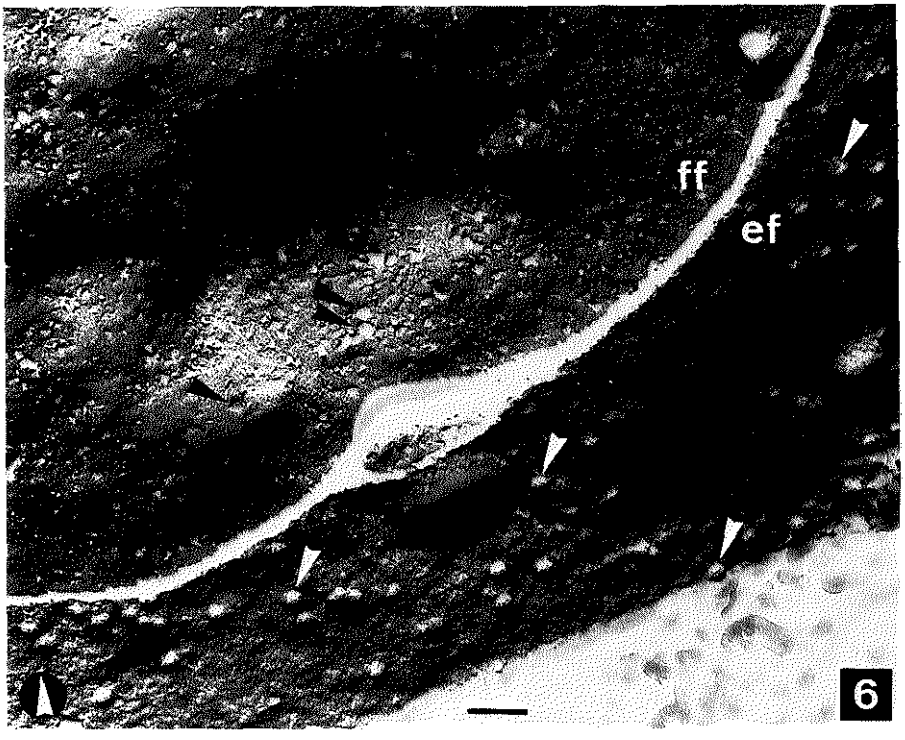

Figure 6. Mouse T-lymphocyte incubated with TYMV–RAMTh conjugate. A random distribution of the label (white arrows) is seen on the etched face (ef) of the cell. On the fracture face (ff) imprints of the virus particles can be seen (black arrows). Etching time was 5 min, at −100°C. The encircled arrow indicates the platinum evaporation direction. Bar = 1000 Å.

In order to investigate differences between the membrane architecture of lymphoid subpopulations, and to study a possible relation between antigenic structures and intramembranous particles, labelling of cells with markers detectable in the electron microscope is required. For SEM studies, several cell-surface labelling techniques have been described in which large virus particles were used as immuno-electron microscopical markers, for example tobacco mosaic virus (Hämmerling *et al.*, 1975, Nemanic *et al.*, 1975) and T4 bacteriophages (Kumon *et al.*, 1976). In this study, we used the freeze-etch technique and selected small virus particles ranging in diameter from about 250–500 Å. This range in diameter of markers enables a high resolution in TEM studies of freeze-etch replicas. Smaller markers (e.g. ferritin) are sometimes difficult to distinguish from contaminating particles which occur in some cases as a result of long etching periods.

B-cells incubated with TYMV–RAMIg conjugate at 4°C for 30 min often showed the marker distributed in aggregations (Fig. 5). As indicated by the results of analytical ultra-centrifugation and negative staining of the conjugate, non-specific aggregation of virus particles during or after the conjugation steps is not likely. Therefore, aggregates

of label on the cell surface may be considered as a result of the interaction of conjugated IgG molecules with surface associated Ig, i.e. as patch formation. Patch formation is a well known phenomenon in B-cells incubated with an anti-Ig antiserum (Bretton, 1972; Raff & de Petris, 1973). Unlike labelled B-cells, T-lymphocytes incubated with TYMV–RAMTh conjugate under the same conditions (i.e. at 4°C) showed a homogeneous distribution of the virus particles (Fig. 6). This observation is in agreement with results obtained in immuno-fluorescence (Raff & de Petris, 1973) and rosetting studies (de Vries *et al.*, 1976). Human mononuclear cells labelled for receptors for the Fc fragment of IgG with AMV and Rabbit IgG anti-AMV also showed patch formation. This observation is consistent with earlier observations on EA rosettes (de Vries *et al.*, 1976).

Control experiments revealed that the antigen binding capacity of the IgG molecules was maintained in TYMV–RAMIg conjugate and in TYMV–RAMTh conjugate, as shown by blocking experiments with non-conjugated RAMIg or RAMTh. In addition, no a-specific binding to lymphoid cells was observed with either glutaraldehyde-activated virus particles or with pure virus particles. However, we found it essential to use distilled glutaraldehyde since earlier experiments in which we conjugated with commercially available glutaraldehyde gave negative results. In enzyme histochemistry too, it is known that enzymes are preserved only after fixation with monomeric glutaraldehyde; the polymeric glutaraldehyde and glutaric acid present in commercial grades of glutaraldehyde reduce their activity (Anderson, 1967).

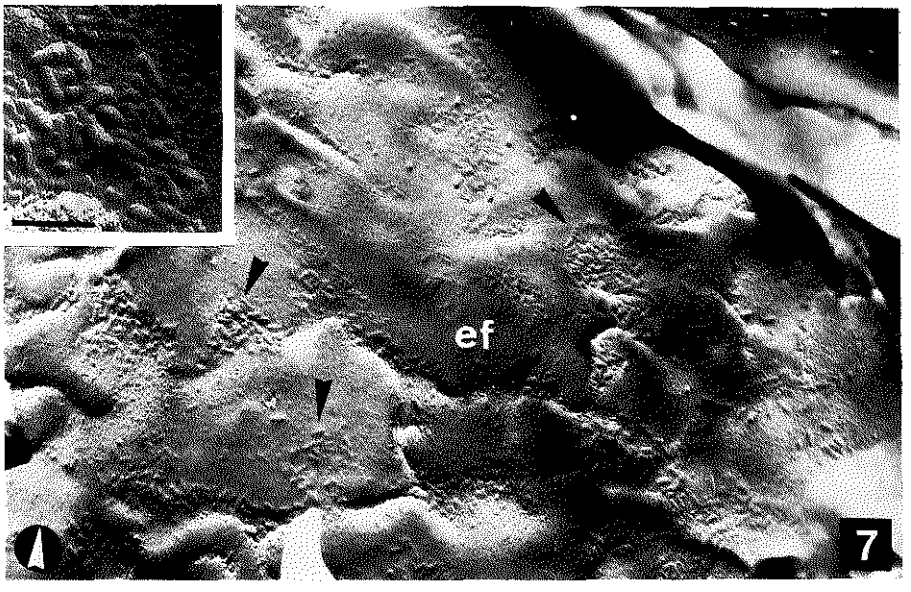

Figure 7. Human mononuclear cell, incubated with rabbit IgG anti-AMV and AMV for the detection of receptors for the Fc fragment of IgG. AMV particles (arrows) can be seen on the etched face (ef). Etching time was 1 min, at −100°C. The encircled arrow indicates the platinum evaporation direction. Inset: higher magnification of AMV particles on the etched face of the cell membrane. Bars = 2500 Å and 1000 Å (inset).

It should also be mentioned that the use of AMV in the two-step conjugation method did not give satisfactory results. In the electron microscope, RAMIg–AMV conjugates did not show the characteristic 250–500 Å particles; instead, most of the particles were spherical. Apparently the AMV is less stable than the TYMV, resulting in deformation of the AMV particles during preparation of the conjugate. AMV particles, however, serve very well in sandwich labelling techniques, using antisera directed against AMV.

Comparison between the virus images of non-conjugated and IgG conjugated particles in negatively-stained preparations revealed the presence of small ellipsoid particles of about 150–200 Å. Since these particles have a similar diameter to those of immunoglobulin molecules (Valentine & Green, 1967), we assume they reflect IgG molecules coupled to the virus particles by means of glutaraldehyde. Incubation of TYMV particles with rabbit anti TYMV IgG that was prepared in the same way as rabbit anti-AMV IgG also showed particles of 150–200 Å associated with the virus particles in negatively stained preparations (results not illustrated). This observation supports the view that ellipsoid 150–200 Å particles indeed represent immunoglobulins.

It may be concluded that the use of different strains of viruses with regard to size and shape like TYMV and AMV permits the demonstration of different specific cell surface determinants on mononuclear cells. In principle, different receptors may be demonstrated on the same cell with the present method. Also, it is possible to investigate a relation between antigenic structures and intramembranous particles, since a replica of the virus particle is visible on the fracture plane of the freeze-etched cell.

Preliminary experiments carried out in our laboratory on lymphoid cell suspensions labelled with TYMV–RAMIg and prepared for scanning electron microscopy (see also, van Ewijk & Mulder, 1976) have revealed spherical particles of similar size as TYMV particles on the surface of lymphoid cells. This indicates that the present method can also be applied for the demonstration of receptors on cells studied in the scanning electron microscope.

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Curriculum vitae

Na het behalen van het diploma HBS-b aan de Gemeentelijke Lorentz-HBS te Arnhem ben ik in 1961 begonnen met de studie biologie aan de Rijksuniversiteit te Utrecht. In de periode na het candidaatsexamen gaf ik gedurende 2 jaar biologielessen aan leerlingen van de Lorentz-HBS te Arnhem en gedurende 1 jaar aan leerlingen van de Rehoboth Kweekschool te Utrecht.

Het doctoraal examen in de biologie, met als hoofdvakken: histologie, Prof. Dr. J.C. van de Kamer; elektronenmikroskopie, Dr. P.F. Elbers, en bijvak: chemische dierfysiologie, Prof. Dr. H.J. Vonk, werd door mij in 1969 met goed gevolg afgelegd.

Hierna trad ik in dienst van de Medische Faculteit Rotterdam, als wetenschappelijk medewerker aan de afdeling Celbiologie en Genetica. Nadat in 1972 de trillingen in de werkruimten van dit instituut tot rust waren gebracht, kon worden aangevangen met het wetenschappelijk onderzoek dat in dit proefschrift wordt beschreven.