Experientia 41 (1985), Birkhäuser Verlag, CH-4010 Basel/Switzerland

Characterization of lymphoid and nonlymphoid cells in the white pulp of the spleen using immunohistoperoxidase techniques and enzyme-histochemistry

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Key words. Spleen; lymphoid and non-lymphoid cells; enzyme histochemistry.

Introduction

The main function of the lymphoid system is to offer an opportunity for immune reactions against foreign substances or organisms. In vitro studies show that both T and B lymphocytes as well as mononuclear phagocytes are essential for most types of immune responses. In vivo the interaction between antigen, T lymphocytes, B lymphocytes and mononuclear phagocytes takes place within a frame-work of reticular cells in the peripheral lymphoid organs; the outcome of these interactions is likely to be determined by the precise micro-environment situation in which they meet[31]. In the peripheral lymphoid organs T and B lymphocytes are located in defined compartments which are also populated by characteristic nonlymphoid cells[26, 50, 70]. The (sub)classes of lymphocytes can be defined by their surface determinants and visualized with immunohistoperoxidase methods. At the light microscopical level nonlymphoid cells are mostly characterized by their enzyme histochemical properties and/or functional capacity. At the moment an increasing number of monoclonal antibodies against antigens present on reticular elements is becoming available[1]. These antigens are mostly shared by several lymphoid and nonlymphoid cells. A useful tool to distinguish between these different cell types is the combination of immunohistoperoxidase techniques and enzyme histochemistry on the same section. The aim of this paper is to illustrate the possibilities of immunohistoperoxidase techniques in morphological studies of the white pulp. A review is presented on the characterization and localization of lymphoid cells and, particularly, nonlymphoid cells in the white pulp, using immunohistoperoxidase techniques and enzyme histochemistry. The antigen-induced transformation of lymphocytes to plasma cells is also described.

Aspects of immunohistoperoxidase and enzyme histochemical techniques

For the characterization of immunoglobulin containing (plasma) cells with anti-immunoglobulin antisera we use tissue fixed for 4 h in a sublimate-formaldehyde solution (6 g $HgCl_2$, 5 ml glacial acetic acid, 10 ml 40% formaldehyde solution and 85 ml distilled water)[5]. The tissue pieces are embedded in low melting point paraffin wax (42–44 °C).

For the demonstration of surface membrane constituents or extracellularly located immune complexes cryostat sections of frozen unfixed tissue are used. For the characterization of surface antigen, cryostat sections are fixed in acetone for 10 min. Both before and after acetone fixation cryostat sections are air-dried for at least 30 min.

In our studies we generally use the indirect immunoperoxidase technique. This procedure is carried out as follows. Before the incubation with the antisera the sections are washed in 0.01 M phosphate buffered saline (PBS, pH 7.4). Sections are then covered with the appropriate dilution of antisera for 30–60 min and, after three washings in PBS, this is followed by incubation for 30 min with horseradish peroxidase (HRP) labeled antibodies against the IgG of the species in which the first antisera were raised. HRP is thereafter revealed by the diaminobenzidine (DAB) method[19] (5 mg DAB in 10 ml Tris-HCl buffer, 0.05 M, pH 7.6, containing 0.01% H_2O_2) for 5 min. The sections are briefly counterstained with haematoxylin, washed in tap water for 15 min, dehydrated and embedded in malinol. During the incubation with the antibody solutions and the DAB solution slides are kept horizontal. All procedures are carried out at room temperature. The same immunohistoperoxidase staining procedure is used for both cryostat and paraffin sections. When using cryostat sections it is advisable to use a two step glutaraldehyde prepared HRP-conjugate rather than a periodate prepared one[3, 4].

For the demonstration of cells involved in immune complex trapping, HRP-anti-HRP complexes are intravenously injected[7]. 24 h later spleen pieces are removed and frozen in liquid nitrogen. Cryostat sections are fixed in Baker's formol for 10 min, incubated with an HRP solution and, after three washings with PBS, stained for peroxidase activity with the DAB method[56]. Immunohistoperoxidase techniques may be successfully combined with histological staining procedures or enzyme histochemistry. It is essential that the immunohistoperoxidase reaction precedes other staining procedure[10, 58]. Immunohistoperoxidase techniques may also be combined with autoradiography on the same tissue section[52]. The combination of these two techniques extends, for instance, the possibilities for lymphocyte migration studies[53]. The principles of double immunoenzymatic labeling to detect two antigens simultaneously in the same tissue section have been recently reviewed[46].

Morphology of the white pulp

The splenic white pulp consists of three major compartments; the periarteriolar lymphocyte sheath (PALS), the follicles and the marginal zone surrounding both PALS and follicles. The PALS consists of an inner and an outer part. The outer part is characterized by concentric sheaths of reticulin fibers[70]. Sections of spleen stained with methyl-green pyronin show that PALS and primary follicles are populated with small lymphocytes with darkly stained nuclei. Follicles may exhibit a folli-

cle center (germinal center) surrounded by a corona of small lymphocytes. This type of follicle is called a secondary follicle. The marginal zone contains predominantly medium-sized lymphocytes. These cells are characterized by a round, pale staining nucleus with one or two nucleoli and a fair quantity of faintly pyroninophilic cytoplasma[36]. The marginal zones are the largest white pulp compartments in the rat[40]. They contain approximately as many lymphocytes as the small lymphocytes zone.

Lymphoid cells

T and B cells reside in different areas of the white pulp as demonstrated by postnatal thymectomy[50, 68] and detection of characteristic membrane antigens by immunohistoperoxidase[27] or immunofluorescence techniques[18, 22]. T cells are mainly present in the central area of the PALS and B cells in the peripheral part of the PALS, follicles and marginal zone. With anti-IgM (anti-μ) and anti-IgD (anti-δ) antibodies it was found in rats that marginal zone B cells show a predominantly $\mu^+\delta^-$ surface phenotype while follicular B cells mainly express μ and δ on their surface[20]. In rats, no dominant $\mu^+\delta^-$ lymphoid areas are present in lymph nodes and Peyer's patches[20]. The medium-sized lymphocytes in the marginal zone exhibit a strong membrane staining for IgM; the follicular small lymphocytes a moderate membrane staining for IgM[9] (fig. 1). Ontogenetic studies of the peripheral lymphoid organs show that the marginal zone, populated by medium-sized lymphocytes with strong IgM membrane staining, develops as a distinct compartment, independently from PALS and follicles, and is exclusively present in the spleen[9, 12]. The characterization of the T cell subclasses in the white pulp is described in the contribution of van Ewijk (this volume).

Nonlymphoid cells

Reticular cells and macrophages occur in all white pulp compartments. Reticular cells which synthesize reticulin fibers can be demonstrated by staining these fibers. Macrophages can be demonstrated with enzyme histochemical methods. In follicle centers are macrophages characterized by the ingested lymphocytes present; these phagocytes are known as tingible body macrophages.

In the white pulp there are nonlymphoid cells with a dendritic morphology. In contrast with reticular cells and macrophages these dendritic cells are present exclusively in defined white pulp areas. The nomenclature of the dendritic cells used in this paper has been recommended recently[66]. The follicles contain nonlymphoid cells which are capable of arresting antigen-antibody complexes at the cell surface; the so-called follicular dendritic cells (FDC). The central area of the PALS contains interdigitating cells (IDC). Another cell type with dendritic morphology can be found between adherent cells of cell suspensions prepared from peripheral lymphoid organs of the mouse. These cells are called lymphoid dendritic cells (LDC) and are confined to the white pulp in the spleen. At the moment there is growing evidence that FDC, IDC and LDC each play their own important role in a variety of immune responses. At the periphery of the white pulp in the rats and mice another nonlymphoid cell type has been found; the so-called marginal metallophils.

Follicular dendritic cells

The first description of antigen localization in the spleen was presented by White[73]. Humphrey and Frank[33] demonstrated that antigens are trapped as antigen-antibody complexes. White investigated antigen trapping in the spleen of chickens. He found trapped antigen located along dendritic cell processes of nonlymphoid cells. He called these cells 'dendritic macrophages'. Using ultrastructural autoradiographic methods and radio-iodinated antigens Nossal et al.[49] and Hanna and Szakal[23] found that antigens, after injection in rats and mice respectively, localized at the surface of nonlymphoid cells with extensively convoluted cell pro-

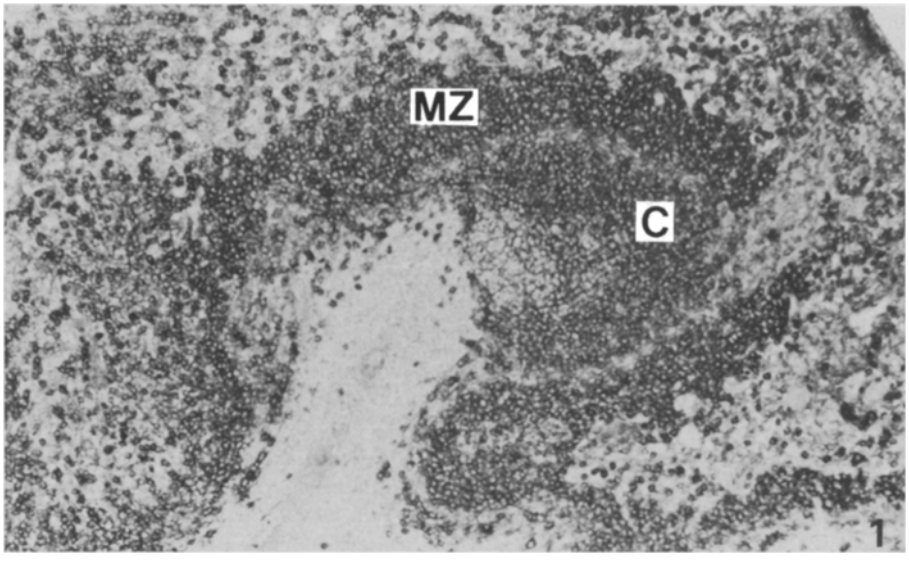

Figure 1. Staining for IgM. Cells with strong IgM membrane staining are located in the marginal zone (MZ) and cells with moderate IgM membrane staining are mainly located in the follicle corona (C). × 100.

cesses. Nossal and colleagues described these cells as 'antigen-retaining dendritic reticular cells'. In mammals these dendritic cells have been found exclusively in the light zone of the follicle center where they are often in close connection with capillaries. These follicular dendritic cells (FDC) show irregularly shaped nuclei with a little heterochromatin while around the nucleus only a scanty cytoplasm is present. The main part of the cytoplasm is situated in the long cell processes. FDC in mammals contain only a few primary lysosomes[26,70]. FDC are characterized by their ability to bind immune complexes. This capability can be demonstrated by peroxidase histochemistry, when peroxidase-anti-peroxidase (PAP) complexes are used as immune complexes[7] (fig. 2). Since peroxidase histochemistry and other enzyme histochemical techniques may be combined successfully, the enzyme pattern of these cells can be demonstrated[10]. With these techniques we found that FDC in rats and mice showed no acid phosphatase activity[10,11]. In mammals FDC originate probably from the reticulum network[21,24,26,67,69]. We found that follicular immune-complex-retaining dendritic cells in chicken spleen, visualized with immunohistoperoxidase techniques at light and electronmicroscopical level, exhibit evident acid phosphatase activity and possess considerable numbers of primary lysosomes but no phagolysosomes[14]. Also in turtles and toads immune trapping cells show acid phosphatase activity and contain a lot of primary lysosomes[38,39]. These lower vertebrate species do not possess follicles. Considering these phylogenetic aspects, the nomenclature of the immune-complex-retaining cells may be considered insufficient and confusing[66]. Our findings suggest that in the phylogeny different cell types, possibly belonging to different cell lines, have the ability to retain immune complexes for a long time on their surfaces. The possible role of the retention of antibody complexed antigen at the cell surface of the FDC in the regulation of the immune response has been recently reviewed[37,51].

Interdigitating cells

Veldman described the occurrence of a characteristic nonlymphoid cell in the paracortex of the lymph node of rabbits[72]. He called these cells interdigitating cells (IDC). They have a clear cytoplasm that extends between the surrounding T lymphocytes and forms interdigitating process with neighboring IDC. IDC have irregularly shaped nuclei with a thin layer of marginally located chromatin. IDC were also detected in the thymus-dependent area of the spleen, the inner PALS[15,47,68,70]. Veerman showed that IDC are monocyte-derived[70] and thus belong to the mononuclear phagocyte system[17,43]. IDC contain only a small amount of carbon after Indian ink injection. Carbon uptake combined with enzyme histochemistry demonstrates that IDC show a spot of nonspecific esterase activity in the same area where carbon is found[11]. In rodents IDC sometimes possess a weak acid phosphatase activity[11,68]. Nonspecific esterase activity and a weak acid phosphatase activity have also been demonstrated in IDC in human spleen[47]. IDC show ATPase activity in human spleen[47] but not in rat spleen[6]. IDC have been shown to express large amounts of Ia antigens[28,41]. Staining for Ia antigens with the immunohistoperoxidase technique combined with demonstration of acid phosphatase activity on the same tissue section reveals the presence of two distinct cell types in the PALS[8]. Round-oval cells with strong acid phosphatase activity representing the macrophages only occasionally showed Ia antigen on a cell process. Strongly Ia positive branched cells only showed no or only weak acid phosphatase activity in the cytocenter. Veldman[72] suggested that the function of IDC is to present antigen to T cells. Further investigations[35] support this view and have shown that IDC are surrounded by T helper cells.

Steinman and Cohn[61] isolated a dendritic cell type from mouse spleen suspensions. This cell type would be confined to the white pulp. These in vitro dendritic cells, which demonstrate a weak carbon uptake and a slight acid phosphatase activity, are highly radio-sensitive. Further investigations[62,63,64] have shown that this cell type expresses large amounts of Ia antigens and is a potent activator of the mixed lymphocyte reaction. Steinman and coworkers consider their dendritic cells as distinct from mononuclear phagocytes and not monocyte derived. Comparison between IDC in vivo and the in vitro dendritic cell reveals general similarities[9,11]. Both cell types possess similar morphology and an identical enzyme histochemical pattern, and are strongly Ia positive. After irradiation both IDC and in vitro dendritic cells can develop phagocytic capacity. In our opinion, the in vitro dendritic cells are identical to the in vivo interdigitating cells.

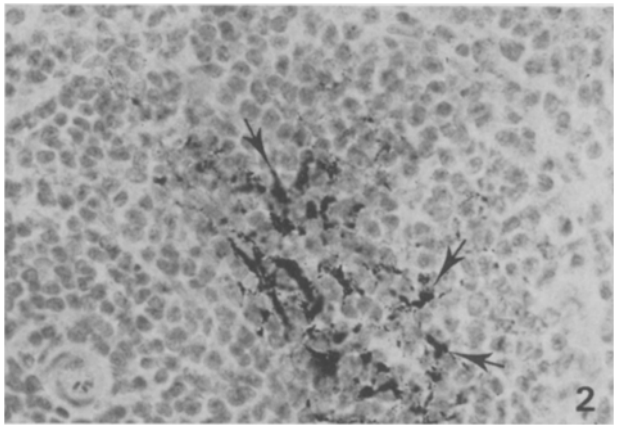

Figure 2. 24 h after i.v. injection of HRP-anti-HRP (PAP) complexes. The complexes are visible as dark strands of granules (arrows) in the follicle. × 350.

Marginal metallophils

Snook[59] described in the rat spleen a rim of cells blackened by Marshall's silver impregnation[45] at the periphery of the white pulp along the inner border of the marginal sinus. These cells are named marginal metallophils on account of their topographical position and staining by silver impregnation. They show an evident acid phosphatase activity but a weak carbon uptake. Marginal metallophils exhibit granular staining with aldehyde fuchsin just like tingible body macrophages[65]. From one comparative study[57] on the splenic white pulp

of different mammalian species, including mice, it was concluded that marginal metallophils are present in rats only. On the contrary, we observed in mice a single line of branched metallophilic cells at the outer border of both follicles and PALS[11]. The line of metallophils can be observed as a distinct structure by its characteristic localization and the orientation of the slender cell processes toward the center of the white pulp. Marginal metallophils in mice show a very strong nonspecific esterase activity by which these cells can be identified easily[11] (fig. 3). The nonspecific esterase activity in marginal metallophils is completely inhibited by the organophosphate inhibitor E 600 in a concentration of 10^{-6} M whereas macrophages in the red pulp and PALS even show evident positive NSE staining with as much as 10^{-3} M E 600. Recent observations suggest that the presence of marginal metallophils is not limited to rodents. Blue and Weiss[2] found macrophages lying on the circumferential reticulum of the marginal sinus in cats. These cells did not ingest thorotrast although it coated their surfaces. The authors suggest that these cells are in some respects similar to cells designated as Snook's marginal metallophils. In chicken spleen we found at the outer border of the Schweigger-Seidel sheath a single line of nonlymphoid cells with evident acid phosphatase activity but without phagocytic capacity[14]. Shortly after injection of Indian ink or of immune complexes these compounds are transiently coated at the cell surface. In chicken spleen these cells have a similar topographical position with respect to the blood circulation and the medium-sized lymphocytes around the Schweigger-Seidel sheath, as marginal metallophils in rats and mice.

The function of marginal metallophils is still unclear. Recent studies of Humphrey[29, 30, 32, 33], however, may be important. He demonstrated that DNP conjugates of neutral polysaccharides were almost exclusively detected in the marginal zone and DNP conjugates of acidic polysaccharides were predominantly localized in the red spleen of the mouse spleen. DNP conjugated neutral polysaccharides ingested and retained exclusively by marginal zone macrophages elicited long lasting IgM-anti-DNP responses, in contrast to conjugates with acidic polysaccharides, ingested by red pulp macrophages. Humphrey suggests that marginal zone macrophages may have a special role in the presenting of thymus-independent antigens to a subclass of responsive B cells without involving T cell help. In cryostat sections of the spleen, the cytoplasm of the labelled marginal zone macrophages appeared to extend between other cells in a dendritic manner. The marginal zone macrophages stained strongly for acids phosphatase and nonspecific esterase activity. Marginal zone macrophages were only obtained in cell suspension after extensive digestion with collagenase, and lymphocytes showed strong adherence to these cells. We obtained marginal metallophils also in spleen suspensions, but only after collagenase digestion, in contrast with red pulp macrophages and Steinman's dendritic cells. In these cell suspensions lymphocytes showed a strong adherence to marginal metallophils (unpublished observations).

When the morphological, enzyme histochemical and in vitro findings are taken together, there are strong similarities between marginal zone macrophages, indicated by selective uptake of fluorescent derivatives of neutral polysaccharides, and marginal metallophils. When marginal metallophils play an essential role in the regulation of the immune response these cells must be considered also as belonging in the group of dendritic cells[66].

Plasmacellular reaction

The antigen-induced transformation of lymphocytes to plasma cells is called the plasmacellular reaction. In a histological study using methyl green-pyronin staining, Langevoort[42] demonstrated that the antibody forming cells in the spleen originate within the PALS. In an elec-

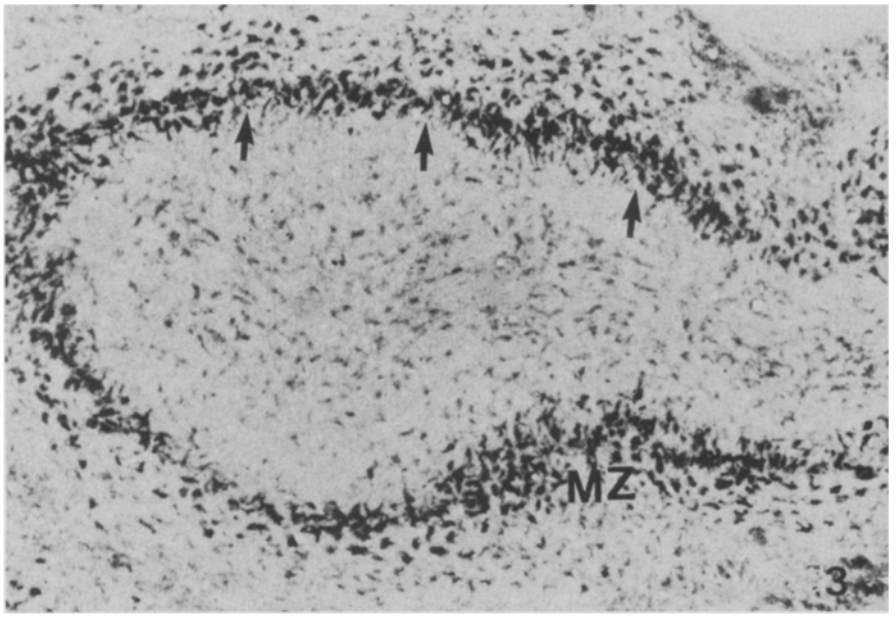

Figure 3. Demonstration of nonspecific esterase activity in mouse spleen. Marginal metallophils show a very strong nonspecific esterase activity (arrows). × 80.

tron microscopic study Veerman[69] observed these cells especially in the outer PALS. Van Ewijk et al.[16] found that after SRBC administration the appearance of immunoglobulin-containing blasts in the outer PALS correlated with the appearance of IgM antibodies in peripheral blood. To detect the development of IgM- and IgG-containing plasmablasts in the white pulp of mice we recently investigated[13] the plasmacellular reaction against both the thymus-dependent antigen SRBC and the thymus-independent antigen LPS by studying the white pulp with the immunohistoperoxidase technique and specific antisera against IgM, IgG, IgG_{2A} and IgG_{2B}. We found that the thymus-independent IgM response to LPS and the thymus-dependent IgM response to SRBC start in the outer PALS respectively 1.0 and 2.5 days after intravenous antigen injection. In contrast to the exclusive localization of the first-appearing IgM-blasts in the outer PALS (fig. 4), the first IgG-blasts appeared 3 days after SRBC administration and were dispersed through the whole PALS. Similar observations are reported in the rat after SRBC administration[58]. In the PALS the numbers of IgM and IgG blasts were strongly diminished respectively 4 and 7 days after SRBC administration. However, increased numbers of IgG blasts were found in the follicles from 5 days onwards in and around the folllicle centers. Especially between 6 and 10 days some follicles contained many IgG blasts (fig. 5). These blasts were located partly in the germinal center but mainly at the border of the follicle center. Thus, there is an IgG blast development early in the immune response in the PALS and later on in the follicle centers. After LPS administration a very strong IgM blast reaction was observed in the PALS between 1.0 and 4.0 days and only a very slight increase of IgG-blasts between 2.5 and 4.0 days. No follicle center reaction occurred after LPS administration.

An interesting finding in this study was the localization of the IgM-blast development in the outer PALS both after administration of a thymus-dependent and a thymus-independent antigen. This finding does not imply that in unstimulated animals the precursors of the IgM-blasts are also located in the outer PALS. Studies of Nieuwenhuis[48] and Veerman[69,71] suggest that the precursors of IgM blasts are the immunocompetent marginal zone lymphocytes. MacLennan and coworkers[44] suggest, however, that the marginal zone lymphocytes might be mainly precursors of IgG_{2C} (rats) and IgG_3 (mice) plasmablasts. The location of these plasmablasts after antigen challenge is still unknown. It would be interesting to know whether the development of IgG_3 blasts runs parallel to, and takes place in the same compartment as the development of IgM blasts or the other IgG subclass blasts. The development of specific antibody containing blast cells can be studied using HRP as antigen[60]. Cells containing anti-HRP can be detected by incubating cryostat sections with HRP followed by peroxidase cytochemistry. However, HRP in itself is a very weak antigen. Intravenous injection of HRP elicits an immune response in the spleen only after subcutaneous injection of HRP together with a strong adjuvant followed by intravenous injection of the antigen. At the moment we are investigating[54,55] the development and localization pattern of specific antibody-containing cells in the spleen during primary and secondary responses against human serum albumin (HSA). Demonstration of the specific antibody-containing cells is performed by incubation of spleen sections with HSA-HRP conjugates, followed by peroxidase cytochemistry. Specific antibody-containing cells are detected in the spleens of rabbits 4 days after injections of HSA. They are detected in the outer PALS and are subsequently also found in the follicles.

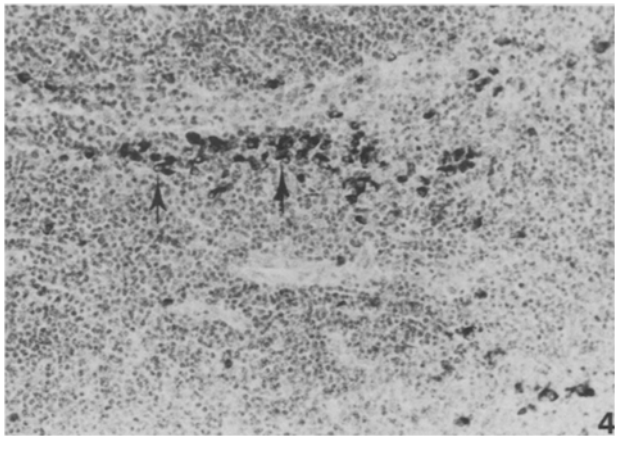

Figure 4. Demonstration of the first IgM blasts (arrows) in the outer PALS 2.5 days after SRBC administration. × 120.

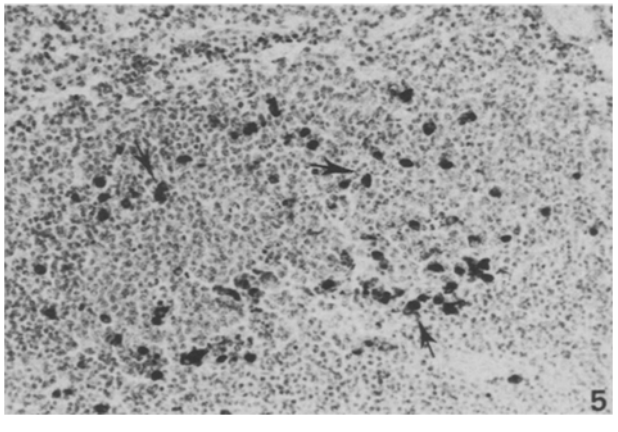

Figure 5. IgG_{2A} blasts (arrows) in and at the border of a follicle center 10 days after SRBC administration. × 150.

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0014-4754/85/020209-07$1.50 + 0.20/0

The role of the spleen in leukemias and lymphomas including Hodgkin's disease

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Key words. Spleen; leukemia; lymphoma; Hodgkin's disease; non-Hodgkin's lymphoma; malignant histiocytosis.

1. Introduction

The spleen as the largest single lymphoid organ in the body is of interest for clinical medicine and pathology, not only because it may serve as a 'showcase' for the hematopoietic and lymphoid tissue, but also because in cases of lymphoma and leukemia it gets involved sooner or later in the majority of patients. In some it may even be the site of the first or only clinical manifestation of hematopoietic neoplasia.

For obvious reasons, it will not be possible in this chapter to treat all the possible clinical, morphological and functional aspects of splenic involvement in hematopoietic neoplasias.

In classical myeloid or lymphoid leukemia the diagnosis is usually established by the examination of peripheral blood and bone marrow. Involvement of the spleen in these diseases is more in the sense of a 'bystander reaction' although splenomegaly may be one of the earliest symptoms. It has usually no decisive influence on the management of the patient.

In hairy-cell leukemia and malignant histiocytosis, however splenomegaly may be the most important symptom, and not infrequently the diagnosis can only be es-