LOCALIZATION AND PHENOTYPICAL CHARACTERIZATION OF MURINE MACROPHAGES

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LOCALIZATION AND PHENOTYPICAL CHARACTERIZATION OF MURINE MACROPHAGES

LOKALISATIE EN FENOTYPISCHE KARAKTERISERING VAN MACROFAGEN IN DE MUIS

PROEFSCHRIFT

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aan de Erasmus Universiteit Rotterdam
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Voor mijn ouders

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1. ORIGIN AND DIFFERENTIATION OF MACROPHAGES

1.1. History

Macrophages are widely distributed throughout the body. They originate from the bone marrow and together with their precursor cells they constitute the mononuclear phagocyte system (Table 1.1). This system consists of monoblasts, promonocytes, and monocytes in the bone marrow, monocytes in peripheral blood, and macrophages in tissues (van Furth, 1972; Lasser, 1983). The more mature cells of the system are able to phagocytize and degrade particles and soluble macromolecules. Moreover, they release many regulatory products. Because of these functions macrophages are important cells in resistance to infection and in regulation of the extracellular environment.

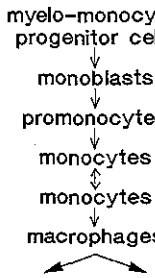
In history, the presence of monocytes in the peripheral blood was first discovered by Shilling in 1912 (Jaffe, 1935). Thirteen years later Lewis (1925) described the differentiation of blood monocytes into macrophages using an in vitro system. In situ this was demonstrated by Ebert and Florey in 1939 who used rabbit ear chambers to show that carbon-loaded monocytes migrated from the blood vessels into the connective tissue and transformed into tissue macrophages (Ebert and Florey, 1939). More recent studies with tritiated thymidine and bone marrow chimeras confirmed these observations (Volkman and Gowans, 1965; Spector and Lykke, 1966).

The first functional property of cells belonging to the mononuclear phagocytic system, namely 'phagocytosis', was observed by Haeckel in 1862. He discovered that injected indigo particles selectively penetrated certain blood cells of the specimen Thetis fimbria and localized near the nucleus. Twenty years later Metchnikoff discovered that two cell types in the blood of a starfish larva were actively involved in phagocytosis. He called these cells microcytes and macrocytes, because of their different sizes. Furthermore, he recognized the ability of such cells to degrade particles like bacteria. This property constitutes an important line of host-defense against infection (Metchnikoff, 1905). Nowadays microcytes are referred to as polymorphonuclear leukocytes or neutrophilic granulocytes and macrocytes as macrophages or mononuclear phagocytes.

Subsequent research further elucidated the secretory function and the role of mononuclear phagocytes in the immune system, as well as many other functions involved in preservation of the integrity of the mammalian body (Takemura and Werb, 1984; Unanue, 1984; Gordon, 1986).

Table 1.1

The Mononuclear Phagocyte System



inflammation	**steady state**	
exudate macrophages	osteoclasts (?)	bone
exudate-resident macrophages	fixed and central macrophages	bone marrow
epithelioid cells	histiocytes	connective tissue
multinucleated giant cells	fixed macrophages	intestine, other organs
	Kupffer cells	liver
	alveolar and interstitial macrophages	lung
	free and fixed macrophages	lymph and lymphoid organs
	veiled cells(?), interdigitating cells(?)	
	microglial cells	nervous system
	free macrophages	serous cavities
	Langerhans cells (?)	skin
	type A cells	synovia

1.2. Macrophage development

In general it is accepted that macrophages belong to the progeny of the hemopoietic stem cell in the bone marrow (Figure 1.1; van Furth and Cohn, 1968). This stem cell undergoes several cycles of proliferation and differentiation during approximately 8 days to deliver progenitor cells committed to the myelo-monocytic lineage. These cells, when stimulated with the appropriate stimulating factors, give rise to the most immature morphologically recognizable mononuclear phagocytes in the bone marrow, which are the monoblasts (Goud et al., 1975) and the promonocytes (van Furth and Cohn, 1968; van Furth and Diesselhoff-den Dulk, 1970). Under normal steady state conditions monoblasts have a cell cycle time of about 12 hours (van Furth, 1981). Each monoblast gives rise to two promonocytes of which each in turn divides after approximately 16 hours to give two monocytes (van Furth, 1981). The newly formed monocytes leave the bone marrow within 24 hours. They remain in the peripheral

blood for a short time of 12 to 32 hours, with an average of 18 hours (Nichols and Bainton, 1973). Monocytes that leave the circulation differentiate into tissue macrophages under the influence of local environmental factors, generally without further divisions. They do not return to the blood stream, but are thought to be disposed off locally by other macrophages or become trapped within the draining lymph nodes and die there. Furthermore, it has been observed that macrophages can leave the body via the lumina of the respiratory and gastrointestinal tracts or in secretory products like milk (Gordon, 1986). Resident macrophages are present in most tissues (Fig. 2) and may live many months, perhaps even years (Shand and Bell, 1972). Under normal circumstances they show little evidence of mitotic activity, but under certain stimulating conditions some may divide. The quantitative significance of local proliferation in the generation of tissue macrophages is a subject of considerable debate. It seems likely that the extent to which local proliferation contributes to the macrophage pool will vary from tissue to tissue. For example, the peritoneal cavity contains a very low percentage of proliferating macrophages (van Furth, 1978; Melnicoff et al., 1988), whereas the lung contains 20 per cent or more (Chen and Lin, 1982; Evans et al., 1987). At least a part of this latter population consists of proliferating immature mononuclear phagocytes derived from the bone marrow (Blussé van Oud Alblas et al., 1983). Furthermore, there is some evidence that a small population of liver macrophages (Kupffer cells) may arise from local macrophage proliferation (Diesselhoff-den Dulk et al., 1979).

In addition to the resident macrophages described above, two other macrophage populations can be distinguished in tissues and cavities, i.e., exudate macrophages and immunologically activated macrophages. These cell types are increased in number after a sterile or non-sterile inflammatory stimulus, respectively. Intraperitoneal injection of a sterile inflammatory agent, such as thioglycollate, results in an increased macrophage turnover with a ten-fold increase of the macrophage influx into the peritoneal cavity (Gordon, 1986). The infiltrating exudate macrophages are less mature compared with the resident macrophages. They retain the ability to proliferate and still possess many of the monocyte characteristics. Macrophages entering sites of bacterial infections become activated to perform particular functions. For example, they may show an increased functional activity in that they present antigens to T lymphocytes, kill tumor cells, and destroy facultative and obligate intracellular parasites (Adams and Hamilton, 1984).

Two other cell classes of the mononuclear phagocyte system often found in an inflammatory exudate are the epithelioid cell and the multinucleated giant cell. Three types of epithelioid cells have been recognized, i.e. the "secretory", the "vesicular" and the "activated" type. "Secretory" epithelioid cells show a high secretory activity. They mainly occur in granulomas with a strong delayed hypersensitivity reaction to the

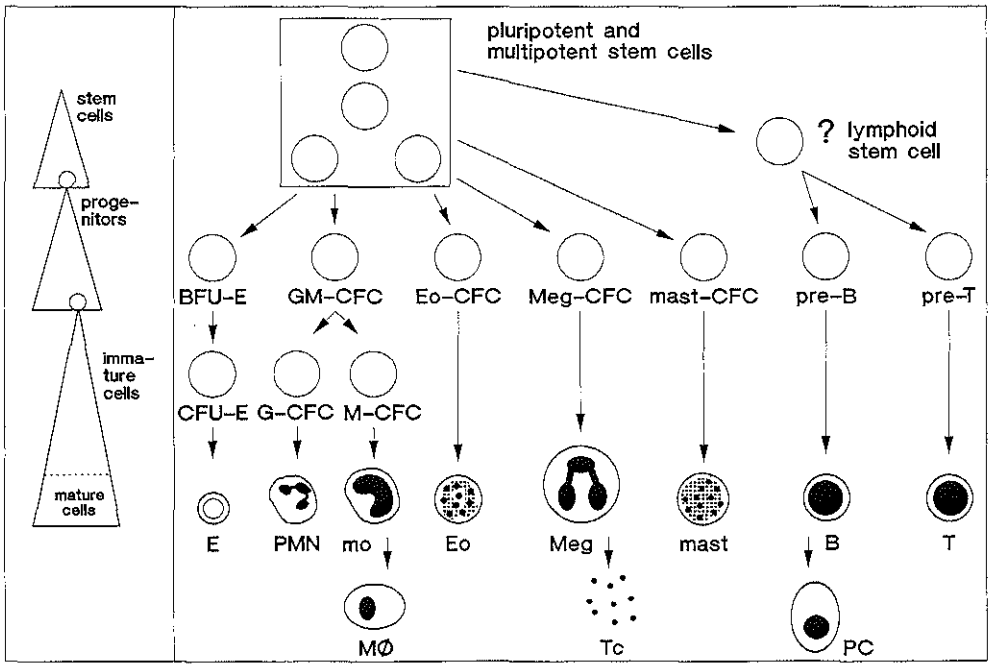


Figure 1.1 Stem cell differentiation in the murine bone marrow.
B, B cell; BFU-E, burst forming units-erythroid; CFC, colony forming cell; E, erythrocyte; Eo, eosinophilic granulocytes; G, granulocyte; GM, granulo-monocytic; M, monocyte; Meg, megakaryocyte; Mϕ, macrophage; PC, plasma cell; PMN, polymorphonuclear granulocyte; T, T cell; Tc, thrombocytes

antigen. The appearance of these epithelioid cells is often associated with an increased fibroblast proliferation and collagen production (Turk and Narayanan, 1982). "Vesicular" epithelioid cells are mainly present in chronic granulomas and probably resemble a degenerable form of "secretory" epithelioid cells. The "activated" epithelioid cells have many characteristics in common with activated macrophages (van Furth, 1980). Their relationship with the afore mentioned epithelioid cells is not clear.

Like epithelioid cells, multinucleated giant cells also show some heterogeneity. Three morphologically different cell types can be distinguished, i.e."Toutan", "Foreign body" and "Langerhans" giant cells. Experimental evidence makes it likely that these variants represent different phases in the life of the same cell (Chambers and Spector, 1982). It is widely accepted that multinucleated giant cells are derived from fusion of monocytes rather than nuclear division. Giant cell formation is frequently caused by poorly soluble antigenic materials. Simultaneous attachment of two macrophages to the same material results in endosome formation by both cells. The endosome margins of these cells meet each other and fusion occurs.

Two possible candidates which may belong to the mononuclear phagocyte system

are the osteoclasts and the dendrocytes. Osteoclasts are multinucleated cells present in the bone marrow that are able to resorb calcified tissue matrices. They are formed by fusion of post-mitotic, mononuclear cells that presumably originate from hemopoietic stem cells (Marks, 1983; Scheven et al., 1986). In culture they are derived from weakly adherent radiosensitive immature mononuclear phagocytes (Burger et al., 1985). This observation, together with the fact that osteoclasts are phagocytic, are arguments to classify them within the mononuclear phagocyte system. However, it is not clear whether osteoclasts are formed directly by fusion of monocytes, or, alternatively, have a precursor cell in common with cells of the monocytic lineage. Dendrocytes will be discussed in more detail in Chapter 4.

1.3. Factors influencing macrophage development

Differentiation of hemopoietic progenitor cells occurs in a complex stromal network consisting of a number of cell types and an intercellular matrix (Weiss, 1976). This so-called "microenvironment" plays an important role in the hemopoietic cell differentiation by means of cellular interactions and the production of short-range humoral factors (Golde and Cline, 1984; Ploemacher et al., 1986; Bagby, 1987). At least part of the differentiation process is under the absolute control of a family of regulators called colony-stimulating factors (CSF). These glycoprotein growth factors are produced by many tissues in the body and they are essential for the survival, growth and differentiation of hemopoietic progenitor cells in vitro (Metcalf, 1984, 1987). At the moment distinct CSF controlling the production of granulocytes and macrophages have been purified and cloned. The prefix of the CSF depends on the predominant type of differentiated cells present in colonies cultured in semi-solid media, i.e. G-CSF stimulates the production of neutrophilic colonies, M-CSF monocytic colonies, GM-CSF neutrophilic, eosinophilic and monocytic colonies and multi-CSF (IL-3) gives rise to neutrophilic, eosinophilic, monocytic, megakaryocytic, mast cell and erythroid colonies. Although both multi-CSF and GM-CSF may induce monocyte/macrophage differentiation via the process of down modulation (Walker et al., 1985; Nicola, 1987), it is thought that survival, proliferation and differentation of mononuclear phagocytes predominantly is regulated by M-CSF (Das and Stanley, 1982; Stanley et al., 1983). This homodimeric glycoprotein consists of two disulfide-bound protein chains of approximately 35 kD (Stanley and Heard, 1977) and the cellular response to M-CSF is mediated via a high affinity cell-surface receptor (Morgan and Stanley, 1984). The purified receptor is a 165 kD glycoprotein with intrinsic tyrosine kinase activity (Yeung et al., 1986, 1987). It may be identical or closely related to the c-fms proto-oncogene

product (Sherr et al., 1985; Sacca et al., 1986; Rettenmier et al., 1986). The number of receptors increases with cell maturation, adherent macrophages displaying 50,000 or more receptors per cell (Nicola, 1987). After binding of M-CSF to its receptor the receptor complex is rapidly internalized and subsequently degraded (Chen et al., 1984a). Depending on the type of macrophage this happens rapidly (peritoneal macrophages) or more slowly (bone marrow derived macrophages) and it has been suggested that the accumulation of intracellular pools of M-CSF receptor may be correlated with the biological response to M-CSF (Stanley and Guilbert, 1981; Guilbert and Stanley, 1986; Guilbert et al., 1986). M-CSF is found in the serum or plasma of normal mice at biologically active concentrations (Bartocci et al., 1986) and appears to selectively regulate mononuclear phagocyte production in vivo (Broxmeyer et al., 1987). In addition to its hemapoietic action, M-CSF can stimulate mouse macrophages to produce plasminogen activator (Lin and Gordon, 1979), prostaglandins (Kurland et al., 1979), interleukin 1 (IL-1; Moore et al., 1980), interferon (IFN; Fleit and Rabinovitch, 1981), oxygen products (Wing et al., 1985), acid ferritin (Broxmeyer et al., 1985) and G-CSF (Metcalf and Nicola, 1985). M-CSF serum levels are regulated by macrophages themselves by receptor-mediated endocytosis followed by intracellular degradation of the growth factor. In this way approximately 94% of the circulating M-CSF is selectively cleared. The system provides a feedback control mechanism whereby the rate of macrophage production is determined by the number of mature macrophages (Bartocci et al., 1987). Other feedback mechanisms in the regulation of macrophage production are executed by some products of mature macrophages. For example, E-type prostaglandins (Pelus et al., 1979), IFN α/β (Moore et al., 1984) and acid isoferritin (Broxmeyer et al., 1985) inhibit CSF-induced proliferation.

During inflammation, cell proliferation is under control of a complex regulatory mechanism in which many factors are involved. The overall reaction results in a recruitment and activation of granulocytes and macrophages which eliminate the inflammatory stimulus. In addition to known hemopoietic growth factors present under steady state conditions, it has been demonstrated that other factors can also have an important role in macrophage production and recruitment. Macrophages present at the inflammatory site temporarily produce a factor, called Factor Increasing Monocytopoiesis (FIM), which, as indicated by its name, stimulate monocyte production in the bone marrow (Van Waarde et al., 1977; Sluiter et al., 1982, 1987).

2. CHARACTERIZATION OF MACROPHAGES

2.1. Morphological and cytochemical characterization

Macrophages are a very heterogeneous population of cells. In general, two types of heterogeneity can be recognized, i.e., inter and intrapopulation heterogeneity (Dougherty and McBride, 1984). Interpopulation heterogeneity refers to differences between populations from different tissue sites. Such populations differ considerably with respect to a large number of characteristics most likely as a result of exposure to local environmental signals. Intrapopulation heterogeneity refers to differences between subpopulations of macrophages obtained from one particular site. This heterogeneity could at least in part be due to differences in the stage of development or the state of activation. As a result, cells may exhibit differences in enzyme activity, expression of cell surface receptors and, ability to phagocytize. Therefore, investigation of macrophages and the use of organ-derived macrophage populations requires adequate characterization of the particular population under study.

Macrophages can be characterized on the basis of their morphological, cytochemical, and functional features together with their surface receptor and antigen expression. However, classification must be based on more than one property, since distinct macrophage subsets may share one or more characteristics. Morphological characterization includes all features seen in light, phase-contrast and electronmicroscopy like for example cell size and shape, the nuclear to cytoplasmic ratio, the staining properties of the cytoplasm and the presence and number of specific cell organelles (Table 2). Although morphological criteria alone are often insufficient for identification of cells, some cell types may be easily recognized by the appearance of specific morphological characteristics, e.g. Birbeck granules in Langerhans cells (Wolff and Stingl, 1983), effete lymphocytes in tingible body macrophages (Chen et al., 1978) and the presence of rod-shaped cytoplasmic inclusions in macrophages of several strains of mice (Berman, 1966; Fedorko, 1975; Marshall et al., 1988) and rabbits (Simon and Burke, 1970).

Other morphological characteristics are often associated with specific macrophage functions. The large vesicular nucleus with prominent nucleoli, the extended and well developed Golgi apparatus, the presence of abundant rough endoplasmatic reticulum and many ribosomes all are indicative of the high metabolic activity of macrophages. Furthermore, the numerous microvilli and irregular shape of the plasma membrane are features of their endocytic property. Especially in immunologically activated macro-

phages the plasma membrane forms many extensions, which constitute a large surface area available for rapid internalization of foreign material and for cell spreading (Mørland and Kaplan, 1977; Cohn, 1978; Nabarra et al., 1978). These membrane movements are related to the presence of a subplasmalemmal network of actin-containing filaments cross-linked by actin-binding protein and myosin (Unkeless et al., 1981; Tycko and Maxfield, 1982; Tycko et al., 1983). The cytoplasm contains large mitochondria, microfilaments and microtubules as well as digestive vacuoles, coated vesicles, and many lysosomes rich in hydrolytic enzymes. The number of lysosomes increases during maturation and is related to the increased endocytic activity of the phagocyte (Cohn and Benson, 1965; van Furth et al., 1970). In contrast to some of their precursor cells, macrophages do not contain primary azurophilic granules (Nichols et al., 1971).

Another approach to characterize macrophages is by determination of the presence of particular enzymes (Table 2.1). Their localization and degree of activity give information about the developmental stage and the immunological or functional state of the macrophage. Reliable markers for most mononuclear phagocytes (MNP) are the nonspecific esterases, which can be demonstrated using α-naphthyl butyrate or acetate as substrate (Yam et al., 1971; Horwitz et al., 1977). The diffuse staining of the cytoplasm is characteristic for MNP and distinguishes them from lymphocytes that show a patchy staining pattern (van Furth, 1980). Additionally, immunocytochemical detection of the enzyme lysozyme may be used for the detection of MNP. All MNP constitutively secrete this enzyme independent of their state of activation (Gordon et al., 1974; Takemura and Werb, 1984).

The intracellular localization of the enzyme peroxidase is indicative of a certain developmental stage. The enzyme is present in the nuclear envelope, endoplasmic reticulum, Golgi apparatus and granules of monoblasts and promonocytes. Monocytes and exudate macrophages only contain peroxidase-positive granules, while resident macrophages lack such granules (Daems et al., 1979). Another marker used to determine the differentiation stage of MNP is the expression of the lysosomal enzyme β-galactosidase. The activity of this enzyme increases during differentiation but enzyme levels are not influenced by exposure to modulating signals (Bursuker et al., 1982). Other enzymes have also proven to be useful markers to distinguish activated macrophages from resident macrophages. The membrane-bound ectoenzymes 5'-nucleotidase (Bursuker and Goldman, 1982, 1983) and NAD nucleotidase (Artman and Seeley, 1979) have a very low activity in activated macrophages, while resident macrophages express a high activity. Other ectoenzymes, like leucine aminopeptidase, alkaline phospho-di-esterase and NAD(P)H-oxidase show an increased activity after macrophage activation (Morahan and Miller, 1984; Ryter, 1985). Macrophage activation

Table 2.1

Morphological and cytochemical characteristics of macrophages

Morphological	Cytochemical
- large vesicular nucleus	- non-specific esterase activity
- prominent nucleoli	- acid phosphatase activity
- well developed Golgi system	- lysozyme activity
- many mitochondria and ribosomes	

also leads to an increased production of lysosomal enzymes (Cohn, 1979). This increment is not identical for all enzymes and differences between e.g., acid phosphatase, cathepsin and β-glucuronidase have been observed (Mørland and Mørland, 1978).

Macrophages possess a variety of receptors, mostly glycoproteins, which enable them to recognize various cell surfaces, extracellular matrices, and soluble molecules. These receptors play an essential role in phagocytosis, receptor-mediated pinocytosis, cell adhesion, and cell-cell interaction. At least three receptor types play an important role in phagocytosis, the receptor for the Fc portion of Ig (FcR; Unkeless and Eisen, 1975; Unkeless, 1980; Unkeless et al., 1981), the type 3 complement receptor (CR3; Silverstein et al., 1977; Ross et al., 1982), and the mannose-fucose receptor (MFR). Both the FcR and CR3 react with opsonized micro-organisms. The MFR has lectin-like properties and binds specific sugars on the surface of micro-organisms. It enhances the attachment of micro-organisms like yeast and bacteria to macrophages (Warr, 1980; Glass et al., 1981; Blackwell et al., 1985). Furthermore, it increases pinocytosis of soluble glycoproteins bearing mannose or fucose residues, such as horseradish peroxidase. Experiments with this latter compound showed that receptor-mediated pinocytosis is much more efficient than fluid phase pinocytosis (Kaplan and Nielsen, 1978; Sung et al., 1983). Receptor-binding allows macrophages to take up different kinds of glycoproteins efficiently that are present in low concentrations. Apart from these receptors, macrophages express receptors for lipoproteins (Fogelman et al., 1985), for modified sugars (Vlassara, 1984), sulphated polysaccharides (Chong and Parish, 1986), fibronectin (Wright and Meyer, 1985), laminin (Bohnsack et al., 1985) and for various peptides, hormones, neurotransmitters and pharmacological agents (Gordon, 1988a).

The functions of macrophages are very diverse and depend on their localization,

differentiation stage and state of activity. In general, three main functions can be observed, 1) phagocytosis and pinocytosis, 2) immunoregulation and 3) secretion of various biologically active products. These features will be discussed in more detail in Chapter 3.

2.2. Immuno-histochemical characterization of macrophages

Monoclonal antibodies (mAb) are a powerful tool to investigate macrophage heterogeneity. Nowadays various monoclonal antibodies against markers present on cells of the monocytic lineage are available (Table 2.2). Their expression varies with the developmental stage and the state of activation of the cells. Furthermore, many markers are expressed by other cell types as well. These features, together with the lack of information on the structure and function of most antigens, make it difficult to classify these markers in certain well-defined categories. Within this thesis, markers will be classified according to their dispersal among macrophages and other cell types. Using this criterium three categories of markers can be recognized:

a. markers exclusively expressed by macrophages or their precursor cells;
b. markers present on the majority of macrophages but also on other cell types;
c. markers present on certain subpopulations of macrophages but also on other cell types.

In the following sections most murine representatives of these categories will be briefly discussed with special emphasis on the markers studied in the Appendix papers. Human markers are extensively reviewed in the literature (Todd et al., 1985; Kreipe et al., 1987) and will only be mentioned if they are relevant for comparison with murine analogues.

2.2.1. Mononuclear phagocyte-specific antigens

A marker present on the cell membrane of most MNP is the F4/80 antigen. This antigen, a glycoprotein of about 160 kD, is recognized by a rat anti-mouse monoclonal antibody of the IgG2b subclass (Austyn and Gordon, 1981). MNP expressing the antigen are present in many tissues, i.e. hemopoietic and lymphopoietic organs (Hume et al., 1983a), skin (Nussenzweig et al., 1981; Hume et al., 1984a), central nervous

Table 2.2

mAb against macrophages

Ag	mol.mass (kDa)	mAb	CD-designation	Ag-function	references
AcM.1 Ag	-	AcM.1	-	-	Taniyama and Watanabe, 1982
asialo-GM1	-	SH34	-	-	Solomon and Higgins, 1987
BMA-1 Ag	# x 38	BMA-1	-	-	Perry et al., 1987a
BM8 Ag	125	BM8	-	-	Malorny et al., 1986
B23.1 Ag	-	B23.1	-	-	LeBlanc and Biron, 1984
CLA T-200 Ly-5	170-220	a.o. 30-G12 YBM/42	CD45	protein tyrosine phosphatase	Ledbetter and Herzenberg, 1979 Watt et al. 1983
DNL3.7 Ag	-	DNL3.7	-	-	Loken et al., 1983
DNL4.4 Ag	-	DNL4.4	-	-	Loken et al., 1983
ER-HR3 Ag	55-67 69-78	ER-HR3	-	-	De Jong et al., 1987
ER-BMDM1	160	ER-BMDM1	-	-	Leenen et al., 1990c
ER-MP12 Ag	140	ER-MP12	-	-	Leenen et al., 1990b
ER-MP20 Ag	14	ER-MP20	-	-	Leenen et al., 1990b
ER-MP23 Ag	38	ER-MP23	-	aminopeptidase	Leenen et al., 1990d
ER-MP54 Ag	90/80/70	ER-MP54	-	-	Leenen et al., 1990b
ER-MP58 Ag	-	ER-MP58	-	-	Leenen et al., 1990b
ER-TR6 Ag	-	ER-TR6	-	-	Van Vliet et al., 1984
ER-TR9 Ag	-	ER-TR9	-	involved in neutral poly-saccharide uptake	Van Vliet et al., 1985
FcRI	2 x 50	3A2	CD64	IgG2a Fc receptor protein kinase	Kagami et al., 1989
FcRII	47-70	2.4G2	CDw32	IgG1/IgG2b Fc receptor	Unkeless, 1979
Forssmann	-	M1/22.25	-	-	Springer, 1980

Table 2.2

mAb against macrophages (continued)

Ag	mol.mass (kDa)	mAb	CD-designation	Ag-function	references
glycolipid		33B12			Sonnenberg et al., 1986
F4/80 Ag	160	F4/80	-	-	Austyn and Gordon, 1981
HSA	-	M1/69 B2A2 J11d	-	-	Springer, 1980 Scollay et al., 1984 Bruce et al., 1981
H-2 Ia	2 x	a.o. M5/114,	-	Ag-peptide binding	Bhattacharya et al., 1981
	25-34	ER-TR1,-2,-3		for presentation	Van Vliet et al., 1984
H-2 I-J	-	JK10-23	-	involved in T_s	Malley et al., 1988
H-2K/D	46 / 12	a.o. M1/42	-	cellular recognition	Springer, 1980
H11 Ag	-	30-H11	-	- induction	Ledbetter and Herzenberg, 1979
IL-2-R	55	3C7 AMT-13	CD25	low affinity interleukin-2 receptor	Ortega et al., 1984 Osawa and Diamantstein, 1984
LFA-1 Ly-15	180 (α)	a.o. M7/14 H35-89.9	CD11a	cell adhesion molecule	Davignon et al.,1981b Pierres et al.,1982
	95 (β)	M18/2	CD18		Sanchez-Madrid et al,1983
Ly-6C	14-17	a.o. Monts-1 6C3	-	signal transduction (?)	Jutila et al., 1988 Dumont et al., 1985
MAA-1 Ag	-	MAA-1	-	-	Fox and Petty, 1984
Mac-1 Ly-40	170 (α)	a.o. M1/70	CD11b	C3bi receptor, cell adhesion molecule	Springer et al., 1979
	95 (β)	M18/2	CD18		Sanchez-Madrid et al.,1983
Mac-2	32	M3/31 M3/38	-	-	Ho and Springer, 1982
Mac-3 LAMP-2	110	M3/84	-	-	Ho and Springer, 1893
Mac-4 54-2 Ag	180	M3/37 54-2	-	-	Springer, 1981 LeBlanc et al., 1980

Table 2.2

mAb against macrophages (continued)

Ag	mol.mass (kDa)	mAb	CD-designation	Ag-function	references
MA158.2	-	158.2	-	-	Koestler et al., 1984
MBR-1 MBR-2 MBR-3 30-E2 Ag	-	MIV 55 MIV 38 14G8 30-E2	-	-	Falkenberg et al., 1989 Leenen et al., 1986a Kung et al., 1982 Ledbetter and Herzenberg, 1979 Martinet al., 1988
MM9 Ag	-	MM9	-	-	Taniyama and Tokunaga, 1983
MOMA-1 Ag	-	MOMA-1	-	-	Kraal and Janse, 1986
MOMA-2 Ag	-	MOMA-2	-	-	Kraal et al., 1988
M43 Ag	-	M43	-	-	Sun and Lohmann-Matthes, 1982
M57 Ag	-	M57	-	-	Sun and Lohmann-Matthes, 1982
M102 Ag	-	M102	-	-	Sun and Lohmann-Matthes, 1982
M143 Ag	-	M143	-	-	Sun and Lohmann-Matthes, 1982
Pgp-1 Ly-24	95	IM7.8.1 AMF-8	CD44	-	Trowbridge et al., 1982 Colombatti et al., 1982
SER	170-185	SER-4	-	R of sialylated glycoconjugates	Crocker and Gordon, 1989
Tf-R	2 x 100	a.o. H129.121	CD71	transferrin receptor	Van Agthoven et al.,1984
TR-1N Ag TR-3N Ag	57 / 46	TR-1N TR-3N	-	-	Papiernik et al., 1987
WE15 Ag	-	WE15	-	-	Taniyama and Tokunaga, 1983
3AE8 Ag	-	3AE8	-	signal transduction (?)	Chen et al., 1984b
7/4 Ag	-	7/4	-	-	Hirsch and Gordon, 1983

- : data are presently unknown
CLA, common leukocyte antigen; HSA, heat stable antigen; Tf-R, transferrin receptor
FcR, Fc receptor; SER, sheep erythrocyte receptor

system (Hume et al., 1983b), intestines (Hume et al., 1984a), kidney (Hume and Gordon, 1983), endocrine organs (Hume et al., 1984b) and other tissues (Hume et al., 1984c). In vitro studies have demonstrated that the expression of the F4/80 antigen during hemopoiesis appears at an early stage in macrophage differentiation. Pluripotent colony and cluster-forming progenitors do not express the F4/80 antigen but, after stimulation with IL-3 or GM-CSF, they give rise to F4/80-positive pre-adherent macrophage progenitors (Hirsch et al., 1981). This enhanced expression of the F4/80 antigen correlates well with increased CSF-1 responsiveness indicative of commitment to the monocytic lineage (Hume et al., 1984d). The observation that the antigen is expressed on the bipotent myelomonocytic cell lines WEHI-3B and FDC-P1 (Dexter et al., 1980) suggests that the F4/80 antigen may appear prior to the granulopoietic or monopoietic commitment (Hume et al., 1985). F4/80 antigen expression is decreased on BCG-activated peritoneal macrophages (Ezekowitz et al., 1981; Ezekowitz and Gordon, 1982) and exudate macrophages (Ezekowitz et al., 1985b). In part this decrement may be explained by the effects of lymphokines such as IFN-γ since exposure of thioglycollate-elicited peritoneal macrophages to these agents resulted in a decreased antigen expression (Berton and Gordon, 1983).

Some lineage-specific markers are restricted to certain subpopulations of monocytic cells. A marker present on both monocytes and resident macrophages is recognized by the mAb MOMA-2 (Kraal et al., 1987). The in situ distribution of the antigen recognized by this mAb is comparable with the F4/80 antigen with the exceptions that microglial cells are MOMA-2-negative and that macrophages in T and B cell regions of lymphoid organs are positive. Furthermore, the marker is weakly present on dendritic cells, Langerhans cells and interdigitating cells (Kraal et al., 1987).

The antibody BM8 recognizes resident macrophages of various tissues including spleen, lymph nodes, thymus, skin and liver (Malorny et al., 1986). Additionally, the antigen is present on exudate macrophages in granulomata induced with complete Freund's adjuvant. In bone marrow cultures stimulated with CSF-1, the number of BM8-positive cells increased gradually in time and reached a plateau at day 8 of culture. At this time 80% of all cells was positive (Malorny et al., 1986).

An opposite relation with macrophage maturity has been observed with the mAb MB-1. This mAb specifically binds to non-adherent macrophage precursors and, due to maturation, the number of positive cells declines with increasing cultivation times (Gordon and Hirsch, 1982).

Another mAb recognizing a subpopulation of macrophages is ER-TR9 (van Vliet et al., 1985). This mAb exclusively stains non-metallophilic macrophages present in the marginal zones of the spleen and the medullary sinusses of the lymph nodes. Positive

cells in the splenic marginal zone are closely associated with B cells.

The metallophilic macrophages adjacent to the marginal sinus are recognized by the mAb MOMA-1 (Kraal and Janse, 1986). A macrophage-specific mAb against the receptor for sheep red blood cells has been described by Crocker et al. (1986).

The mAb SH34 recognizes the asialo GM1 glycoprotein expressed by resident alveolar macrophages (Akagawa et al., 1981) and activated peritoneal macrophages (Akagawa and Tokunaga, 1982; Solomon and Higgins, 1987).

2.2.2. Antigens present on the majority of macrophages but also on other cell types

An antigen present on all leukocytes including MNP is the common leukocyte antigen T-200. Proteins precipitated with antibodies against this antigen range in molecular weight from 170 kD to 220 kD (Trowbridge, 1978). This antigen is recognized by the monoclonal antibodies 30-G12 (Ledbetter and Herzenberg, 1979), 13/2 (Trowbridge, 1978), YBM/42 (Watt et al., 1983) and 3A35 (Le Corre et al., 1987).

Another group of antigens present on most leukocytes in both mouse (Sanchez-Madrid et al., 1983a) and man (Sanchez-Madrid et al., 1983b) are leukocyte differentiation antigens. Together with the Very Late Antigens (VLA1 - VLA5) and the cyto-adhesin molecules they are the products of the integrin supergene family. This family encodes for a variety of adhesion receptors that have the tripeptide arginine-glycine-aspartic acid as a recognition site (Ruoslathi and Pierschbacher, 1987; Ginsberg et al., 1988; Hemler, 1988).

The leukocyte differentiation antigens include three structurally and functionally related members, i.e. the leukocyte function-associated antigen-1 (LFA-1), the complement type III (C3bi) receptor (CR3, Mac-1) and the p150.95 protein. These different surface molecules share a common β-subunit of 95 kD which is non-covalently associated with distinct α-subunits of 175 kD, 165 kD and 150 kD, respectively (Sanchez-Madrid et al., 1983b). These α-subunits are for 33-50% identical in amino acid sequences (Springer et al., 1985). Various monoclonal antibodies are raised against the different subunits. Especially the application of mAb against the distinct α-units has provided much information about the exact distribution and function of these antigens.

The LFA-1 antigen is expressed at the surface of T and B lymphocytes, natural killer cells, monocytes, macrophages and granulocytes, but is absent from non-hemopoietic cell types (Davignon et al., 1981a; Beatty et al., 1983; Campana et al., 1986). Recent studies indicate that the antigen is involved in interactions between T lymphocytes and monocytes (Keizer et al., 1985; Dougherty and Hogg, 1987).

Furthermore, LFA mediates the lytic activity of cytotoxic T cells (Davignon et al., 1981a; Krensky et al., 1984) and natural killer cells (Hildreth et al., 1983; Miedema et al., 1984; Schmidt et al., 1985). In the mouse the α-subunit of the LFA-1 is recognized by the mAb M7/114 (Davignon et al., 1981a), FD441.8 (Sarmiento et al., 1982), and H35-89.8 (Pierres et al., 1982). Human analogues for this mAb are TS1/12, TS1/22, TS1/122, TS2/4, TS2/6 and TS2/14 (Sanchez-Madrid et al., 1983b).

The CR3 antigen is present on monocytes, macrophages, granulocytes, and natural killer cells, (Springer et al., 1979; Ault and Springer, 1981). It mediates granulocyte adherence (O'Shea et al., 1985; Springer and Anderson, 1986a), and the attachment and phagocytosis of various microbes (Klebanoff et al., 1985; Mosser and Edelson, 1985; Bullock and Wright, 1987), bacterial wall lipopolysaccharides (Wright and Jong, 1986) and C3bi coated particles (Beller et al., 1982; Wright et al., 1983). The murine CR3 α-subunit is recognized by mAb M1/70 (Springer et al.,1979) that also cross-reacts with the human CR3 (Beller et al., 1982). Other mAb that bind to the α-unit of the human CR3 are OKM1 (Breard et al., 1980), Mo-1 (Todd et al., 1981), OKM9 (Wright et al., 1983; Sanchez-Madrid et al., 1983b), OKM10 (Wright et al., 1983; Sanchez-Madrid et al., 1983b), 60.1 (Beatty et al., 1983) and LM2/1.6.1 (Miller et al., 1986).

The antigen p150.95 has a more restricted distribution. It is mainly found on monocytes and macrophages (Hogg et al., 1986), but also on granulocytes (Lanier et al., 1985; Schwarting et al., 1985). Cytotoxic T cells (Van Wauwe et al., 1980; Hildreth and August, 1985) express only low amounts of this antigen. Like the CR3 antigen, p150.95 is involved in granulocyte adherence (Dongworth et al., 1985; Sanchez-Madrid et al., 1983b) and C3bi binding (Malhotra et al., 1986). Furthermore, mAb against the antigen are able to inhibit lysis of target cells by cytotoxic T cells in a manner similar to anti-LFA-1 mAb (Keizer et al., 1987a). In monocytes the antigen is involved in processes like migration, chemotaxis, adhesion and phagocytosis and therefore it is likely that p150.95 is one of the major adhesion-associated molecules on these cells (Keizer et al., 1987b). No mAb are available yet against the murine p150.95 α-subunit but 441.8 (Sarmiento et al., 1982), and H35-89.8 (Pierres et al., 1982). Human analogues for this mAb are TS1/12, TS1/22, TS1/122, TS2/4, TS2/6 and TS2/14 (Sanchez-Madrid et al., 1983b).

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Since the β-subunit is present in all three antigens, mAb against this subunit interfere with many of the mentioned functions. Especially, cell adherence, aggregation and migration are affected (Anderson et al., 1986; Springer and Anderson, 1986b). In the mouse this β-subunit is recognized by the mAb M18/2 (Springer et al., 1984). Human analogues include CLB54 (Miedema et al., 1984), MHM-23 (Hildreth et al., 1983) and TS2/4 (Sanchez-Madrid et al., 1983b).

The clinical importance of these antigens has recently been recognized. Since various chemo-attractants that are released during an inflammatory response stimulate ('upregulate') the expression of CR3 and p150.95, it has been suggested that these antigens play an important role in the inflammatory process (Springer et al., 1984, 1986; Anderson et al., 1986). Additionally, it has been demonstrated that persons with a congenital inability to express the β-chain show a marked impairment of surface expression of all three heterodimers. These patients suffer from bacterial infections, show a lack of pus formation and have a persistent granulocytosis (Springer et al., 1984; Anderson et al., 1985).

In addition to the receptor-like molecules described above, lympho-hemopoietic cells, including cells of the monocytic lineage, possess various other receptors recognized by mAb. Macrophages express receptors for the constant region of IgG. The analysis of these so-called Fc receptors (FcR) has been complicated by the presence of distinct receptors with specificity for different subclasses of IgG.

Competition experiments, using aggregated IgG and immune complexes of different subclasses, indicate that there are at least three different FcR for IgG on macrophages (Diamond and Sharff, 1980; Diamond and Yelton, 1981). The trypsin-sensitive FcRI (Unkeless and Eisen, 1975) binds specifically immunoglobulins of the IgG2a subclass and is involved in antibody-dependent cellular cytotoxicity (Steplewski et al., 1983). In man, the FcRI binds the Fc part of the human IgG1 and IgG3 in its monomeric configuration, and the murine IgG2a and IgG3 (Lubeck et al., 1985; Rosenfeld et al., 1985). The murine FcRI is recognized by mAb 3A2 (Kagami et al., 1989) and human analogue by the mAb 32 (Anderson et al., 1986; Anderson and Looney, 1986) and 10.2 (Dougherty et al., 1987). Two other murine FcR are trypsin-resistant, i.e. FcRII that binds immunoglobulins of the IgG1 and IgG2b subclasses (Unkeless et al., 1979; Looney et al., 1986) and FcRIII that binds aggregated IgG3 (Diamond and Yelton, 1981). Both receptors are involved in the endocytosis of immune complexes (Kurlander and Batker, 1982; Kurlander et al., 1984; Clarkson et al., 1986). In man, like FcRI, the FcRII shows affinity for IgG1 and IgG3 but only in the form of immune complexes and with a much lower affinity (Looney et al., 1986). Additionally, the human FcRII binds murine IgG1 and IgG2b. The murine FcRII receptor is recognized by the mAb 2.4G2 (Unkeless et al., 1979) and 6B7C (Puré et al., 1987), and its human analogue by the mAb IV3 (Anderson et al., 1986; Looney et al., 1986) and CIKM5 (Hogg and Horton, 1987). Despite the similarities between murine and human FcR there are also some discrepancies. For example the murine FcRIII has no human analogue. Instead, some human lympho-hemopoietic cells bear the FcR_{lo} receptor. Within the monocytic lineage macrophages are the only cells that express the receptor. Its precise specificity has not yet been well delineated but some studies indicate a preferential binding to IgG1 (Kulczycki, 1984; Anderson and Looney, 1986). FcR_{lo} is recognized by the mAb 3G8 (Fleit et al., 1982), 4F7 (Fleit et al., 1982), VEP13 (Rumpold et al., 1982), Leu-11a, Leu-11b (Philips and Babcock, 1983) and B73.1 (Perussia et al., 1983a,b).

Another group of antigens present on some cells of the immune system including monocytes and macrophages are the products of the class II major histocompatibility complex gene family. In the mouse they encode the Ia antigens and in man the HLA-D antigens (Kaufman et al., 1984). These antigens play an important role in antigen presentation, the activation of T cells and self-recognition (Unanue and Allen, 1987). MAb directed against these determinants can be used to interfere with these functions or to identify MNP involved in these functions (Kedar and Steinberg, 1987). Many mAb are raised against both murine and human MHC class II antigens. Some anti-Ia mAb are M5/114 (Bhattacharya et al., 1981), 11-5.2 (Rhodes and Agger, 1987), 10-2.16 (Vogel et al., 1983), B21-2 (Nussenzweig et al., 1980) and 2E8 (Nussenzweig et al., 1981), and some anti-HLA-D mAb are BL2 (Cohen et al., 1981), OKIa1 (Reinherz et al.,

1979), 9.3F10 (Van Voorhis et al., 1983) and L203 (Lampson and Levi, 1980).

Macrophage antigens with a much wider distribution than all above mentioned antigens are Mac-2 and Mac-3. These markers were originally described as macrophage-specific (Ho and Springer, 1982, 1983), but later studies indicated that they were present on many other cell types (Flotte et al., 1983). The Mac-2 antigen is a polypeptide of 32 kD that is recognized by the mAb M3/38 and M3/31 (Ho and Springer, 1982). It is present on monocytes (Nibbering et al., 1987a), elicited peritoneal macrophages (Ho and Springer, 1982), Langerhans cells (Haines et al., 1983), dendritic cells (Flotte et al., 1984), and some epithelia and endothelia (Flotte et al., 1983). In bone marrow cultures stimulated with CSF-1 it has been demonstrated that the immature non-adherent cell fraction contained little or no Mac-2-positive cells, whereas nearly all adherent cells were positive (Walker et al., 1985). Also the Mac-3 antigen, a polypeptide of 110 kD that is recognized by the mAb M3/84 (Ho and Springer, 1983), is present on cells in various tissues. The tissue distribution resembles that of Mac-2 and includes the epithelia and endothelia of the small intestine, the lung, the liver and the kidney (Flotte et al., 1983). This ubiquitous presence of the antigen in various tissues may be understood as the Mac-3 antigen is identical to the lysosomal membrane protein LAMP-2 (Chen et al., 1985a,b).

2.2.3. Antigens present on macrophage subsets and other cells

A number of mAb has been described which discriminate between resident and activated macrophages. Most of these mAb show cross-reactivity with other cell types, especially polymorphonuclear neutrophilic granulocytes. Despite this, these monoclonal antibodies may provide important information about the mechanisms which underlie the processes of macrophage activation and differentiation. MAb produced against macrophages in different stages of activation are 54-2 (LeBlanc et al., 1980), M3/37 (Springer, 1981), AcM.1 (Taniyama and Watanabe, 1982), MM9 and WE15 (Taniyama and Tokunaga, 1983), and 7/4 (Hirsch and Gordon, 1983). MAb 54-2 and M3/37 both recognize the Mac-4 antigen. The AcM.1 antibody recognizes activated macrophages induced by pyran and Corynebacterium parvum but not thioglycollate or peptose peptone-elicited macrophages (Taniyama and Watanabe, 1982). The antigen recognized by mAb 7/4 has a similar distribution since it is present on C. parvum and BCG-activated macrophages, but not thioglycollate-elicited macrophages (Gordon et al., 1985). In contrast, the mAb MM9 and 54-2 recognize epitopes that are restricted to the thioglycollate or peptose peptone-elicited macrophages. WE15 reacts with both groups of macrophages (LeBlanc et al., 1980; Taniyama and Tokunaga, 1983). MAb 54-2 also

reacts with marrow-derived macrophages and mast cells (Katz et al., 1981), whereas 7/4 binds to polymorphonuclear neutrophils (Hirsch and Gordon, 1983).

Two of the above described antibodies, i.e. AcM.1 and WE15, are capable of inhibiting complement-mediated tumor cell killing by macrophages (Taniyama and Watanabe, 1982; Taniyama and Tokunaga, 1983). Other mAb reactive with tumoricidal macrophages from BCG-infected mice are 2E1.2D5 (Someya, 1985) and 1A2.10D (Someya, 1986). Cells that could be labeled with 2E1.2D5 or 1A2.10D were more effective in tumor cell killing than negative cells (Someya, 1986). Furthermore, the mAb MA158.2 preferentially binds to tumoricidal macrophages (Koestler et al., 1984). However, all antigens detected by these mAb appeared not to participate in the tumor cell killing mechanism since antibody binding itself had no effect on the cytolytic activity of the labeled macrophages. Three other mAb associated with macrophage functions were described. Antibodies M43, M57 and M102 react with macrophages that are cytotoxic after lymphokine activation, kill antibody-coated tumor cells and express natural killer cell activity, respectively (Sun and Lohmann-Matthes, 1982). In man, a mAb A1-3 is described that detects an antigen present on activated monocytes and macrophages (Hancock et al., 1986) and it inhibits monocyte procoagulant activity (Ewan et al., 1986).

3. FUNCTIONS AND LOCALIZATION OF MACROPHAGES

3.1. General functions of mononuclear phagocytes

In the former chapters it is outlined that MNP are heterogeneous with regard to their cell size, morphology, enzyme content and surface antigens. This variability probably resembles the numerous functions that are performed by MNP. Most of these functions can be classified within three general categories, i.e. phagocytosis, immunoregulation and secretion of biologically active products (Table 3.1).

Table 3.1

Functional properties of mononuclear phagocytes

* phagocytosis and pinocytosis	* immunoregulation	* secretion
- micro-organisms	- T and B cell development	- complement factors
- effete cells	- antigen processing	- enzymes
- debris	- antigen presentation	- bioactive lipids
		- plasma proteins
		- hemopoietic growth factors

Phagocytosis is the ability of MNP to ingest foreign material like micro-organisms, tumor cells and effete cells. MNP are attracted to an infected focus by e.g. bacterial products, complement factors and antigen-antibody complexes via a process termed chemotaxis (Carr, 1978). Phagocytosis occurs in a number of succesive phases starting with a recognition phase followed by an attachment, movement and ingestion phase (Steinman et al., 1983). Generally, phagocytic cells recognize invading micro-organisms only when the latter are coated with certain plasma proteins, the so-called opsonins. Well-known opsonins are the cleaved parts of the C3 complement factor and the various IgG. As mentioned before, MNP possess specific receptors for these components, the FcR and CR. Both receptor types exhibit distinct functional properties. Binding of opsonized particles to Fc receptors constitutively results in phagocytosis and respiratory burst activity, whereas binding to complement receptors not necessarily leads to phagocytosis and no oxygen radicals are produced (Wright and Silverstein, 1983; Wright and Griffin, 1985). Occasionally, opsonin-independent sugar-specific recognition can occur by means of the lectin-like mannose-fucose

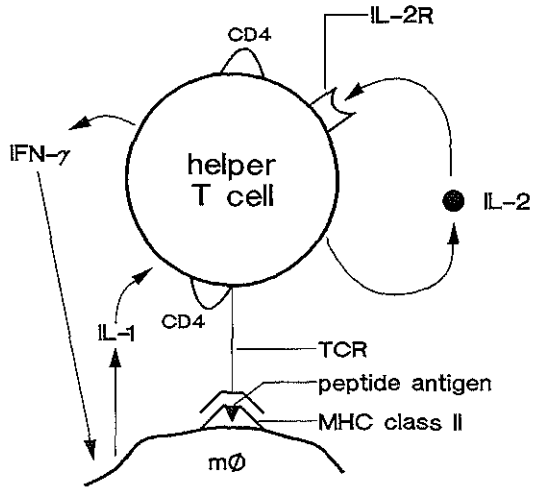

Figure 3.1. Antigen presentation and T cell activation by macrophages.
CD, cluster domain; IFN-γ, gamma interferon; IL, interleukin; IL-R, interleukin receptor; MHC, major histocompatability complex; mϕ, macrophage; TCR, T cell receptor

receptor (MFR). Ligands for MFR include mannan, a mannose rich cell wall component of yeast, zymosan, and poorly defined glycoproteins on parasites such as Leishmania donovani (Blackwell et al., 1985; Ezekowitz et al., 1985a). After attachment of the micro-organisms to the cell surface by one of these mechanisms, the so-called movement phase starts. The bacteria are surrounded by protoplasmic protrusions of the macrophage. It is proposed in the so-termed "zipper hypothesis" that ingestion only occurs if receptors interact sequentially around the full circumference of the target, guiding membrane flow before fusion (Silverstein et al., 1977). When the macrophage pseudopodia meet, the bacteria are ingested and enclosed in a vesicle, the phagosome. Further digestion involves oxygen-dependent and independent mechanisms. The former mechanism includes the release of oxidative metabolites derived from the respiratory burst (Babior, 1984) and oxidative capacity (Beaman and Beaman, 1984). The latter process includes acidification of the phagocytic vacuole by fusion with lysosomes (Gabig and Babior, 1981).

The involvement of macrophages in the regulation of immune responses is well recognized. Besides their function in the first-line host defence they play an important role in B and T cell development and differentiation (Heinen and Tsunoda, 1987; Quesenberry et al., 1987). Furthermore, one of their main functions is the presentation of antigens to cells of the immune system (Figure 3.1; Allen and Unanue, 1984; Unanue, 1984). Presentation of antigens to B cells is a relatively simple process. The

naive antigen is recognized by the surface Ig of the B cell, whereafter plasma cell differentiation and the subsequent production of immunoglobulin starts. In contrast, presentation of antigen to T cells requires biochemical modifications of the antigen by macrophages, the expression of Ia surface molecules and the production of interleukin-1 (IL-1). The biochemical changes that a protein must undergo in order to be expressed on the cell surface of an antigen presenting cell is termed "antigen-processing". This process is complex and still not fully unravelled. Various patterns of antigen-processing have been recognized probably due to distinct mechanisms of protein degradation. Part of the antigen is recycled to the cell surface. This can happen immediately or after a certain processing period. Within the latter process the modified antigen is presented in close association with MHC class II antigens and can be recognized by T lymphocytes. This form of antigen-processing is inhibitable with the pH-increasing lysosomotropic agents chloroquine and ammonium chloride. In contrast, directly recycled antigens are not associated with Ia molecules and the process is not influenced by lysosomotropic agents. Antigens processed in this manner are a source of molecules available for other antigen-presenting cells and B cells. After re-binding of the antigen to antigen-presenting cells they do not necessarily have to be reprocessed and presented to T cells (Ziegler et al., 1987). Not all of the antigen will be recycled to the cell membrane. In vitro metabolic studies indicated that part of the antigen was released as soluble molecules in the culture medium. The release of these peptides was a temperature-dependent process unaffected by chloroquine or ammonium chloride.

An important factor secreted by macrophages during antigen presentation to T cells is IL-1. The molecule is present in two forms termed IL-1α and IL-1β (Auron et al., 1984; LoMedico et al., 1984) that have identical activities and bind to the same receptor on the target cell membrane (Dower et al., 1985; Kilian et al., 1986). IL-1 acts on T cells in two ways. It induces the expression of receptors for interleukin-2 (IL-2) and simultaneously stimulates the production of this T cell growth factor (Lowenthal et al., 1986). Furthermore, it has been demonstrated that a membrane-bound form of IL-1 is directly involved in the cellular interactions between macrophages and T cells during antigen presentation (Kurt-Jones et al., 1986). Not only macrophages but also various other cell types are able to process and present antigens (Poulter, 1983; Unanue and Allen, 1987). At least in part they use similar mechanisms as macrophages (Chain et al., 1986).

Another function of macrophages in the cellular immune response is the production of complement factors. MNP have the potency to synthesize all complement components and it has been postulated that MNP execute many functions of the system at a local level. These include activation of complement, opsonization,

chemotaxis, anaphylaxis, and lysis of micro-organisms (Daha, 1985; Johnson and Hetland, 1988). Besides complement components many other substances may be produced and secreted by macrophages like enzymes, enzyme inhibitors, plasma proteins, growth factors, extracellular matrix proteins, bioactive lipids, steroid hormones and reactive oxygen intermediates (reviewed by Davies and Bonney, 1979; Takemura and Werb, 1984; Nathan, 1987). Where relevant for this thesis, these factors will be discussed in more detail in the following paragraphs.

3.2. Functions of exudate and activated macrophages

After stimulation with an appropriate inflammatory agent, macrophages can become activated. Activation occurs at a local level after monocytes are attracted to a lesion. The process involves multistep, complex phenomena induced by a diverse group of immunomodulators. Distinct stimuli can promote successively higher levels of activation (Cohn, 1978; Hirsch and Gordon, 1983) and therefore multiple mediators at a site would be more effective than any one acting alone. The number of stimuli, their concentration, macrophage susceptibility, and other variable conditions result in a large functional heterogeneity. For this reason, activation of macrophages must always be defined with respect to a certain function, e.g. antigen presentation, tumoricidal or bactericidal activity. Despite the diversity among macrophages resulting from the activation process, there is general agreement in categorizing them in (a) exudate or inflammatory macrophages and (b) activated macrophages.

Exudate macrophages occur in non-immunologically mediated inflammatory sites. Compared with resident macrophages they are enlarged in size. Furthermore, the expression of various surface receptors is elevated, for example FcRI and FcRII (Ezekowitz and Gordon, 1984), the transferrin receptor (Hamilton et al., 1984) and the activated form of CR3 (Griffin, 1984). They exhibit high secretory activity but do not possess enhanced microbicidal or tumoricidal activity (Cohn, 1978; Adams and Hamilton, 1984; Takemura and Werb, 1984).

Activated macrophages occur in non-sterile inflammatory sites and develop from resident or exudate macrophages after stimulation with a specific class of lymphokines, termed macrophage activating factors (MAF). These factors are produced by antigenically stimulated T cells and induce antigen non-specific activation of macrophages (Simon and Sheagren, 1972; David, 1975). It has been demonstrated that an important MAF is IFN-γ (Roberts and Vasil, 1982; Schreiber et al., 1983). This factor is able to activate macrophages in vivo and in vitro to perform various functions (Murray et al.,

1985). Other biochemically defined MAF are tumor necrosis factor (TNF-α; de Titto et al., 1986; Shparber and Nathan, 1986) and lymphotoxin (TNF-ß; Shparber and Nathan, 1986). Traditionally the performance of enhanced microbicidal (Mackaness, 1969; Nathan, 1983) and tumoricidal (Adams and Nathan, 1983) activity has been designated as activation of macrophages, but other functions, like for example antigen presentation and enhanced chemotactic or phagocytic activities, should be included (Adams and Hamilton, 1984). Tumor cell lysis is one of the best investigated functions of activated macrophages (Adams and Hamilton, 1984, 1987) and therefore offers a good model to study the complexity of the activation process. Young mononuclear phagocytes from sites of inflammation, but not resident macrophages, are susceptable to MAF and are termed "responsive". After exposure to MAF the macrophages become "primed" and gain responsiveness for second signals as endotoxin, crude lymphokines or the supernatant of tumor cells (Figure 3.2; Hamilton and Adams, 1987). MAF lowers the dose requirements for a second signal. On the molecular level the process involves alterations in intracellular calcium levels (Somers et al., 1986) and possibly also

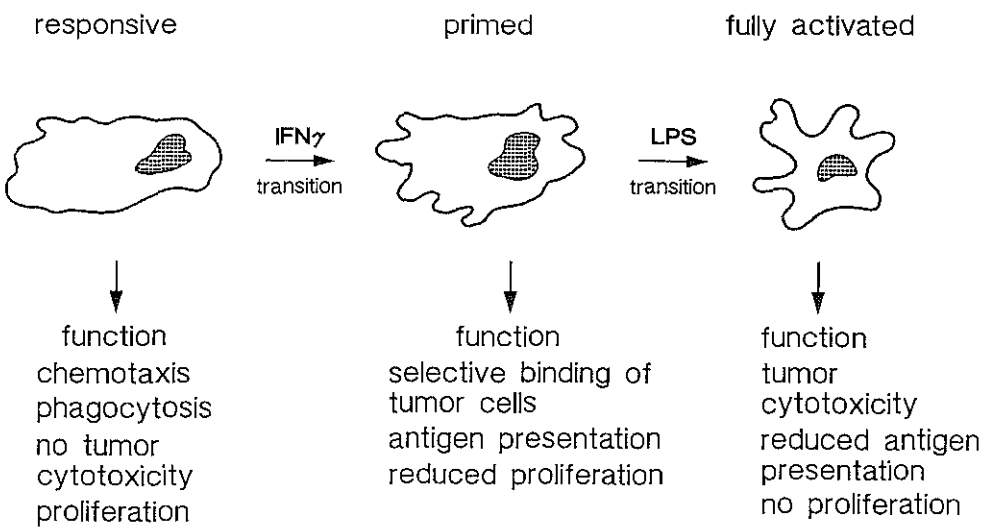

Figure 3.2 Current view on the multi-step process of macrophage activation.
IFNγ, gamma interferon; LPS, lipopolysaccharide

activation of protein kinase C (Hamilton et al., 1985). Furthermore, two-dimensional gel electrophoresis demonstrates the synthesis of new proteins. In mice, extra surface proteins of 38, 42, 65, 75, 80, 85 and 120 kD are expressed (Johnston et al., 1987a, b). Likely, expression of these proteins could has association with the activation

process although further investigation is needed to clarify this.

Activated macrophages can display two general ways of cytotoxicity on target cells. The first, termed cytostasis, is the inhibition of cell proliferation by interruption of DNA synthesis. This is a cell cycle-independent phenomenon and it is speculated that macrophages interfere with target cell iron metabolism, especifically by making iron unavailable to enzymes that require it, such as aconitase and ribonucleotide reductase (Hibbs et al., 1984).

The second type of cytotoxicity is the killing of cells. Involved killing mechanisms include the secretion of cytolytic proteinase (Adams et al., 1980), arginase (Currie, 1978), hydrogen peroxide (Nathan and Cohn, 1980), and the elaboration of mediators such as TNF (Beutler and Cerami, 1986) and IL-1 (Onozaki et al., 1985; Lachman et al., 1986).

3.3. Localization and function of tissue macrophages

3.3.1. Macrophages in the bone marrow

Macrophages are more or less evenly distributed throughout the bone marrow. They stain positively for both acid phosphatase and α-naphthyl-butyrate esterase (Bainton, 1985). Furthermore, they express the macrophage antigen F4/80 and FcRII, but not Mac-1 (Hume et al., 1983b, 1984c; Crocker and Gordon, 1985). Part of them are Ia-positive (Crocker and Gordon, 1985). The majority of the macrophages is located at specific histologically defined sites, i.e. against the subendosteal bone surface (Hume et al., 1984c), in the parasinal locations of the hemopoietic cords among the hemopoietic cells (Weiss, 1976; Lichtman, 1981; Hume et al., 1983b), within the lumen of the vascular sinuses (Weiss, 1976) and as the central cell in erythroblastic (Bessis and Breton-Gorius, 1962; Ben-Isay and Yoffey, 1972) and myelomonocytic islands (Hume et al., 1983a; Crocker and Gordon, 1985).

An important function of macrophages in the hemopoietic cords is the phagocytosis of erythroblastic and megakaryocytic nuclei, and aging or damaged platelets, erythrocytes and leukocytes (Cline, 1975). Parasinal macrophages regularly extend into the sinusoidal lumen and thus monitor cellular traffic into and out of vessels (Weiss and Sakai, 1984). Central macrophages play a regulatory role in erythropoiesis and granulopoiesis by cellular interactions and the production of hemopoietic mediators (Cline and Golde, 1979). Macrophages in erythroblastic islands possess a specific lectin-like receptor with specificity for sialylated components on erythroid cells (Crocker and

Gordon, 1986). They synthesize ferritin (Doolittle and Richter, 1981), transferrin (Haurani et al., 1973) and haptoglobin (Palmer and Schueler, 1976) and thus are a local source of iron for developing normoblasts (Carr, 1978). Additionally, central macrophages exhibit burst promoting activity (BPA) by secreting a factor that influences progenitor cell proliferation (Ploemacher et al., 1979; Zuckerman, 1980). This factor is probably identical to multi-CSF or interleukin 3 (Dexter, 1984; Nicola and Vadas, 1984). Terminal differentiation of the erythroid progenitor cells may be mediated by macrophage-derived erythropoietin (Rich, 1987). Other factors produced by bone marrow macrophages have regulatory effects in the myelopoiesis. Stimulating factors include multi-CSF (Rich, 1986) and GM-CSF (Nicola and Vadas, 1985; Rich, 1986). Inhibiting factors are IFN-α/β and PGE (Moore et al., 1984). Besides the described effects of macrophages on the myeloid and erythroid development there are indications that macrophages also influence B cell development (Heinen and Tsunoda, 1987; Quesenberry et al., 1987). Furthermore, it is well recognized that macrophages produce IL-1 and TNF, monokines that can stimulate other cells to produce GM-CSF (Nathan, 1987; Golde and Gasson, 1988) and BPA (Zuckerman et al., 1985).

3.3.2. Macrophages in the thymus

Macrophages are present in all compartments of the thymus, i.e. the cortex, the medulla and the corticomedullary zone (Beller and Unanue, 1980; Duijvestijn et al., 1982). Within these regions they show a marked heterogeneity with respect to their morphology and expression of macrophage antigens. Macrophages of the outer cortex, integrated in or just beneath the capsule and adjacent to the cortical trabeculae stain positively with mAb F4/80 (Hume et al., 1983a), MOMA-2 (Kraal et al., 1987) and ER-MP23 (Leenen et al., 1990b). The majority of these cells also is Ia-positive (Zepp et al., 1984). In contrast, these cells do not stain with the macrophage marker BM8 (Malorny et al., 1986). Towards the deeper parts of the cortex and the corticomedullary zone the number of F4/80 and Ia-positive cells decreases (Hume et al., 1983a; Zepp et al., 1984). MOMA-2-positive cells are abundant in both regions (Kraal et al., 1987). Macrophages located in these areas branch between the thymocytes and surround them with their cytoplasmic protrusions forming typical thymocyte clusters. Part of the cortical and cortico-medullary macrophages stains positively for periodic acid Schiff (PAS). The PAS-positive inclusions in these cells ar large heterolysosomes derived from phagocytized lymphocytes (Weiss and Sakai, 1984; Mueller et al., 1987).

Thymic medullary macrophages are distributed in a scattered pattern. F4/80-posi-

tive cells have a more rounded morphology and are often located in the vicinity of blood vessels (Hume et al., 1983a). These perivascular F4/80-positive macrophages also stain with Mac-1 (Flotte et al., 1983). More recent studies indicate that this latter antigen is expressed by the majority of the thymic macrophages (Nabarra and Papiernik, 1988). Most if not all medullary macrophages have shown to be MOMA-2--positive (Kraal et al., 1987), approximately 50% is Ia-positive (Nabarra and Papiernik, 1988) and only few are BM8-positive (Malorny et al., 1986).

It is likely that thymic macrophages play an important role in T cell selection and differentiation. The exact mechanism by which macrophages are involved in this process is still unknown. It has been demonstrated that macrophage products like thymocyte growth factor (Beller and Unanue, 1977) and IL-1 (Gallily et al., 1985) are involved in these processes. Furthermore, specific cell-cell interactions between stromal cells and thymocytes are important (Kyewski et al., 1982). In radiation chimeras, replacement of host thymocytes by injected bone marrow cells follows a characteristic pattern. Donor-derived T cells appear first as a cluster around Ia-negative macrophages and later around cortical Ia-positive epithelial or Ia-positive medullary dendritic cells indicating a defined sequence of lympho-stromal interactions during intrathymic maturation (Kyewski et al., 1982).

Isolation of thymic reticulum cells and subsequent inoculation of thymocytes on these cells provides a good model to investigate the thymocyte-stromal cell interactions. Phenotyping of the isolated cells indicated that many adherent cells had macrophage characteristics. These include the property of phagocytosis and high enzyme contents of acid phosphatase, nonspecific esterase and 5'nucleotidase (Papiernik et al., 1983). However, many cells possess the enzyme α-mannosidase, a characteristic they have in common with dendritic cells (Papiernik et al., 1983). Part of the cells are Ia-positive (Papiernik et al., 1983; Zepp et al., 1984). It has been demonstrated that cultured thymic macrophages are able to induce lymphocyte proliferation. Whether this stimulation is restricted to more mature medullary thymocytes (Papiernik et al., 1983) or also include younger cortical thymocytes (Zepp et al., 1984) is still a matter of debate. Distinct enrichment factors for the various adherent cell types in these isolation procedures may explain these differences. It is obvious that stimulation of the thymocytes only occurs when they are in close contact with the adherent cell. Treatment of adherent cells with cytochalasin B, a compound that prevents macrophage-lymphocyte interaction completely by inhibition of cytoskeleton modelling, abolishes the stimulatory effect (Zepp et al., 1984). In addition, it has recently been described that mAb against the Ia, Mac-1 and LFA-1 molecules also inhibit adherent cell-induced T cell proliferation (Papiernik and El Rouby, 1988). Furthermore, as a result of the macrophage-lymphocyte interaction the IL-2 receptors

on the lymphocytes are upregulated which is suggestive for an involvement of IL-1 in the process (Papiernik and El Rouby, 1988).

3.3.3. Macrophages in the spleen

Within the spleen macrophages are present in both the red and the white pulp. Red pulp macrophages are abundant and more or less evenly distributed. They are heavily labeled with F4/80 (Hume et al., 1983a; Witmer and Steinman, 1984), BM8 (Malorny et al., 1986) and MOMA-2 (Kraal et al., 1987), but are negative for Mac-1 (Flotte et al., 1983; Witmer and Steinman, 1984). Although less well investigated, the functions of these macrophages partially overlap with those described for bone marrow macrophages. They are present as central macrophages in hemopoietic islands, perform a scavenger function by phagocytosis of red blood cells (Orlic et al., 1965) and possess the lectin-like receptor that binds sialylated components on erythropoietic cells (Crocker and Gordon, 1986). Furthermore, it has been suggested that they regulate cellular traffic in the red pulp of the spleen (Weiss and Sakai, 1984).

White pulp macrophages are localized in the marginal zone, the periarterial lymphatic sheath (PALS) and the follicle. Within the marginal zone the marginal zone macrophages have been recognized (Humphrey and Grennan, 1981). Marginal zone macrophages show a low NSE activity and are highly phagocytic. They are large and stain positive for acid phosphatase (Matsuno et al., 1986). They specifically express the antigen recognized by mAb ER-TR9 (Dijkstra et al., 1985; van Vliet et al., 1985) but lack the F4/80 antigen (Witmer and Steinman, 1984; Hume et al., 1985). In contrast, a second, minor macrophage population of F4/80-positive cells is present in the marginal zone. The cells are small, phagocytic and exhibit a low acid phosphatase activity. These characteristics are similar to those of the red pulp macrophages (Matsuno et al., 1986; Kotani et al., 1985). The precise function of macrophages in the marginal zone is not clear. In mice, marginal zone macrophages are highly phagocytic. In this way they probably perform an important scavenger function. The observation that carbon-loaded macrophages migrate from the marginal zone to the germinal center of the follicle is suggestive of a contribution of marginal zone macrophages in the antigen transport and presentation to B cells in the follicle (Kotani et al., 1985). Furthermore, there are some indications that marginal zone macrophages have a special role in presenting thymus-independent type 2 antigens to specific B cells located in the marginal zone (Humphrey and Grennan, 1981; van Vliet et al., 1985). However, more recent data contradict these results (Claassen et al., 1986a; Kraal et al., 1989).

At the border of the marginal sinus and the PALS the marginal metallophils are localized (Snook, 1964). They are easily characterized by their strong NSE activity and their small phagocytic capacity. Furthermore, they exclusively stain with the mAb MOMA-1 although they also are weakly positive for Mac-1 (Kraal and Janse, 1986). Like marginal zone macrophages they lack the F4/80 antigen (Witmer and Steinman, 1984; Hume et al., 1985). Most, if not all, other macrophages in the periarterial lymphatic sheath stain positively for mAb MOMA-2 (Kraal et al., 1987). In particular those which are located around the central arteriole additionally express antigens recognized by mAb F4/80, M1/70 and 2.4G2 (Witmer and Steinman, 1984). Their function still is unclear but it is likely that they are involved in the processing and presentation of antigens to B cells. The function of marginal metallophils is also rather speculative. It has been suggested that they are involved in antigen presentation (Kraal and Janse, 1986), the detoxification of endotoxins (Eikelenboom et al., 1978), and the direction of lymphocyte traffic in the spleen (Brelinksa and Pilgrim, 1982).

Macrophages present within germinal centers can be subdivided into two populations. First, the classical tingible body macrophages can be recognized. They are MOMA-2-positive (Kraal et al., 1987), but are F4/80 and Mac-1-negative (Witmer and Steinman, 1984). It has been suggested that they develop from the marginal metallophils (Groeneveld et al., 1986). Second, a macrophage-type can be recognized that, as was demonstrated after carbon injection, migrated from the marginal zone (Kotani et al., 1985). The exact function of both follicular macrophage populations is unknown. It has been speculated that tingible body macrophages play a role in eliminating abnormally programmed B lymphocytes by somatic mutation or heavy chain class switching (Nieuwenhuis and Opstelten, 1984) and that the marginal zone-derived macrophages are involved in antigen transport, processing and presentation (Kotani et al., 1985).

3.3.4. Macrophages in the lymph nodes

Macrophages are present in all parts of the lymph nodes, i.e. the cortex, the paracortex and the medulla. They enter a lymph node with the afferent lymph, pass the subcapsular sinusoid endothelium and migrate through the paracortex to the medullary cords and sinuses (Hendriks et al., 1980). The decrement of macrophages in lymph nodes deprived of afferent lymphatic vessels indicate that lymph node macrophages do not replicate (Hendriks et al., 1980). Cortical macrophages are abundant in the area adjacent to the subcapsular sinus. They stain positively with F4/80 (Hume et al., 1983a), M1/70 (Witmer and Steinman, 1984), ER-TR9 (van Vliet et al., 1985) and

MOMA-2 (Kraal et al., 1987) but not with BM8 (Malorny et al., 1986). Macrophages in the paracortex are only recognized by MOMA-2 but not by other macrophage markers. After antigenic stimulation the tingible body macrophages of the follicles were also heavily stained with MOMA-2 (Kraal et al., 1987). Additionally, these cells stained with the mAb 2.4G2 (Witmer and Steinman, 1984).

The medullary macrophages of the lymph node stain positively with many macrophage markers. They are positive for F4/80 (Hume et al., 1983a), ER-TR9 (Van Vliet et al., 1985), BM8 (Malorny et al., 1986) and MOMA-2 antigens (Kraal et al., 1987).

The function of macrophages present in the region adjacent to the subcapsular sinus is that of scavenging and clearing antigens from the afferent lymph (Hendriks et al., 1980; Witmer and Steinman, 1984). After endocytosis the antigens are processed. By migration of macrophages from the subcapsular sinus to the paracortex or the follicles they are transported to precommitted lymphocytes which are triggered to execute an appropriate immune response. Furthermore, it has been speculated that macrophages are involved in the regulation of lymphocyte influx and efflux by affecting the endothelial cells of the high endothelial venule (Hendriks et al., 1980). The function of tingible body macrophages within the follicle centre is probably analogous to that described for the spleen, i.e. removal of effete and abnormally programmed B lymphocytes.

3.3.5. Macrophages in the liver

The liver contains the largest number of macrophages in the body (Lee et al., 1985). These cells, called Kupffer cells, together with endothelial cells form the sinusoidal lining of the liver. Kupffer cells stain positively with most macrophage markers including F4/80 (Hume et al., 1984a), BM8 (Malorny et al., 1986) and MOMA-2 (Kraal et al., 1987). The markers are not evenly distributed over the population since it has been demonstrated that in comparison to BM8 only a small number of Kupffer cells express the F4/80, Mac-2 and Ia (M5/114) antigens (Nibbering et al., 1987c). However, the BM8 antigen expression may be overestimated since in another study it has been demonstrated that most, if not all, Kuppfer cells are positive for B21/2 (anti-Ia) and 2.4.G2 (Lepay et al., 1985). Kupffer cells lack the Mac-1 antigen (Lepay et al., 1985; Nibbering, 1987c).

Kupffer cells play an essential role in the elimination of antigens derived from the portal circulation, primaryly through the process of phagocytosis (Crofton et al., 1978). Isolated Kupffer cells have been shown to present soluble antigens to T cells in vitro and to function as accessory cells for mitogen-induced T lymphocyte proliferation

(Richman et al., 1979; Rogoff and Lipsky, 1979). Recently, it has been demonstrated that liver sinusoidal lining cells, including Kupffer cells, are also able to take up, process and retain antigen in vivo (Rubenstein et al., 1987). After isolation, they are capable to present the antigen-primed T cells although less effectively than a reference population of peritoneal-exudate macrophages. The difference in the antigen presenting capability of peritoneal and liver macrophages cannot be explained by a deficiency of IL-1 production since it has been demonstrated that liver sinusoid lining cells secrete IL-1 (Rubenstein et al., 1987). Compared with peritoneal macrophages Kupffer cells release elevated levels of this interleukin (Leser et al., 1982) and it has been speculated that this mediator is involved in the macrophage-mediated regulation of hepatocyte cytochrome P450 levels and protein synthesis (Keller et al., 1985; Peterson and Renton, 1986). Moreover, it has been demonstrated that Kupffer cells are two to three times more phagocytic than peritoneal macrophages (Laskin et al., 1988). However, they produce less reactive oxygen intermediates and hydrogen peroxide (Laskin et al., 1988; Lepay et al., 1985). A low hydrogen peroxide production correlates often with a high apoprotein E secretion (Werb et al., 1985). Indeed, apoprotein E is one of the major secretory products of Kupffer cells (Lepay et al., 1985). This suggests that Kupffer cells may play a significant role in hepatic lipoprotein metabolism.

3.3.6. Macrophages in the lung

Pulmonary macrophages are present in two anatomically distinct compartments. The alveolar macrophages predominantly reside in the air spaces, whereas the interstitial macrophages are located in the lung parenchyma. Alveolar and interstitial macrophages account for 60 and 40 per cent of the lung's macrophage population, respectively (Bowden and Adamson, 1980). It has been speculated that the interstitial macrophage is an intermediary stage between monocytes and alveolar macrophages (Bowden and Adamson, 1972). However, more recent data show that part of the alveolar macrophage population is self sustaining (Saywer, 1986; Shellito et al., 1987) and that the predominant source of alveolar macrophages are the monocytes from the blood with a smaller proportion arising from interstitial macrophages (Bowden and Adamson, 1980; Blussé van Oud Alblas et al., 1983). There are indications that the expression of macrophage markers differs between both populations. Over 85% of all alveolar macrophages obtained by lung lavage showed an intense expression of the macrophage markers T-200, Mac-2 and Mac-3, a moderate expression of F4/80 and a low expression of Mac-1 and FcRII (Nibbering et al., 1987a). Only around 30% of the cells were weakly positive for Ia. In contrast, interstitial macrophages were much more

variable in their staining pattern. Compared to alveolar macrophages they showed a more intense expression of the F4/80, FcRII, Ia and Mac-1 antigens but a lower expression of the Mac-2 antigen (Nibbering et al., 1987b). Some care should be taken by the interpretation of these data since the interstitial macrophages were cultured for two days which may influence their phenotype.

The alveolar macrophage acts as the lung's first cellular line of host defense in sequestering inhaled foreign material by phagocytosis and subsequent translocation to the regional trachea bronchial lymph node (Harmsen et al., 1985). Studies on the role of alveolar macrophages as accessory cells in antigen-induced lymphocyte activation lead to controversial conclusions. Some studies have shown that alveolar macrophages suppress lymphocyte proliferation in response to antigens and mitogens (Laughter et al., 1977; deShazo et al., 1983), but other studies have shown the opposite (Ettensohn and Roberts, 1983; Toews et al., 1984). Furthermore, it was demonstrated that at low ratios of alveolar macrophages to T lymphocytes, antigen presentation is normal, whereas at high ratios of macrophages to T lymphocytes, antigen presentation is suppressed (Rich et al., 1987). A reasonable explanation for these findings is obtained from a recent study which demonstrates that Ia-positive dendritic cells and not macrophages were the antigen presenting cells (Holt et al., 1988). Antigen presentation by dendritic cells in vitro was inhibited by the presence of low numbers of macrophages. Moreover, in vivo depletion of alveolar macrophages by dichloro-methylene-diphosphonate greatly enhanced the pulmonary immune response (Thepen et al., 1989). In relation to this view it is of interest that alveolar macrophages show a decreased IL-1 activity when compared to monocytes (Wewers et al., 1984). This phenomenon can be explained by the production of high PGE_2 levels by alveolar macrophages (Monick et al., 1987). In addition to their immunological function, alveolar macrophages are involved in lung surfactant turnover (Dougherty and McBride, 1984). Like the alveolar macrophages, interstitial macrophages also have an important function in the immune response. It has been demonstrated that these cells are phagocytic, cytotoxic and immunoregulatory (Holt et al., 1985; Chandler et al., 1986).

3.3.7. Macrophages in the gastro-intestinal tract

It was estimated by Lee et al. (1985) that the gastro-intestinal tract contains one of the largest macrophage pools of the body. This pool can be subdivided into two main categories, i.e. macrophages present in the non-lymphoid parts and those in lymphoid parts of the tract. The former category mainly consists of macrophages in the lamina

propria, submucosa (LeFevre et al., 1979) and muscle layers (Mikkelsen et al., 1985, 1988), whereas the latter category includes macrophages of the tonsils and Peyer's patches.

Within the lamina propria macrophages occur in the villous cores and loose connective tissue surrounding the crypts of Lieberkühn. They are observed more frequently in the apical regions of the villi, often adjacent to blood vessels or in close association with plasma cells (Sawicki et al., 1977; Hume et al., 1984a). Additionally, more elongated macrophages are located just beneath the epithelium (Hume et al., 1984a). In studies using tritiated thymidine, it was estimated that the turnover of macrophages in the lamina propria is very low (Sawicki et al., 1977). Although most of these macrophages express the pan-macrophage marker F4/80 (Hume et al., 1984a), they vary in the expression of Ia molecules (Mayrhofer et al., 1983; Selby et al., 1983; Wilders et al., 1983), and the presence of the enzymes acid phosphatase (Wilders et al., 1983; Sminia et al., 1986) and membrane adenosine triphosphatase (Selby et al., 1983). Little is known about the precise functions of macrophages in the non-lymphoid regions of the intestine. They probably play an important role in the first line host defense by phagocytosis of bacteria (Beeken et al., 1987; Wells et al., 1987) and
the production of antibacterial enzymes such as lysozyme (Spencer et al., 1986). Another activity of intestinal macrophages may be the control of epithelial function (Hume et al., 1984a, 1985). Furthermore, the migration of macrophages into the epithelium and their release into the lumen may provide a potential excretion mechanism of indigestible material (LeFevre et al., 1979).

Since Peyer's patches represent the major component of the gut-associated lymphoid tissue they will be discussed here in more detail. Macrophages are present in all regions of the Peyer's patch. They occur within the epithelium, the subepithelial space, the mid-dome region, the germinal centres and close to the serosal border (LeFevre et al., 1979). Differences in morphology (Hammer et al., 1983) and enzyme expression of e.g. the enzymes acid phosphatase (Sminia et al., 1983) and nonspecific esterase by the various macrophages are suggestive of the existence of distinct sub-populations (LeFevre et al., 1985).

The functions of the Peyer's patch macrophages are only partly understood. It is obvious that Peyer's patch macrophages are able to phagocytize and to transport the phagocytized material to the mesenteric lymph node. For example, it has been observed that ingested inert particles like carbon, exclusively pass through the specialized epithelium of Peyer's patches (Joel et al., 1978; Lause and Bockman, 1981; LeFevre et al., 1985) and are phagocytized by macrophages in subepithelial region. After longer exposure to the carbon, carbon-loaded macrophages were present in the

other regions of the Peyer's patch and the mesenteric lymph node. After termination of the particle ingestion the carbon resided for the longest period in the basal macrophages (Joel et al., 1978). These observations suggest that the distribution of carbon in these tissues at least in part is due to migration of subepithelial macrophages.

The property of antigen presentation by Peyer's patch macrophages is a matter of considerable debate. Early studies indicated that within the Peyer's patch little antibody synthesis occurred and it was postulated that this was due to a lack of accessory function by Peyer's patch macrophages (Kagnoff and Campbell, 1974; Challacombe et al., 1979). More recent studies demonstrated that cells obtained from enzymatically disrupted Peyer's patches of various species are able to present antigens to T cells. Part of these cells probably are macrophages since they phagocytize (Richman et al., 1981; MacDonald and Carter, 1982), and are positive for acid phosphatase (Sminia et al., 1983), nonspecific esterase (Lause and Bockman, 1981; Richman et al., 1981; Wilders et al., 1983) and ATP-ase (Wilders et al., 1983). Later studies indicate that non-adherent dendritic cells, rather than macrophages, are the main antigen presenting cells in these sites (Barr et al., 1983; Spalding et al., 1983). The observed antigen presentation in the above studies could be explained by a contamination of the macrophages with these dendritic cells. Furthermore, it has been demonstrated that, compared with peritoneal macrophages, Peyer's patch macrophages showed a low expression of all types of Fc receptors as well as Ia, but express normal Mac-1 levels (Vetvicka et al., 1987).

3.3.8. Macrophages in the kidney

Within the last few years it has become obvious that the number of macrophages in the kidney has been underestimated. In a quantitative study with F4/80 it has been demonstrated that the kidney contains considerable numbers of macrophages (Lee et al., 1985). These macrophages are located throughout the renal interstitium with the highest concentration present in the medullary interstitium. Such cells tend to spread in the plane of the basement membrane of the tubuli and parallel the medullary rays. Within the interstitium of the cortex, macrophages occur in direct physical contact with the outer surface of the proximal and distal tubules and in particular, the Bowman's capsule. No F4/80-positive cells have been observed in the glomeruli (Hume and Gordon, 1983). Using three cytoplasmic markers specific for monocytes/macrophages a similar distribution in human kidneys could be observed. However, in this study it was shown that macrophages were also present in the

glomeruli (Marshall and Maciver, 1984). In rats, interstitial "dendritic" cells can phenotypically be subdivided in distinct subpopulations, i.e., a $W3/25^+Ia^-$, $W3/25^+Ia^+$ and a small $W3/25^-Ia^+$ population. $W3/25^+Ia^-$ and $W3/25^+Ia^+$ cells are abundant in the renal medulla and cortex, respectively (Steiniger et al., 1984). Since W3/25 is present on rat macrophages (Mayrhofer et al., 1983), it is likely that most cortical macrophages are Ia-positive and most medullary macrophages Ia-negative. Indeed, such MHC class II distribution has been observed (Bohman et al., 1988).

The precise role of macrophages in the kidney is not completely understood. They are thought to participate as antigen-processing and presenting cells in renal diseases like immunologically mediated tubulo-intestinal nephropathies and allograft rejection (Gelfand et al., 1979; Harry et al., 1984). If macrophages are identical to the renomedullary type 2 interstitial cells, as is suggested (Hume and Gordon, 1983), than they probably also perform an important antihypertensive function (Pitcock, 1988). Additionally, the observation that splenic and hepatic macrophages are able to produce erythropoietin under hypoxic conditions (Rich, 1987) together with the observation that the erythropoietin-producing cells of the body are located in the renal tubule interstitium (Schuster et al., 1987) make medullary macrophages important candidates for this function.

3.3.9. Macrophages in the nervous system

Bone marrow chimera and monocyte transfer experiments indicate that the microglial cells of the nervous system are bone marrow-derived cells that probably arise from blood monocytes (Ling et al., 1980; Oehmichen et al., 1979; Oehmichen, 1982). A possible monocytic origin is in good agreement with the observation that microglial cells of the retina possess the pan-macrophage marker F4/80 (Hume et al., 1983b). In later studies it has been demonstrated that microglial cells in other regions of the brain also express this marker. Two populations of F4/80-positive macrophages can be observed. The first population is associated with the connective tissue of the choroid plexus, ependyma and leptomeninges. The second population is present in both the grey and white matter of the nervous tissue. Cells of this latter population additionally express the CR3 and FcRII as is demonstrated with mAb M1/70 and 2.4G2 (Perry et al., 1985). In rats, part of these cells express the CD4 antigen (Perry and Gordon, 1987). Furthermore, isolated and cultured cells of microglial origin show various monocytic characteristics such as expression of nonspecific esterase and secretion of lysozyme (Zucker-Franklin et al., 1987).

The function of macrophages in the nervous tissue is still under investigation. It has

been suggested that macrophages surrounding the ventricular spaces play a role in the removal of debris from the ventricles by phagocytosis (Bleier et al., 1982). Macrophages associated with the leptomeninges are thought to be involved in the transport of immunoglobulins across the blood brain barrier (Nyland, 1982). Moreover, macrophages probably have an important function in the developing brain by phagocytizing degenerated nervous cells. These cells are chemotactic for macrophages (Perry et al., 1985). This phenomenon may explain that macrophages are attracted to sites of peripheral nerve injury. Here they are involved in nerve regeneration by the clearance of myelin and the secretion of growth factors that stimulate Schwann cell proliferation and neuron elongation (Perry et al., 1987b). The observation that cultured microglial cells are able to produce IL-1 is suggestive of an immunoregulatory role of these cells in the brain (Giulian et al., 1986).

4. DENDROCYTES

Dendritic cells (DC) were first recognized as a novel cell type isolated from and specific for lymphoid organs (Steinman and Cohn, 1973). Later studies indicated that they could also be isolated from many other organs (Klinkert et al., 1982; Sertl et al., 1986). DC per definition are isolated cells that must be studied in vitro. Together with their in situ counterparts (Table 4.1) they are termed 'dendrocytes' (DDC) according to Austyn (1987).

It is still questionable whether DDC are constituents of the mononuclear phagocyte system or whether they are derived from a distinct lineage. Like MNP, they are bone marrow-derived (Steinman and Nussenzweig, 1980) and from ontogenetic studies it has been suggested recently that they emerge as a separate lineage (Janossy et al., 1986; Van Rees et al., 1987). However, other studies indicate that some macrophage-specific markers show a considerable overlap with the DDC population. This latter observation, together with the notion that DDC share functions with macrophages are reasons to pay some more attention to this cell type in this thesis.

DDC are irregular in shape, have an oval or irregular-shaped nucleus and their cytoplasm contains many electron-dense mitochondria that are easily distinguished from the less dense mitochondria of macrophages. Their cytoplasm contains sparse rough endoplasmatic reticulum but the smooth endoplasmatic reticulum is well developed (Austyn, 1987). In contrast to macrophages, DDC are poorly phagocytic and contain no or only few endocytic vesicles and lysosomes. They stain weakly, if at all, for acid phosphatase, endogenous peroxidase and nonspecific esterase (Witmer and Steinman, 1984). Isolated DDC do not proliferate in culture. However, in bone marrow cultures they develop from precursors and can be maintained for longer periods (Bowers and Berkowitz, 1986).

Phenotypically, DC differ in their antigen expression when compared to other DDC. DC do not express the pan macrophage marker F4/80 (Austyn, 1987; Rhodes and Agger, 1987) and the FcRII (Austyn, 1987). However, they stain positive for Mac-1 (Schuler and Steinman, 1985), Mac-2 and Mac-3 (Rhodes and Agger, 1987). Additionally, they constitutively express the common leukocyte antigen, T-200, and MHC class II (Ia) antigens.

In contrast to DC, dendrocytes present in pulmonary tissues (Sertl et al., 1986) do express the FcRII. Moreover, Langerhans cells in the skin also possess FcRII and additionally express F4/80 (Hume et al., 1983a). The latter antigen has also been observed on DDC in the muscularis externa of the small intestine (Mikkelsen et al.,

1988).

At the moment only a few dendrocyte-specific mAb are available. Murine DDC are recognized by mAb 33D1 (Nussenzweig et al., 1982), NLDC-145 (Kraal et al., 1986) and MIDC-8 (Breel et al., 1987). Their rat analogues specifically stain with IF119 (Nagelkerken et al., 1987).

Nowadays it is well understood that DDC play an important role as accessory cells in the immune

Table 4.1

Localization of dendrocytes

Type	Localization in situ
Interdigitating cells	T cell areas of spleen, lymph node, Peyer's patch, thymic medulla
Veiled cells	Peripheral lymph of afferent lymphatics, Central lymph of thoracic duct after mesenteric lymphadenectomy of rats
Langerhans cells	Epidermis of skin
Interstitial dendrocytes	Interstitial connective tissue of non-lymhoid tissues except brain

response. In vitro, they are able to present antigens as is indicated by their property to induce T cell proliferation in the allogeneic mixed leukocyte reaction (Steinman and Nussenzweig, 1981). In contrast to macrophages and B cells which are other cells involved in antigen presentation, DC were able to bind T cells in an antigen-independent manner (Inaba and Steinman, 1986). This observation suggests that DC are especially important for the induction of a primary immune response. DDC also are able to induce an immune response in vivo. DDC from sensitized rats are able to transfer allergic encephalomelitis to naive animals (Knight et al., 1983). Furthermore, it has been demonstrated that DDC can trigger graft rejection (Lechler and Batchelor, 1982). Treatment of donors with cyclophosphamide and total body irradiation eliminate DDC and achieve prolonged survival of allografts (McKenzie et al., 1984).

5. PURPOSE OF THE THESIS AND INTRODUCTION TO THE EXPERIMENTAL WORK

The aim of the experimental work described in this thesis is to localize and characterize phenotypically the distinct macrophage subpopulations present in murine organs in detail. To this end an immunohistochemical approach was chosen. A panel of macrophage and dendrocyte-specific monoclonal antibodies was used and their staining pattern in various murine organs was investigated.

Chapters 6 and 7 focus on the fixation method used in this study. A novel method was developed based on the fixation properties of diazotized pararosanilin. This reagent possesses three diazonium groups that are able to react with various amino acids. The binding of a single pararosanilin molecule to amino acids of distinct proteins results in cross-linking. It was demonstrated that the hexazonium salt of pararosanilin has very good preservative properties on the tissue morphology of frozen sections from murine lympho-hemopoietic (**Chapter 6**) and other organs (**Chapter 7**) without affecting the antigenicity of these tissues.

Using this fixation method, the macrophage populations of the main lymphoid organs, the gastro-intestinal tract and the kidney were investigated. **Chapter 8** focusses on the distribution of macrophages and dendrocytes in the spleen. The compartmentalization of this organ facilitates the determination of their precise localization. It is demonstrated that specific subpopulations of macrophages reside in the splenic red pulp, the marginal zone, the follicles and the peri-arteriolar lymphatic sheath (PALS).

Chapter 9 describes the distinct macrophage subpopulations of the gastro-intestinal tract. Their distribution in the oesophagus, the stomach, the duodenum, the jejunum, the ileum, the caecum and the rectum was investigated. To obtain more insight in the precise location of these populations each part was further subdivided into regions, i.e., the epithelium, the lamina propria, the submucosa, and the muscularis externa. It was demonstrated that there are relatively few macrophages present in the oesophagus and the stomach. Their numbers gradually increase towards the caecum. Between the distinct regions of each part little variation is observed in the number and phenotype of the macrophages.

The macrophages of the kidney are described in **Chapter 10**. For the determination of the precise location of these cells, a distinction was made between the cortex and the inner and outer medulla. If present, a tubulous and an interstitial component was distinguished in these compartments. Macrophages appeared to be mainly present in the cortical and medullary interstitium and were especially abundant in the outer

medulla. With exception of a small macrophage subpopulation in the outer medulla little variation was observed in the phenotype of most macrophages.

In **Chapters 11, 12 and 13** results are presented obtained with a macrophage specific monoclonal antibody raised in our own laboratory. The histological distribution of cells stained by this monoclonal antibody, ER-HR3, in fetal and adult mice is described in **Chapter 11.** Moreover, the light microscopic and electronmicroscopic characteristics of ER-HR3-positive cells are described. Information on the isolation and purification of the antigen recognized by ER-HR3 is given in **Chapter 12.** Its possible involvement in processes of differentiation and activation are reported and other functional aspects are discussed. In **Chapter 13** the functions of macrophages in the erythropoiesis are briefly reviewed. The possibility that the ER-HR3 antigen is involved in the erythropoiesis was investigated and is discussed in this paper.

6. IMPROVED FIXATION OF FROZEN LYMPHO-HEMOPOIETIC TISSUE SECTIONS WITH HEXAZOTIZED PARAROSANILIN

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SUMMARY

In this study a simple single-step fixation method is introduced using the hexazonium salt of pararosanilin as preservative agent. Tissue preservation by this method was shown to be superior to the commonly used acetone fixation. Pararosanilin fixation caused a minimal loss of antigenicity as was demonstrated using twenty-three monoclonal antibodies directed against lympho-hemopoietic and stromal cells.

INTRODUCTION

Phenotypical analysis using monoclonal antibodies has become a major technique in the characterization of tissues and cells. One of the most important factors in the localization of antigens is adequate fixation. In general, fixation should lead to (1) adequate preservation of tissue morphology and (2) good preservation of the physicochemical stucture and immunologic integrity of the antigen. The first condition often requires strong fixation whereas the second condition is best accomplished with minimal of fixation. The optimal fixation condition therefore consists of a compromise between these contradictory requirements, and may differ depending on the nature of the antigen.

Common fixatives used for immunofixation of cells from lympho-hemopoietic organs are glutaraldehyde and paraformaldehyde (Van Ewijk et al., 1980, Leenen et al., 1985), ethanol (Hancock et al., 1982), acetone (Van Ewijk et al., 1981; Judd and Britten, 1982) and periodate-lysine-paraformaldehyde (Poppema et al., 1981; Hancock et al., 1982). More recently a good preservation of morphology has been reported with the use of some of these fixatives in combination with paraffin (Stein et al., 1985; Sato et al., 1986) or plastic embedding (Viale et al., 1985). The main drawbacks of these methods are the need of special equipment and the time consuming procedure.

In this study we describe a simple and fast single-step fixation method for frozen tissue sections using the hexazonium salt of pararosanilin. This hexozonium salt, which is commonly used in enzyme histochemical procedures, appears to have good fixation properties without affecting antigenicity. For comparison we used acetone-fixed frozen sections.

Mice. Male (CBA/Rij x C57BL/Rij)F1 mice were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands, and kept under conventional conditions. Mice were killed by exposure to carbon dioxide and the spleen, mesenteric lymph node and thymus were excised.

Monoclonal antibodies. The used monoclonal antibodies and their specificities are summarized in Table 6.1. The hybrid cell lines M1/70, M3/38, M3/84, M5/114 and 30-G12 were derived from the American Type Culture Collection.

Pararosanilin solution. Hexazotized pararosanilin was prepared as described by Burnstone (1962) with a minor modification. One volume of a 4% (w/v) pararosanilin in 2M HCl solution was added to one volume of a 4% (w/v) $NaNO_2$ in distilled water. From this solution 0.8 ml was added immediately to 10 ml distilled water.

Tissue preparation. Organs were embedded in Tissue-tek II (Miles Laboratories, Inc., Naperville, MI., USA) on a specimen stub and frozen on solid carbon dioxide. Five micron frozen sections were cut on a cryostat (Leitz, Germany; model 1720) and collected on microscope slides precoated with a solution containing 0.1% gelatin and 0.01% chromium potassium sulphate. After cutting, sections were air-dried and subdivided into four groups. The first group was fixed in acetone and air-dried. To investigate the effect of the fixation time, sections were fixed for 5 sec or 10 min with this fixative. The second group was fixed for 2 min with the pararosanilin solution and then washed with phosphate-buffered saline (PBS), pH 7.8. The third group successively underwent both procedures. After fixation the slides were placed in PBS supplemented with 0.02% (w/v) gelatin and 0.05% (v/v) Tween-20 (PBT). The fourth group underwent no fixation and was immediately placed in PBT.

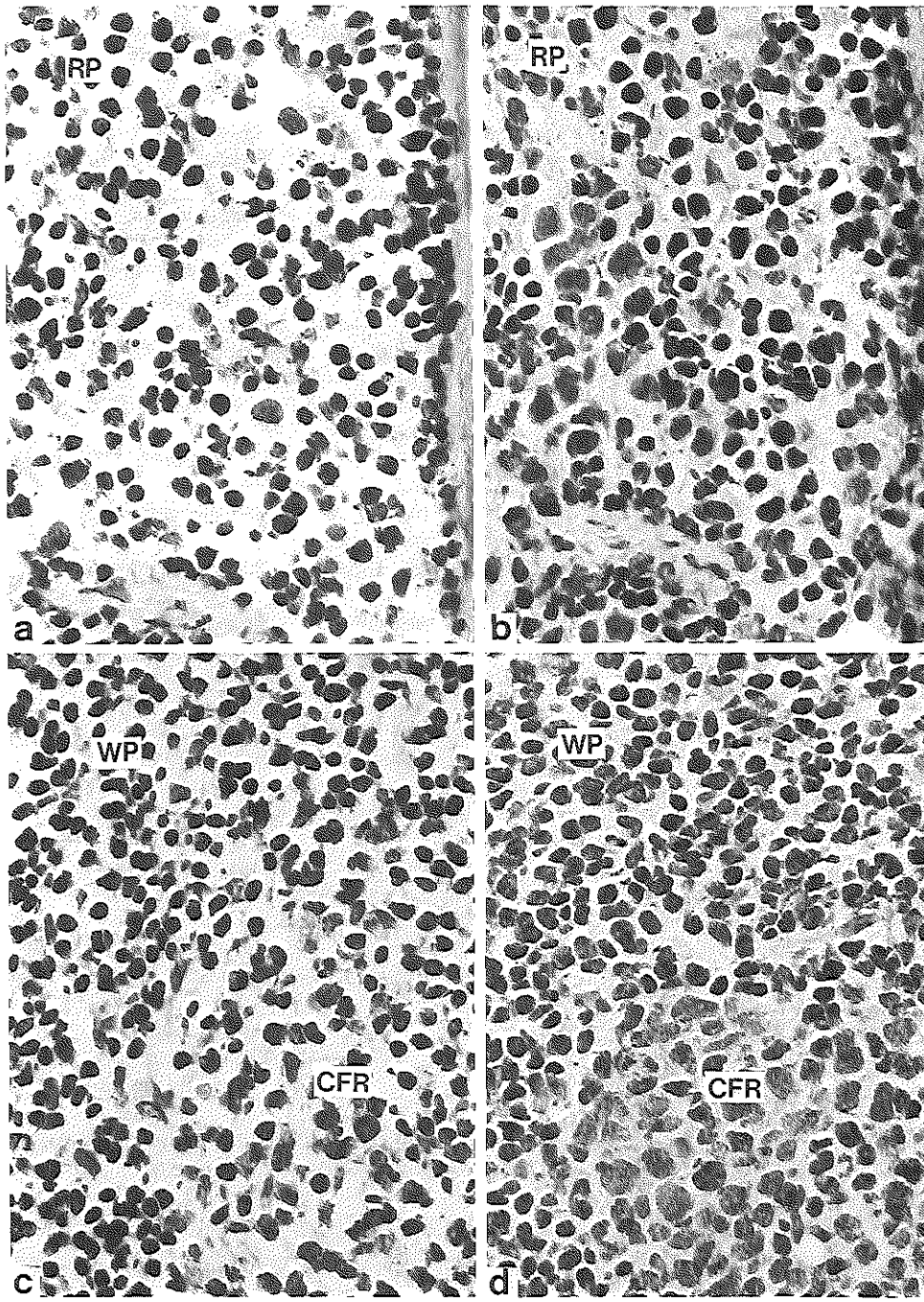
Staining method. The sections were overlaid with 70 μl of the first stage antibody and incubated for 1 hr at room temperature. During incubation the sections were kept in moist chambers to prevent air drying. After rinsing with PBT the sections were overlaid with 70 μl of the conjugate solution and incubated for 1 hr at room temperature. Horseradish peroxidase-coupled rabbit-anti-rat Ig (Dakopatts, Copenhagen, Denmark) was used as a conjugate in a 1:40 dilution. Normal mouse serum at an end concentration of 2% (v/v) was added to avoid background staining. Sections were

Table 6.1
Specificities of the monoclonal antibodies (Mab) used for antigen detection

Mab	Antigen	Specificity	References
M1/42	H-2 K/D	Class I MHC	Springer, 1980
M5/114	H-2 $Ia^{b,d,q}$ - $Ie^{d,k}$	Class II MHC	Bhattacharya et al., 1981
RA3 6B2	B-220	B cells	Coffman, 1982
53-7.3.13	Lyt-1	T cells	Ledbetter et al., 1979
53-6.7.2	Lyt-2	T cells	Ledbetter et al., 1979
H129.19.16	MT4	T cells	Pierres et al., 1984
F4/80	F4/80 Ag	murine mϕ	Hirsch et al., 1981
ER-HR1	ER-HR1 Ag	stromal cells in lympho-hemopoietic organs	Piersma et al., 1985
ER-HR2	ER-HR2 Ag	stromal cells in lympho-hemopoietic organs	Piersma et al., 1985
ER-HR3	ER-HR3 Ag	murine mϕ	De Jong et al., 1987
ER-TR6	ER-TR6 Ag	mϕ, IDC, T cell	Van Vliet et al., 1984
ER-TR9	ER-TR9 Ag	marginal zone mϕ and medullary lymphnode mϕ	Van Vliet et al., 1985
M1/70	Mac-1, CR3	non-fixed mϕ, granulocytes, NK-cells	Springer et al., 1979
M3/38	Mac-2	mϕ, dendritic cells, epithelium	Ho et al., 1982 Flotte et al., 1983
2.4G2	FcRII	mϕ, PMN, B cells	Unkeless, 1979
M3/84	Mac-3	mϕ, dendritic cells, epi- and endothelium	Flotte et al., 1983 Ho et al., 1983
59-AD2.2	Thy-1	T cells, stem cells, myeloid progenitors	Basch et al., 1982 Ledbetter et al., 1979
30-C7	Lgp100a	T and B cells, hemopoietic progenitors	Ledbetter et al., 1979 Miller et al., 1985
H129.37	LFA-1	T and B cells, PMN, mϕ	Pierres et al., 1982
MEL-14	MEL-14	homing receptor on recirculating B and T cells	Gallatin et al., 1983
ER-PT1	ER-PT1 Ag	hemopoietic precursors, mature granulocytes and erythrocytes	Leenen et al., 1987
RB6 8C5	GR-1	granulocytes	Holmes et al., 1986
30-G12	T-200	all leukocytes	Ledbetter et al., 1979

Explanation of abbreviations: mϕ, macrophages; IDC, interdigitating cells; NK cells, natural killer cells; PMN, polymorphonuclear neutrophils; MHC, major histocompatibility complex.

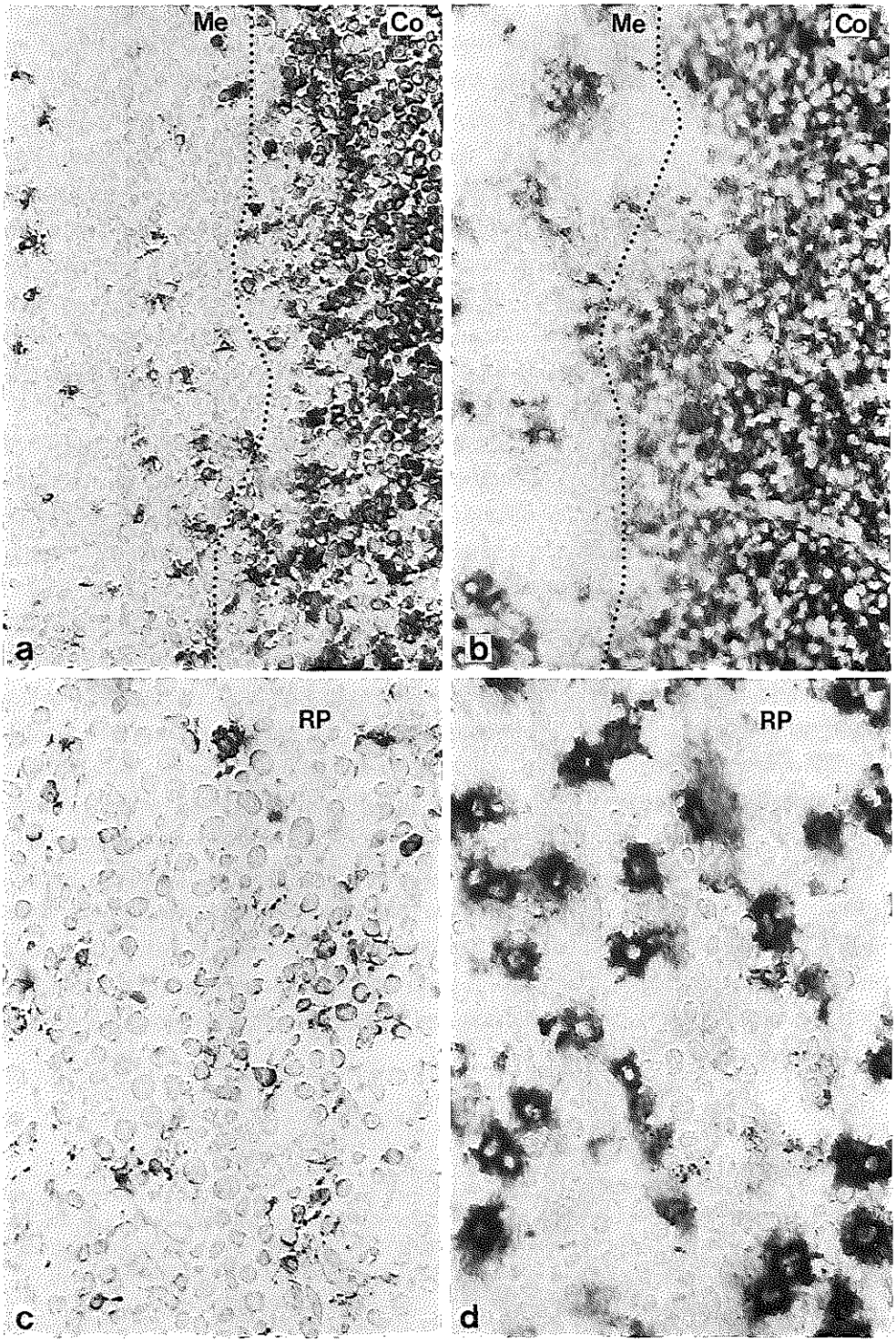
rinsed with PBT and enzyme activity was visualized by incubation with a diaminobenzidine (Sigma Chemical Co., St. Louis, MO, USA) solution (1 mg diaminobenzidine/ml PBS supplemented with 20 µl 1% (v/v) H_2O_2) at room temperatuur. After dehydration the sections were embedded in Entellan mounting medium (Merck, Darmstadt, FRG) and coverslipped.


RP
RP
a
b
WP
WP
CFR
CFR
c
d

Quantitative immunoassay. For a quantitative determination of antibody binding the method according to Leenen et al. (1985) was used with minor modifications. Cells from the adherent macrophage cell lines WEHI-3 and AP284-D4 were diluted to a concentration of 2 x 10^6 and 10^6 cells/ml, respectively. Ten μl aliquots of each cell suspension were plated onto a 60 well Terasaki tray (Greiner, Nürtingen, West Germany) using a Hamilton multichannel dispenser. The cells were cultured overnight at 37°C in α-medium (α-modification of Dulbecco's minimal essential medium) supplemented with 10% (v/v) FCS. The formed monolayers were washed with cold PBS supplemented with 0.02 M NaN_3 (PBA) and used immediately. Cells of the non-adherent W1C3 cell line (Leenen et al., 1990a) were coated on the Terasaki tray with 0.01% (v/v) glutaraldehyde (Polyscience Inc.) in PBS for 25 min at 4°C. The cells were washed with PBA and used in the immunoassay.

The deleterious effect of pararosanilin on antigen detectability was assessed by quantitative measurement of antibody binding to cells fixed with increasing fixative concentrations. Different concentrations of pararosanilin or 0.05% glutaraldehyde were transferred from a masterplate to a single Terasaki tray (7 μl per well) in one step by using a Biotech replicator (Biotech, Basel, Switzerland). Cells were fixed at 4°C for 2 min with pararosanilin or for 25 min with glutaraldehyde and washed twice with PBT. Five μl aliquots of the hybridoma supernatant were added to each well except for the blanks and negative controls which were filled with PBT and Y3 myeloma culture supernatant, respectively. After incubation for 1 h on ice, the plates were washed several times with cold PBT and 7 μl of the second-stage antibody, sheep-anti-rat immunoglobulin conjugated to Escherichia coli β-galactosidase (Amersham, United Kingdom) was added. After several washings with PBT, the trays were incubated for 1 h at 37°C with the fluorogenic substrate 4-methylumbelliferyl galactopyranoside dissolved in a 50 mM potassium phosphate buffer (pH 7.8) supplemented with 4 mM $MgCl_2$ and 10 nM 2-mercaptoethanol. The reaction was stopped by adding 5 μl 0.5 M sodium bicarbonate buffer (pH 10.4) to each well. The generation of the fluorescent product methylumbelliferone was measured by using a scanning microfluorometer (Josselin de Jong et al., 1980).

Figure 6.1. Acetone (a,c) or pararosanilin-fixed (b,d) frozen section of the spleen stained with eosin-hematoxylin. Note the dark nuclei and the cytoplasmic protrusions in the aceton-fixed section, and the diverse cell types, the different sizes of the nuclei and the presence of nucleoli in the pararosanilin-fixed section. CFR, follicle centre reaction; RP, red pulp; WP, white pulp. Magnification x 650.


Me
Co
a
Me
Co
b
RP
c
RP
d

Fixation

The property of pararosanilin to conserve frozen tissue sections was investigated by microscopic examination of eosin-hematoxylin-stained and unstained pararosanilin-fixed sections of lympho-hemopoietic organs. The tissue integrity of these sections, as judged by structure conservation and cellular morphology, was determined with the use of a light and phase contrast microscope. For comparison, cold acetone-fixed sections were used.

The morphology of lympho-hemopoietic tissue sections fixed with cold acetone for 10 min was poor (Figs. 6.1a, c). Especially the stromal sinusoidal regions within these sections, i.e., the red pulp of the spleen and the medulla of the mesenteric lymph node were conserved poorly. Within the eosin-hematoxylin-stained sections this was reflected by little eosin staining due to a loss of cytoplasm caused by tissue shrinkage and cell damage. Furthermore, morphological differences between the distinct cell types were difficult to observe since most nuclei contained dense chromatin. Judgement of unstained sections with the use of the phase contrast microscope confirmed these results. Stromal regions in acetone-fixed sections showed a mazed character while most of the cellular cytoplasm was retracted around the nucleus with the exception of few elongated protrusions (not shown). Fixation of tissue sections with acetone for only 10 sec did not improve the tissue morphology of these stromal regions. However, regions with a high cell density such as the splenic white pulp, the lymphoid paracortex and the thymic cortex showed an improved morphology.

In contrast to acetone, pararosanilin had excellent fixation properties. Pararosanilin-fixed frozen tissue sections showed a very good morphology (Figs. 6.1b,d). The tissue structure was well preserved and little or no shrinkage was observed. This resulted in an increased eosin staining since all cell bodies were kept intact and show well spread cytoplasm. Also the morphology of the cells and their nuclei was improved greatly when compared to acetone fixation. Nuclear details, like the presence of nucleoli, could easily be noted facilitating the recognition of distinct cell types. Phase contrast microscopic analysis of unstained sections confirmed the observation that after pararosanilin fixation cells were undamaged. Sections consisted of a continuous layer of intact cells without any shrinkage artefacts (not shown).

Figure 6.2. Acetone (a,c) or pararosanilin-fixed (b,d) frozen section of the thymic cortico-medullary junction (a,b) and the splenic red pulp (c,d). Thymic and splenic sections were immunostained with 53-6.7.2. (anti Lyt-2) and the 59-AD.2.2 (anti-Thy-1), respectively. Co, cortex; Me, medulla; RP, red pulp. Magnification x400 (a,b) or x650 (c,d).

Successive fixation of frozen sections with pararosanilin and acetone led to a preservation of the tissue morphology that was intermediate to the results obtained with either pararosanilin or acetone alone. Again cell shrinkage occured but to a lesser extent than in acetone-fixed sections.

Antigen preservation

a. Qualitative immunohistolological studies

Besides morpholofical fixation, the property of pararosanilin and acetone to preserve tissue antigens was investigated. The staining intensity of the enzymatic reaction and the antigen distribution in the different subregions of the lympho-hemopoietic organs both were used as descriptive parameters and were scored in a

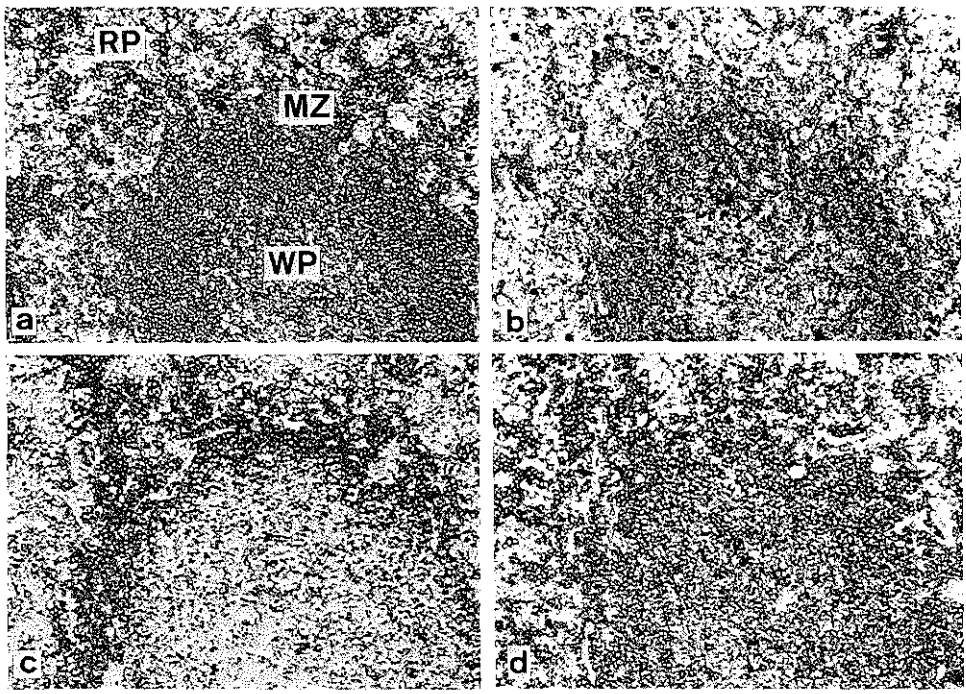


Figure 6.3. Tissue morphology and T-200 (detected with mAb 30-G12) distribution of spleen sections fixed for (a) 5 seconds with acetone, (b) 10 minutes with acetone, (c) 2 minutes with pararosanilin or (d) 2 minutes with pararosanilin with a PBS-prewash of 10 seconds. MZ, marginal zone; RP, red pulp; WP, white pulp. Magnification x150.

Table 6.2
Staining intensity and distribution of antigens in the murine spleen following acetone or pararosanilin fixation

Mab	Intensity		Red pulp		White pulp T		White pulp B	
	ace-tone	pararo-sanilin	ace-tone	pararo-sanilin	ace-tone	pararo-sanilin	ace-tone	pararo-sanilin
M1/42	++[a]	++[a]	+[b]	++[b]	+++[b]	++[b]	+++[b]	++[b]
M5/114	++	+++	+	+	++	++	+++	+++
RA3 6B2	+++	++	+	++	+	+	+++	++
53-7.3.13	+++	+++	+	++	+++	+++	+	+
53-6.7.2	+++	+++	++	++	+++	+++	+	+
H129.19.16	+++	++	+	+	+++	+++	+	+
F4/80	+	++	++	+++	-	-	-	-
ER-HR1	+	+++	++	+++	-	-	-	-
ER-HR2	+	+++	++	+++	-	-	-	-
ER-HR3	++	+++	++	+++	-	-	-	-
ER-TR6	++	+	++	++	++	+	++	+
ER-TR9	+++	+++	-	-	-	-	-(mz+)	-(mz+)
M1/70	++	+++	++	++	+	+	+(mz+)	+(mz+)
M3/38	+	++	+	++	++	++	+(mz+)	+(mz+)
2.4G2	++	+	++	++	-	-	+(mz+)	+(mz+)
M3/84	+	++	+	+++	+	+	+	+
59-AD2.2	+++	+++	+	++	+++	+++	+	+
30-C7	-/+	-/+	-	++	++	++	++	++
H129.37	+	+	+	++	+++	+++	+++	++
MEL-14	+	+	+	+	+++	+++	++	++
ER-PT1	-/+	+	++	++	-	-	-	-
RB6 8C5	+++	+++	++	++	-	-	-(mz+)	-(mz+)
30-G12	+++	+++	++	+++	+++	+++	+++	+++

a. +++ = strong; ++ = moderate; + = weak; - = negative
b. +++ = many; ++ = moderate; + = few; - = no cells
mz, marginal zone.

semiquantitative manner. Results obtained on spleen sections were depicted in Table 6.2. Thymus and lymph node sections showed similar results (data not shown). With exception of the monoclonal antibodies RA3 6B2, H129.19.16, ER-TR6 and 2.4G2, pararosanilin fixation did not affect the overall staining intensity when compared to acetone fixation. However, within the different subregions of the lympho-hemopoietic organs the staining was rather variable. Especially, the cell dense lymphoid regions of the splenic white pulp, the thymic cortex and the lymphoid paracortex were stained less intensively after pararosanilin fixation. In contrast, the less dense stromal areas of the lympho-hemopoietic organs were heavily stained.

Although the general distribution of the tissue antigens as inferred from acetone-fixed sections was not altered, the number of positive cells in the dense lymphoid regions was somewhat reduced when some anti-B and/or T cell antibodies were applied (Table 6.2; compare Fig. 6.2a with 6.2b and Fig. 6.3a with 6.3d). In contrast, stromal regions often showed an increased number of positive cells with various monoclonal antibodies (Table 6.2; compare Fig. 6.2c with 6.2d).

Rinsing the sections with PBS for ten seconds just before the pararosanilin fixation recovered both the staining intensity and the number of positive cells in the dense lymphoid regions (Fig. 6.3d) without affecting these parameters in the stromal regions.

b. Quantitative ELISA studies

The effects of pararosanilin fixation on tissue antigens were also investigated in a quantitative manner. The binding of various monoclonal antibodies to the cell lines W1C3, WEHI-3 and AP284-D4 was analysed with the use of a micro-ELISA system. Compared to unfixed cells only minor differences in binding were observed after pararosanilin-fixation. These differences varied for each monoclonal antibody and cell line, e.g. 59-AD2.2 binding was slightly reduced on WEHI-3 but increased on W1C3 (Fig. 6.4).

It has been shown previously that antigen detection on cells of these cell lines was optimal using 0.05% glutaraldehyde (Leenen et al., 1985). Therefore, this fixative at this concentration was used for comparison. It was demonstrated here that pararosanilin slightly reduced the binding capacity of the monoclonal antibodies to the cell lines tested when compared to glutaraldehyde-fixed cells (Fig. 6.4).

DISCUSSION

Diazonium salts are commonly used in enzyme histochemical reactions to form insoluble coloured precipitates with phenolic compounds made free from the substrate by the enzymatic reaction. In tissue, diazonium salts can bind with proteins e.g. on the phenol group of tyrosine, the indole group of tryptophan and the imidazole group of histidine (Burnstone, 1962; Pearse, 1968). The hexazonium salt pararosanilin, introduced by Davis and Ornstein (1959), contains three reactive diazonium groups and thus, theoretically, must be able to crosslink proteins. In the present study we investigated the use of this compound as a fixative in immunohistological studies by determination of its ability to conserve frozen tissue sections of various lympho-hemopoietic organs without loss of antigenicity.

We demonstrated that diazotized pararosanilin indeed had very good fixative properties. Especially the splenic red pulp and the medullary parts of the mesenteric lymph node and the thymus were excellently preserved and the tissue morphology of these regions was strongly improved when compared to acetone-fixed sections (Fig. 6.1). Furthermore, the overall distribution and antigenicity of most antigens as referred to acetone fixation was not altered (Table 6.2). Thus, T cell and B cell markers still were confined to their specific sites. However, the staining intensity in regions with high cell density, i.e. the splenic white pulp, the lymphoid paracortex and the thymic cortex appeared to be reduced (Figs. 6.2a,b, 6.3a,b). In contrast, the number of positive cells in the less dense stromal regions of these organs increased. These results give the impression, that the distribution of some antigens is inversed compared to that in acetone-fixed sections. This inversion was abolished completely by rinsing the sections with PBS just before pararosanilin fixation (Fig. 6.3). These results may be explained by our finding that pararosanilin prevents cell shrinkage. In dense regions, cells were packed together to such extent that epitopes present on the cellular membranes were poorly attainable for their antibodies. Further support for this view was obtained by our observation that successive fixation of tissue sections with pararosanilin and acetone resulted in cell shrinkage accompanied by a restoration of the staining intensity in dense lymphoid regions. Moreover, it is not likely that pararosanilin causes structural alterations in the antigens since cells present in regions with low cell density showed no reduction of their staining intensity. Additionally, pararosanilin fixation had no effect on the antibody binding when compared to unfixed cells as tested in our micro-ELISA system (Fig. 6.4). In contrast, antibody binding was increased after glutaraldehyde fixation. This increment may improve antigen detection, but also may introduce false positives as was observed with the monoclonal antibody 2.4G2 on W1C3 cells (Fig. 6.4).

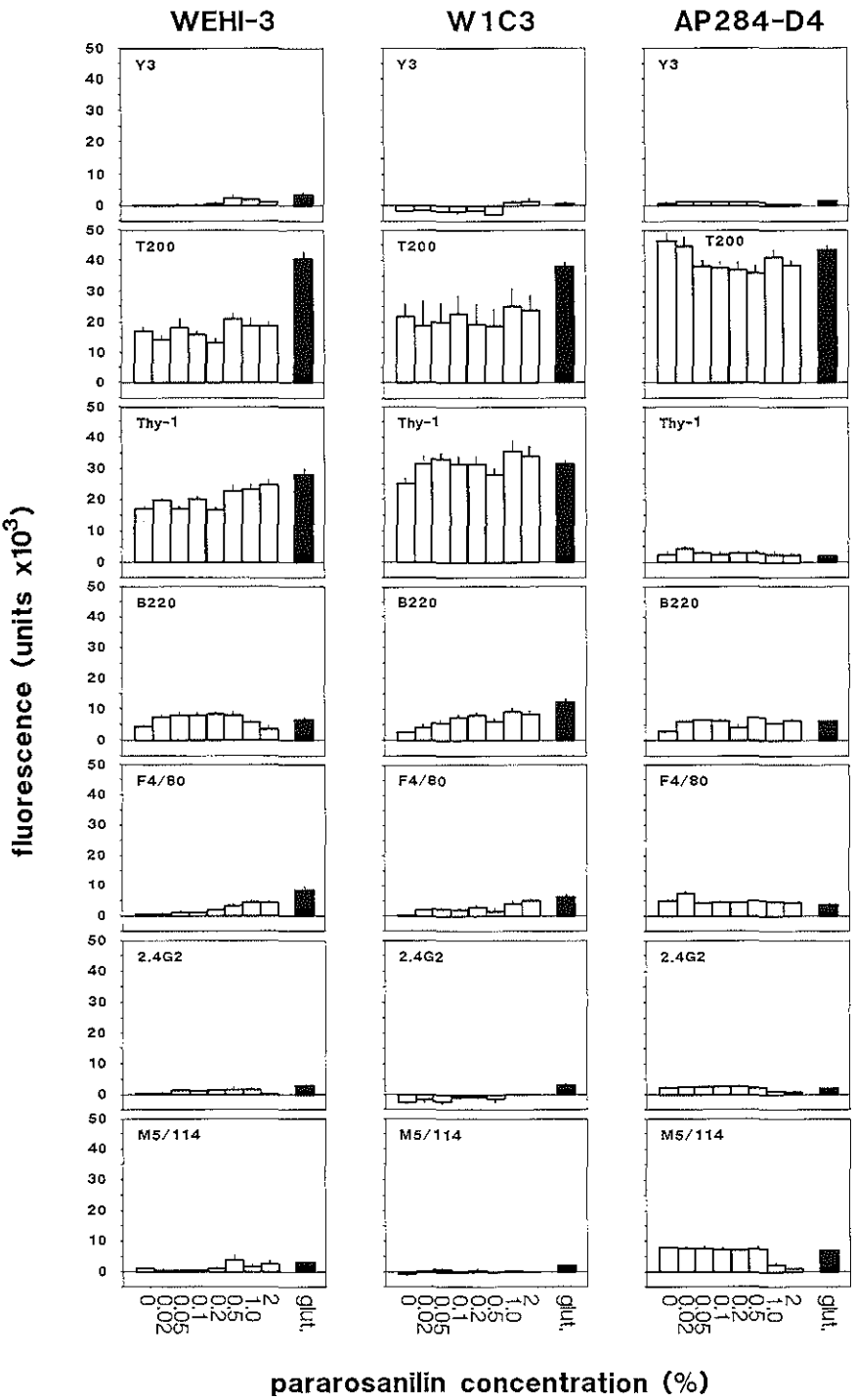


Figure 6.4. Effect of increasing concentrations pararosanilin on the binding of leukocyte-specific antibodies to WEHI-3, W1C3 and AP284-D4 cells. Glutaraldehyde (0.05%) is used for comparison.

Other investigators also have demonstrated that glutaraldehyde influences the detectability of antigens (Drover and Marshall, 1986).

Noteworthy is the modification that we made on the preparation of the pararosanilin solution as described by Burnstone (1962). We used distilled water instead of a phosphate buffer to dilute the diazotized pararosanilin. Utilization of the phosphate buffer resulted in a red-brown precipitate of the tissue. This background staining interfered with the antigen detection in weakly staining sections. Moreover, sections fixed with pararosanilin in phosphate buffer showed autofluorescence which prohibits the subsequent use of fluorescence techniques after pararosanilin fixation. Dilution of diazotized pararosanilin in distilled water completely abolished these effects.

In conclusion, it is demonstrated that pararosanilin has excellent preservative properties for frozen tissue sections. Especially, the morphology of the stromal regions was greatly improved when compared to conventional acetone fixation. As a consequence, immunostaining with various monoclonal antibodies resulted in an increased number of positive cells and improved antigen localization. Thus, acetone fixation may lead to an underestimation of this number in stromal regions. Rinsing the sections with phosphate buffered saline for 10 seconds before pararosanilin fixation makes the method also applicable for the detection of antigens in the dense lymphoid regions of lympho-hemopoietic organs. This adaption has no consequence for the tissue morphology which remains superior to acetone fixation.

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7. IMMUNOFIXATION OF FROZEN SECTIONS FROM MURINE ORGANS USING PARAROSANILIN HEXAZONIUM CHLORIDE

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SUMMARY

Adequate interpretation of the immunohistological staining of frozen sections is often hampered by the poor morphological preservation of the tissues. Although modification of conventional methods like paraffin and plastic embedding greatly improve the tissue morphology, these methods are time consuming and many epitopes loose their antigenic properties. In this study we introduce a simple single-step fixation method with hexazotized pararosanilin. It was demonstrated that pararosanilin fixation of frozen sections results in a very good morphological preservation of many tissues without loss of immunoreactivity.

INTRODUCTION

Fixation is one of the most important technical factors influencing the localization of tissue antigens with monoclonal antibodies. Fixatives must cause good preservation of both the tissue morphology and antigenicity. In general, acceptable tissue morphology is obtained under strong fixation conditions, whereas antigen preservation demands mild fixation conditions. Optimal fixation therefore consists of a compromise between this "structure versus antigenicity" contradiction. Various fixation procedures have been used by several investigators to fulfill this condition (Eldred et al., 1983; Judd and Britten, 1982; Hancock et al.,1982; Schmitt and Schmidt, 1986; Szendroi et al., 1983).

In general, acetone (Judd and Britten, 1982; Schmitt and Schmidt, 1986), ethanol (Szendroi et al., 1983) and periodate lysine-paraformaldehyde (Hancock, 1982) are preferred fixatives. Although acceptable antigen preservation is obtained with these fixatives, the tissue morphology is suboptimal. More recently, improved tissue morphology without the loss of antigenicity was obtained with the use of some of these fixatives in combination with paraffin (Stein et al., 1985) or plastic (Beckstead, 1985) embedding. Two main drawbacks of these methods are the time consuming procedures and the need of special equipment.

In this report we introduce a simple single-step fixation procedure with hexazotized pararosanilin. The aim of this study is to demonstrate the good fixation properties of this compound with respect to the morphological preservation of sections from various murine organs without the loss of antigenicity.

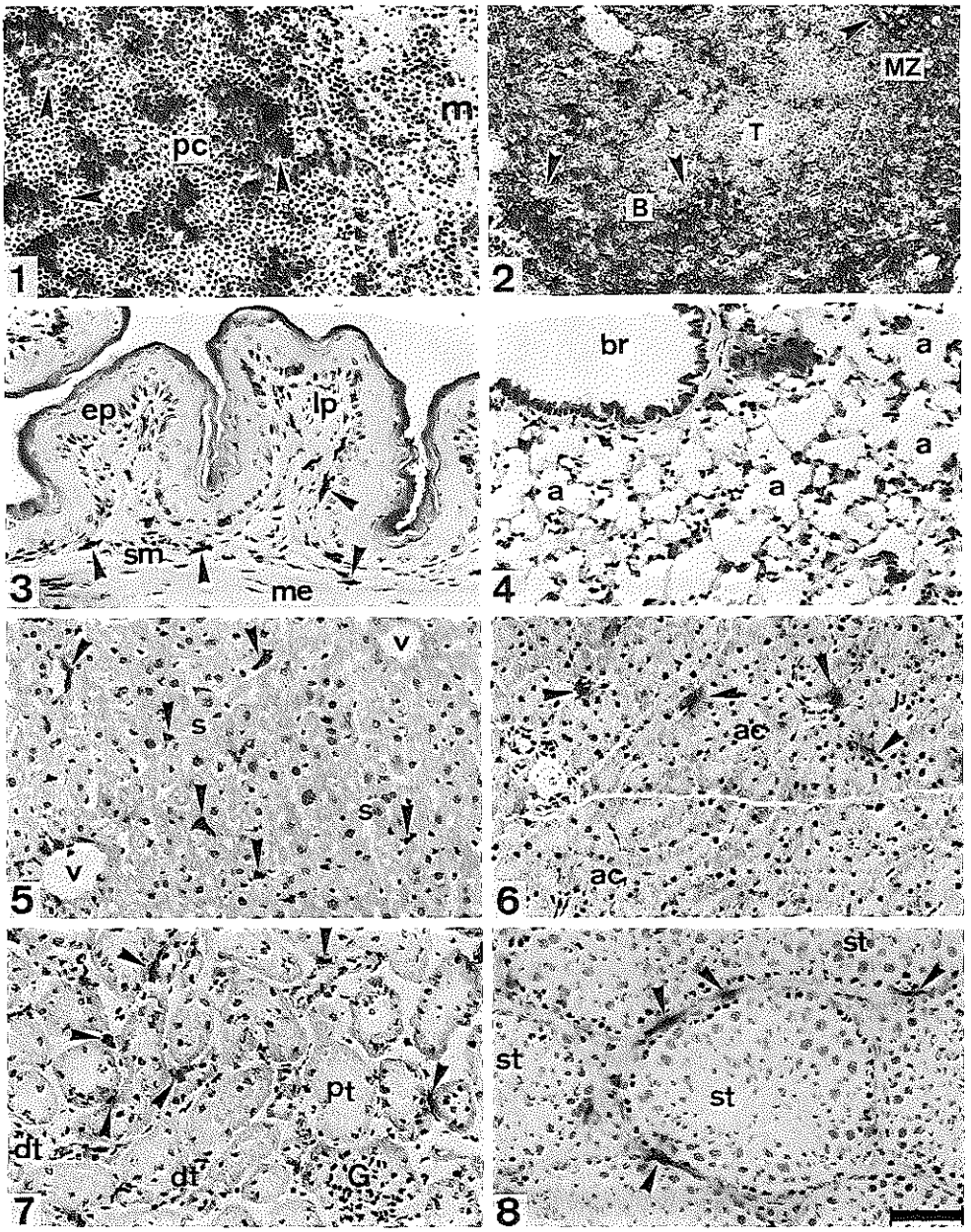
MATERIALS AND METHODS

Mice. Male (CBA/Rij x C57BL/Rij)F1 mice were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands, and kept under conventional conditions. Mice were killed by exposure to carbon dioxide and the stomach, intestine, pancreas, liver, testis, kidney, lung and skin were excised.

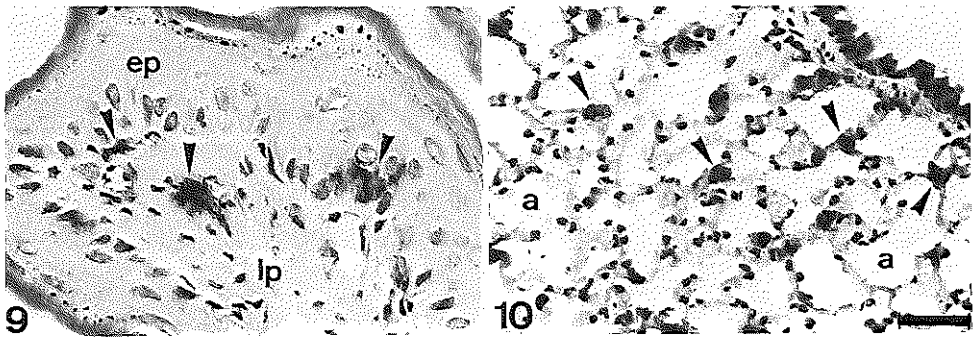
Staining method. Organs were dissected and 5 x 5 mm pieces were embedded in Tissue-tek II (Miles Laboratories Inc., Naperville, MI., USA) on a specimen stub and frozen on solid carbon dioxide. Five μm thick frozen sections were cut on a Leitz cryostat (model 1720, Leitz, West Germany) and collected on microscope slides precoated with a solution containing 0.1% gelatin and 0.01% chromium potassium sulphate. The slides were air-dried and either stored at -20°C or used immediately. The sections were fixed for 2 min with a pararosanilin solution which was essentially prepared as described by Burnstone (1962). In short, one volume of 4% (w/v) pararosanilin (Merck, Darmstadt, FRG) in 2M HCl was mixed with one volume of 4% (w/v) $NaNO_2$ in distilled water. From this solution 0.8 ml was added to 10 ml distilled water. After fixation the slides were washed with phosphate-buffered saline, pH 7.8 (PBS), supplemented with 0.02% (w/v) gelatin and 0.05% (v/v) Tween-20 (PBT). Subsequently, the sections were overlaid with 70 μl of the rat monoclonal antibody M5/114 (anti-Ia; Bhattacharya et al., 1981). Sections were incubated for 1 hr at room temperature. During incubation the sections were kept in moist chambers to prevent air drying. After rinsing with PBT, the sections were overlaid with 70 μl of the conjugate solution and incubated for 1 hr at room temperature. This solution contained a 1:40 diluted horseradish peroxidase-conjugated rabbit-anti-rat Ig (Dakopatts, Copenhagen, Denmark) supplemented with normal mouse serum at an end concentration of 2%. Sections were rinsed with PBT and peroxidase activity was visualized by incubation with a diaminobenzidine (Sigma, St. Louis, MO, USA) solution (1 mg/ml PBS supplemented with 20 μl 1% (v/v) H_2O_2). After rinsing in tap water, the sections were counterstained with hematoxylin, dehydrated, embedded in Entellan (Merck) mounting medium and coverslipped.

RESULTS

The morphological preservation of sections from various murine organs after pararosanilin fixation is illustrated by Figs. 7.1-7.10. The quality of fixation was



Figures 7.1-7.8. Pararosanilin-fixed frozen sections of the mesenteric lymph node (Fig. 7.1), spleen (Fig. 7.2), oesophagus (Fig. 7.3), lung (Fig. 7.4), liver (Fig. 7.5), pancreas (Fig. 7.6), renal cortex (Fig. 7.7) and testis (Fig. 7.8), immunostained with the M5/114 monoclonal antibody. Sections were counterstained with hematoxylin. Ia-positive cells are indicated with arrows.
a, alveoli; ac, acinar tissue; br, bronchiole; B, B cell area; c, central vene; dt, distal tubules; ep, epithelium; G, glomerulus; lp, lamina propria; m, medulla; me, muscularis externa; mz, marginal zone, pc, paracortex; pt, proximal tubules; s, sinusoids; sm, submucosa; st, seminiferous tubules; T, T cell area. Original magnification x150. Bar = 90 μm.



Figures 7.9 and 7.10. High-power magnification of pararosanilin-fixed sections of the oesophagus (Fig. 7.9) and the lung (Fig. 7.10). For abbreviations and counterstain see legend Figs. 7.1-7.8. Original magnification x250. Bar = 50 μm.

assessed by observing the continuity of the cells and their cytoplasmatic and nuclear integrity. Furthermore, the preservation of the tissue architecture was noted. In general, the tissues were preserved very well and their morphology approached that of sections from paraffin embedded organs. Little or no shrinkage occurred and all stereotypic cell types present in the various organs could easily be recognized. Additionally, good preservation of the cell nuclei was obtained. This allowed the observation of nuclear details like the presence of nucleoli.

The distribution pattern of the Ia-antigen recognized by mAb M5/114 is compatible with the known distribution of the antigen and corresponds closely to that demonstrated in acetone-fixed frozen sections (results not shown). Localization of the antigen was very precise and positive cells could easily be distinguished from the surrounding cells.

DISCUSSION

The aim of the current investigations was to determine the fixative properties of hexazotized pararosanilin on frozen sections of various murine organs. This diazonium salt, introduced by Davis and Ornstein in 1959 in enzyme histochemistry, contains three reactive diazonium groups. These groups are able to react with various amino acids (Burnstone, 1962; Pearse, 1968) and in this manner the compound can crosslink proteins. This characteristic probably underlies its fixative properties. In a previous study (De Jong et al., 1990c) we demonstrated that the diazonium salt improves the tissue morphology when compared with acetone-fixed sections. In addition, we

showed that pararosanilin had little or no effect on the antigenicity of various antigens. However, this investigation was restricted to lympho-hemopoietic organs which possess a unique histological structure that may be not representative for many other organs. In the present study we demonstrated that hexazotized pararosanilin is a potent fixative for all organs tested and has excellent preservative properties. Notably, the compound did not alter or reduce the detectability of the Ia antigen. This observation is indicative of the mild effect of pararosanilin on the antigenicity since it has been shown that Ia antigens are sensitive to denaturation by various fixatives (Walker et al., 1984).

Preliminary studies with human tissue specimens indicate that pararosanilin improves the tissue morphology of human spleen and kidney, when compared with conventional acetone fixation (Dr. P.G.J. Nikkels, personal communication). This observation together with the short fixation time and the exact localization of the antigen makes the method a good choice for routine diagnostic screening.

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8. IMMUNOPHENOTYPICAL CHARACTERIZATION OF MACROPHAGE AND DENDROCYTE SUBPOPULATIONS IN THE MURINE SPLEEN

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Submitted for publication.

SUMMARY

In this study we utilized a number of macrophage and dendritic cell-specific monoclonal antibodies to unravel the macrophage and dendrocyte heterogeneity in the murine spleen. We demonstrated that these non-lymphoid cells exhibit unique, characteristic phenotypes and that phenotypically distinct cells occupy distinct microanatomical sites. These phenotypical differences are suggestive of a functional diversity of the various subpopulations. Furthermore, we observed a considerable overlap in marker expression between macrophages and dendrocytes. Based on these findings, we argue that macrophages and dendrocytes belong to the same hemopoietic lineage.

INTRODUCTION

Recent studies indicate that macrophages and dendrocytes - i.e. Langerhans cells, veiled cells and interdigitating or dendritic cells (Austyn, 1987) - occupy specific anatomical locations within the various peripheral lymphoid organs (Veerman and van Ewijk, 1975; Witmer and Steinman, 1984; Buckley et al., 1987). This non-random spatial distribution is most likely indicative of a functional specialization of the various subpopulations.

Particularly, the spleen has been chosen by many investigators as a model to study this assumption (Claassen et al., 1986a, Groeneveld et al., 1986; van Vliet et al., 1985). This organ contains many macrophages and is well compartmentalized (Veerman and van Ewijk, 1975; van Ewijk and Nieuwenhuis, 1985). The splenic red pulp is densely occupied with stromal macrophages. Part of these cells function as scavengers in the bloodstream (Hume et al., 1983; Gordon et al., 1988b). Other red pulp macrophages perform essential functions as central macrophages in hemopoietic islands (Crocker et al., 1988) or as regulators of cellular migration (Weiss and Sakai, 1984).

Like the macrophages of the red pulp, the splenic white pulp macrophages may also vary in their function when present at distinct locations. For example, tingible body macrophages, present in the germinal centers, are thought to be involved in the elimination of aberrantly programmed B lymphocytes (Nieuwenhuis and Opstelten, 1984). Furthermore, marginal zone macrophages are highly phagocytic (Matsuno et al., 1986) and were initially thought to play a specific role in presenting thymus-independent type 2 antigens (TI-2) to specific B cells located in the marginal zone (Humphrey and Grennan, 1981; van Vliet et al., 1985). However, it has recently been demonstrated that specific elimination of marginal zone macrophages did not influence

TI-2 immune responses (Kraal et al., 1989). Marginal metallophils, at the border of the marginal sinus and the periarterial lymphatic sheath (PALS), are likely involved in antigen presentation (Kraal and Janse, 1986; Kraal et al., 1988). These cells may also direct lymphocyte traffic through the spleen (Brelinska and Pilgrim, 1982).

Dendrocytes also vary in their function depending on their localization in distinct compartments. In the PALS, interdigitating cells (IDC) reside (Veldman, 1970), which in vitro give rise to the dendritic cells (DC) (Breel et al., 1987; Austyn, 1987). These cells are specialized as accessory cells for induction of immune responses in resting T cells.

Taken together, these data indicate that macrophages and dendrocytes play pivotal roles in various processes taking place in the spleen. Most importantly, these accessory functions appear to be related to particular macrophage and dendrocyte subpopulations present at specific lymphopoietic and hemopoietic microenvironments. Unraveling the accessory cell heterogeneity in the spleen thus aids to gain insight in the different functions performed by these cells in situ.

Recently, we extended the available panel of monoclonal antibodies (mAbs) against DC and macrophage antigens (de Jong et al., 1990a, Leenen et al., 1990c; 1990d). Moreover, we developed an improved fixation procedure to detect cellular determinants in frozen tissue sections (de Jong et al., 1990c). These new mAbs and fixation procedure are applied in the present paper to study the heterogeneity of the mononuclear phagocytes in the spleen and to further elucidate the relation between macrophages and dendrocytes.

MATERIALS AND METHODS

Mice. Male and female BALB/c mice were bred at our facility, and kept under conventional conditions. Mice were sacrificed by exposure to carbon dioxide and the spleen was removed.

Monoclonal antibodies. Monoclonal antibodies used in the present study and their specificities and references are given in Table 8.1. MAbs were applied either as undiluted hybridoma culture supernatants or as optimally diluted purified antibody.

Tissue preparation and immunostaining. Organs were embedded in Tissue-Tek II (Miles Laboratories, Inc., Kankakee, IL, USA) on a specimen stub and frozen immediately. Five μm sections were cut on a cryostat (model 1720, Leitz, FRG) and collected on microscope slides precoated with a solution containing 0.1% gelatin and

Table 8.1.

Specificities of the monoclonal antibodies (mAbs) used for antigen detection

MAb	Antigen	Specificity	References
BM8	BM8 Ag	Mϕ	Malorny et al., 1986
ER-BMDM1	Mϕ amino-peptidase	Mϕ, dendrocytes, microvilli	Leenen et al., 1990c
ER-HR3	ER-HR3 Ag	Mϕ	de Jong et al., 1987; 1990a
ER-MP23	ER-MP23 Ag	Mϕ	Leenen et al., 1990d
ER-TR9	ER-TR9 Ag	Mϕ	van Vliet et al., 1985
F4/80	F4/80 Ag	Mϕ	Austyn and Gordon, 1981
MIDC-8	MIDC-8 Ag	dendrocytes	Breel et al., 1987
MOMA-1	MOMA-1 Ag	Mϕ	Kraal and Janse, 1986
MOMA-2	MOMA-2 Ag	Mϕ	Kraal et al., 1987
Monts-4	Monts-4 Ag	Mϕ, IDC	Jutila (pers. commun.)
M1/70	Mac-1, CR3	non-fixed Mϕ, granulocytes, NK cells, DC	Springer et al.,1979
M3/38	Mac-2	Mϕ, dendrocytes, epithelium	Ho and Springer, 1982 Flotte et al., 1983
M5/114	H-2 Ia	immunologically reactive cells	Bhattacharya et al., 1981
NLDC-145	NLDC-145 Ag	dendrocytes	Kraal et al., 1986 Breel et al., 1987

CR3, complement receptor type 3; DC, dendritic cells; IDC, interdigitating cells; Mϕ - macrophages

0.01% chromium potassium sulphate. Slides were air-dried and used immediately or stored at -20 °C. Prior to use, sections were fixed for 2 min. in hexazotized pararosanilin (de Jong et al., 1990c) prepared as follows: one volume of a 4% (w/v) pararosanilin solution in 2M HCl was added to one volume of 4% (w/v) $NaNO_2$ in distilled water. From this mixture 0.8 ml was immediately diluted with 10 ml distilled water.

After fixation, sections were washed with phosphate-buffered saline, pH 7.8 (PBS) supplemented with 0.05% (v/v) Tween-20 (PBS-Tw). The sections were overlaid with 70 µl of the first stage monoclonal antibody and incubated for 1h at room temperature. During incubations the sections were kept in a moist chamber to prevent air drying. After rinsing the sections with PBS-Tw, they were overlaid with 70µl of optimally diluted rabbit-anti-rat Ig conjugated to horseradish peroxidase (Dakopatts, Copenhagen, Denmark) and incubated for 1h at room temperature. Normal mouse serum (2%; v/v) was added to avoid nonspecific binding of the conjugate. Sections were rinsed with PBS-Tw and binding of the conjugate was visualized by incubation with a diaminobenzidine (Sigma, St. Louis, USA) solution (1 mg DAB/ml PBS supplemented with 20 µl 1% (v/v) H_2O_2). The sections were briefly counterstained with hematoxylin and

embedded in Entallan mounting medium (Merck, FRG) after dehydration and cover-slipped.

Enzyme histochemistry. Acid phosphatase and nonspecific esterase activities were demonstrated as described by Burnstone (1962) with Naphthol-AS-BI phosphate (Sigma) and α-naphthyl acetate (Sigma) as substrates, respectively. In both procedures hexazotized pararosanilin was used as diazonium salt.

RESULTS

In order to facilitate the identification of distinct macrophage and dendrocyte subpopulations, we defined a number of microanatomical sites at which these cell types are present. This definition is primarily based on the compartmentalization of the spleen as described previously (van Ewijk and Nieuwenhuis, 1985).

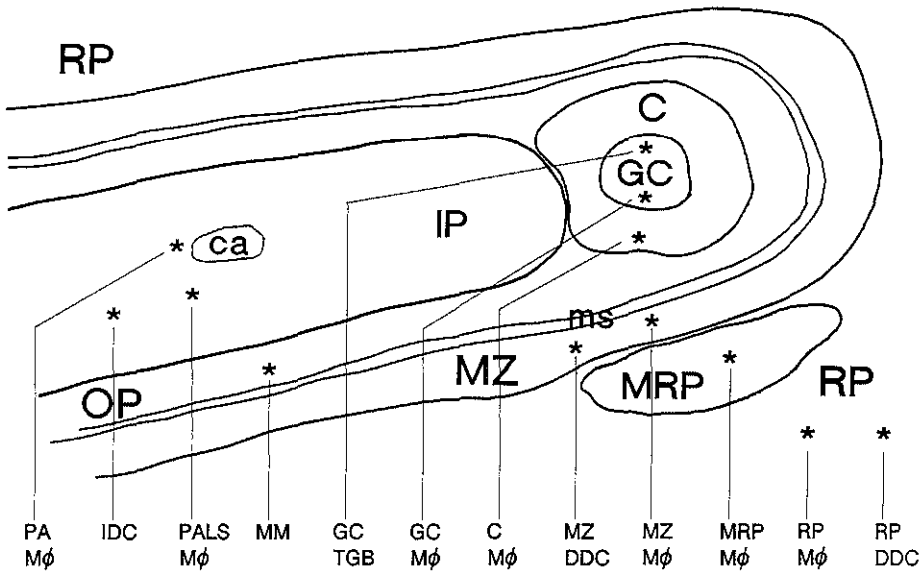
In this study, the location of the various subpopulations of macrophages and dendrocytes in four splenic compartments - i.e. PALS, follicle, marginal zone and red pulp - is described. The localizations and complete phenotypes of the distinctive mononuclear phagocyte subpopulations are presented in Figure 8.1. Individual subpopulations are shown in the Figures 8.2 and 8.3 by immunostaining with a characteristic mAb.

Localization of macrophages and dendrocytes in the splenic PALS

In close association with the central arteriole, 'peri-arteriolar macrophages' were recognized by mAb ER-BMDM1 (Fig. 8.2a). Furthermore, two other non-lymphoid cell types in the inner PALS were recognized, i.e. the NLDC-145-positive interdigitating cells (Fig. 8.2b) and the MOMA-2-positive macrophages (Fig. 8.2c). In the outer PALS, strongly nonspecific esterase-positive marginal metallophils were the predominant non-lymphoid cell type. They were located at the follicular site of the marginal sinus and merged between the coronal B lymphocytes. Marginal metallophils were specifically stained by mAb MOMA-1 (Fig. 8.2d).

Localization of macrophages and dendrocytes in the splenic follicle

The large tingible body macrophages of the germinal centre were easily recognized by their content of effete lymphocytes. Moreover, these cells stained clearly with mAb Monts-4 (Fig. 8.2e). Another macrophage population in the germinal centre was also recognized by this mAb. Cells of the latter population were small and exhibited acid



mAb												
NLDC-145	-	+	-	-	-/±?	-	-	+	-	-	-	+
MIDC-8	-	+	-	-	-	-	-	±	-	-	-	+
ER-BMDM1	++	+	++	-?	-	+	+	+	-?	+	s++	?
ER-TR9	-	-	-	-	-	-	-	-	+	-	-/s+?	-
Monts-4	-	+	+	++	+	+	+	+?	++	+	±	?
ER-MP23	-	-	-	-	-	-	-/s+?	-	-	-	-/s+?	-
M5/114	±	+	+	+	+	+?	+?	+	-	?	s±	+
M1/70	-	-	-	-	-	-	-	-	s±	-	-	-
M3/38	+	+	+?	+	?	+	+	?	+	+	+	?
MOMA-1	-	-	-	++	-	-	?	?	-	-	±	?
MOMA-2	±	-	+	+	+	+?	?	?	-	+	+	?
BM8	-	-	-	-	-	-	-	-	-	+	++	?
F4/80	-	-	-	-	-	-	-	-	-	+	+	?
ER-HR3	-	-	-	-	-	-	-	-	-	?	s++	?
Enzyme												
NSE	-	-	±	++	+	±	±	?	±	-	s±	-
AcPh	s±?	+	+	+	+	+	+	?	-	±	s±	?

Figure 8.1. Localization and phenotype of the macrophages and dendrocytes present at specific micro-anatomical sites.

++, strongly positive; +, moderately positive; ±, weakly positive; s, subpopulation; ?, difficult to determine conclusively.

ca, central arteriole; IP, inner PALS; OP, outer PALS; C, corona; GC, germinal centre; ms, marginal sinus; MZ, marginal zone; MRP, marginal red pulp; RP, red pulp; PA Mϕ, peri-arteriolar macrophages; IDC, interdigitating cells; PALS Mϕ, Mϕ located in the peri-arteriolar lymphocyte sheath; MM, marginal metallophils; TGB, tingible body Mϕ; DDC, dendrocytes

NSE, nonspecific esterase; AcPh, acid phosphatase.

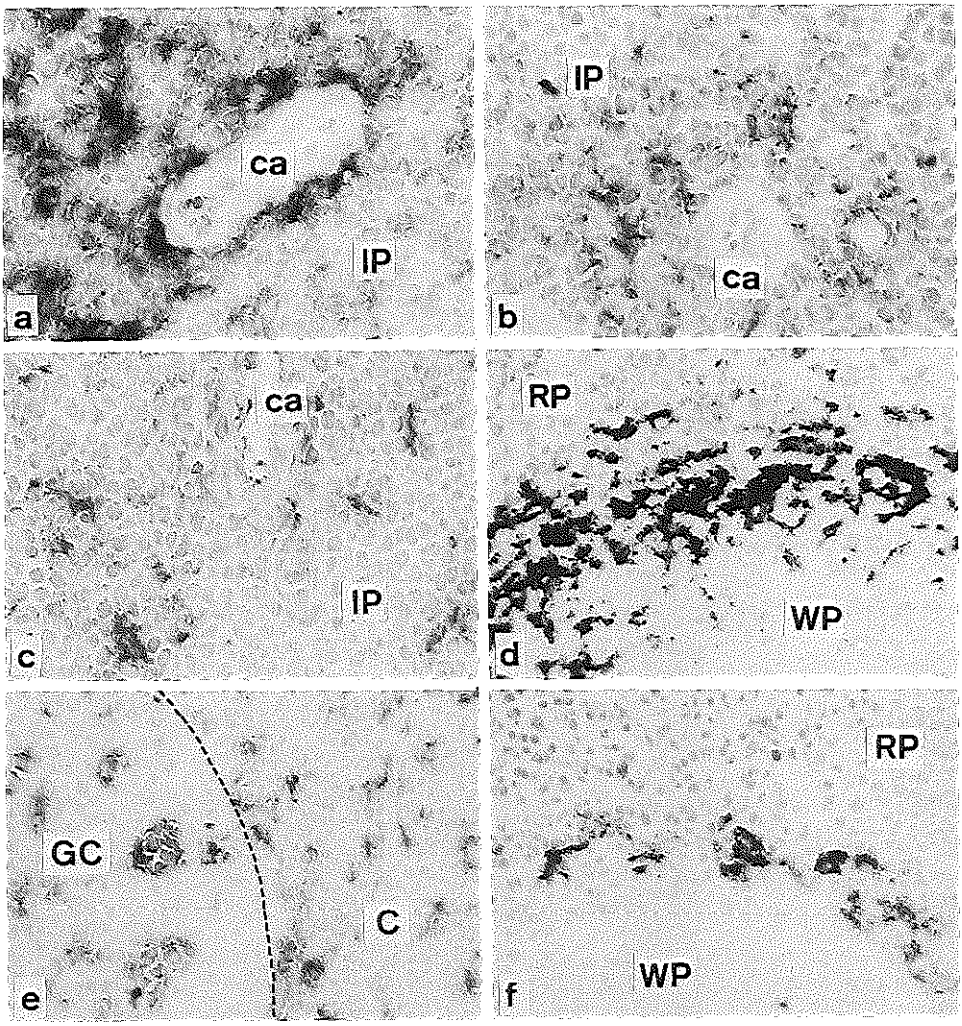


Figure 8.2. Macrophage and dendrocyte subpopulations in the splenic white pulp. (a) peri-arteriolar macrophages stained with mAb ER-BMDM1, (b) IDC stained with mAb NLDC-145, (c) PALS macrophages stained with mAb MOMA-2, (d) marginal metallophils stained with mAb MOMA-1, (e) tingible body macrophages, follicular macrophages and corona macrophages stained with mAb Monts-4, (f) marginal zone macrophages stained with mAb ER-TR9. Original magnifications x 320.
ca, central arteriole; IP, inner PALS; WP, white pulp; RP, red pulp; GC, germinal centre; C, corona.

phosphatase activity. They were termed follicular macrophages. The coronal macrophages, present within the surrounding B cell corona exhibited a comparable phenotype, which also included the Monts-4 marker (Fig. 8.2e).

Localization of macrophages and dendrocytes in the marginal zone

The large marginal zone macrophages, located at the red pulp-side of the marginal sinus were specifically stained with mAb ER-TR9 (Fig. 8.2f). In addition to these cells,

a small NLDC-145-positive population of cells was observed which are referred to asmarginal dendrocytes (data not shown).

Localization of macrophages and dendrocytes in the splenic red pulp

Using our mAb panel, the non-lymphoid cells of the splenic red pulp could be subdivided into at least four subpopulations. One of these subpopulations was located in close association with the marginal zone. Therefore, cells assigned to this population were termed marginal red pulp macrophages. They were recognized for example by mAb ER-BMDM1 (Fig. 8.3a). Other macrophages were scattered throughout the red pulp. They were considered as a single macrophage population by other investigators but staining with the various mAbs revealed that distinct subpopulations consist within the red pulp macrophage population. Thus, F4/80 (Fig. 8.3b) and BM8 (Fig. 8.3c) recognized all red pulp macrophages, whereas ER-HR3 (Fig. 8.3d) and ER-BMDM1 (Fig. 8.3e) recognized a major and a minor subpopulation, respectively. ER-BMDM1-positive cells were present in the central red pulp, but the majority of the ER-HR3-positive cells was located in a zone just beneath the splenic capsule. In addition, a very small population of NLDC-145 positive cells was demonstrated in the red pulp (Fig. 8.3f).

DISCUSSION

In this paper we report on the phenotypical heterogeneity of macrophages and dendrocytes present in the murine spleen. For the characterization of these cells we utilized a number of macrophage and dendritic cell-specific monoclonal antibodies.

Our results, summarized in Figure 8.1, show that macrophages and dendrocytes present at different microanatomical sites partially overlap in their marker expression. However, phenotypical differences between both cell types were also noticed. Nearly all dendrocytes were recognized by mAb NLDC-145 and MIDC-8, and macrophages by mAb MOMA-2 and/or F4/80. Although we were unable to determine the exact location of some markers due to overstaining of adjacent cells, we conclude from Figure 8.1 that, when compared to macrophages, the marker expression of dendrocytes at distinct microanatomical sites was rather constant.

The present study especially refines the information on the presence of macrophage subpopulations in the red pulp. In former studies, positively stained red pulp macrophages have been observed with F4/80 (Hume et al., 1983), BM8 (Malorny et al., 1986), MOMA-2 (Kraal et al., 1987) and ER-HR3 (de Jong et al., 1990a). However, the distinct subpopulations were not identified in these studies. In the present study,

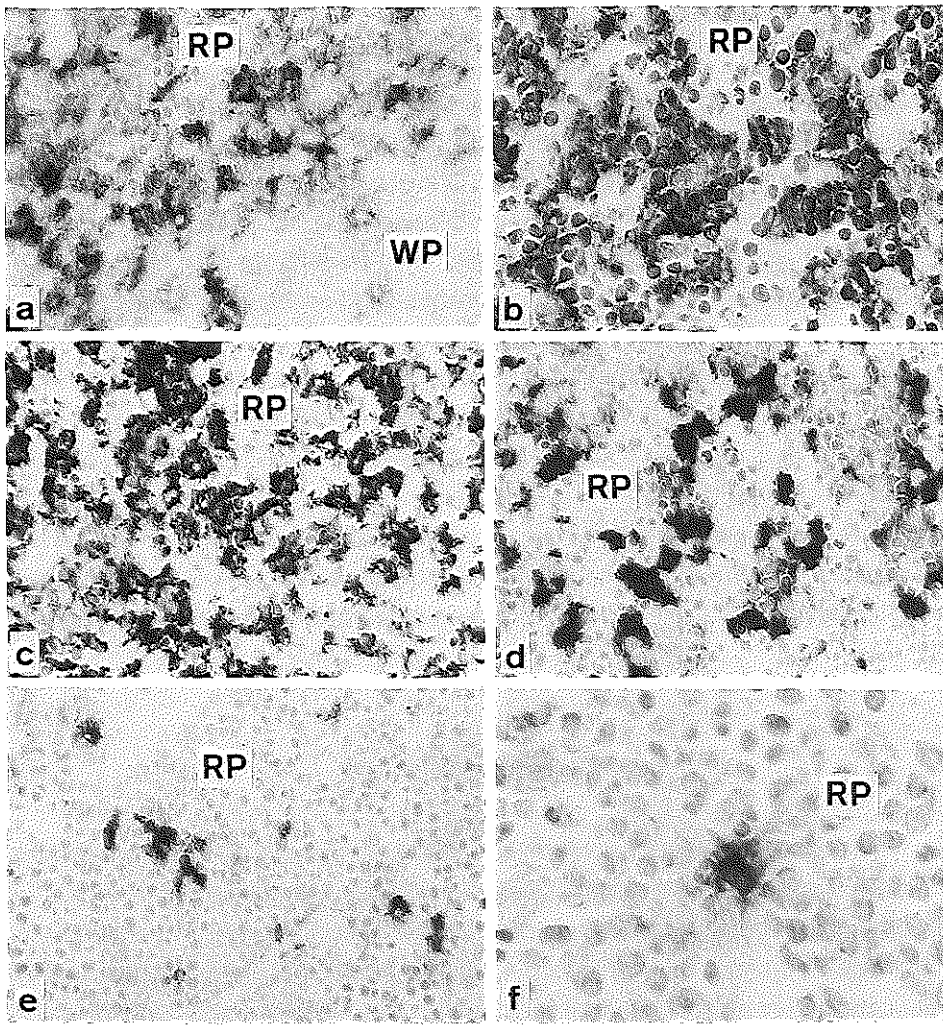


Figure 8.3. Macrophage and dendrocyte subpopulations in the splenic red pulp. (a) marginal red pulp macrophages stained with mAb ER-BMDM1, (b) red pulp macrophages stained with F4/80, (c) red pulp macrophages stained with BM8, (d) red pulp macrophages stained with ER-HR3, (e) red pulp macrophages stained with ER-BMDM1, (f) red pulp dendrocyte stained with NLDC-145. Original magnifications: x 320 (a, c, d, e); x 510 (b); x 800 (f).
RP, red pulp; WP, white pulp.

we observed at least four subpopulations: (i) a major F4/80+, BM8+, ER-BMDM1+, M3/38+ population in close association with the marginal zone, (ii) a smaller F4/80+, BM8+, ER-HR3+ population mainly located near the capsule, (iii) a minor F4/80+, BM8+, ER-BMDM1+ population in the central red pulp and (iv) a remaining population of F4/80+, BM8+, MOMA-2+ cells scattered throughout the red pulp. Double staining procedures are presently ongoing in order to determine a further immunophenotypical

distinction between these populations. Our study further indicated that the expression of the markers detected by F4/80, BM8 and ER-HR3 was confined to cells of the red pulp suggesting that these antigens were involved in functions performed exclusively in this compartment.

The absence of F4/80+ macrophages in the white pulp contrasts with the results of studies by other investigators (Hume et al., 1983a; Witmer and Steinman, 1984). They observed F4/80+ cells around the central arteriole of the inner PALS. In our study we identified these peri-arteriolar macrophages with mAbs ER-BMDM1, MOMA-2 and M5/114, but not F4/80. Possibly, differences in the methodology explain the observed discrepancy with regard to F4/80 detection. However, acetone fixation as applied by Witmer and Steinman (1984) instead of pararosanilin fixation of the tissue sections did not alter our results (data not shown). Another explanation for the observed discrepancy could be the existence of local differences in the concentration of microenvironmental cytokines. In this context, it has been demonstrated that interferon-γ-activated macrophages show a decreased expression of the F4/80 antigen after exposure to these factors (Berton and Gordon, 1983).

Noteworthy, follicular dendritic cells were not recognized by any of the mAb from our panel. This finding supports the notion that follicular dendritic cells have an origin and function different from other dendrocytes. Such cells are not bone marrow-derived; rather they originate from fibroblastic reticulum cells in situ (Humphrey and Grennan, 1981).

Our study also indicate that macrophages and dendrocytes share a number of antigens such as ER-BMDM-1 Ag, Monts-4 Ag, Ia and Mac-2 (Fig. 8.1). Therefore, both cell types are most probably closely related and may originate from the same progenitor cell. So far, the classification of macrophages and dendrocytes has been a matter of considerable debate. The results of ontogenetic studies (Janossy et al., 1986; van Rees et al., 1988) indicating that both cell types must be regarded as separate lineages, have been contradicted by other observations. A direct relationship between the two cell types has originally been suggested by Hoefsmit et al. (1982). These investigators observed Birbeck granule-containing veiled cells and IDC within the draining lymph and lymph node of the skin, respectively. They proposed that these cells originate from epidermal Langerhans cells (LC). This proposal is strengthened by the observations that both LC (Katz et al., 1979) and IDC (Barclay, 1981) are bone marrow-derived, a feature which they have in common with monocytes. Noteworthy, there are several other indications that LC have a monocytic origin: both cells exhibit ATPase and nonspecific esterase activity (Wolff and Stingl, 1983). Moreover, these cells possess type II Fc- and C3bi receptors (Austyn, 1987) and express the macrophage markers Mac-2 and Mac-3 (Haines et al., 1983), F4/80 Ag (Hume et al.,

1983a), BM8 Ag (Malorny et al. 1986) and ER-HR3 Ag (de Jong et al., 1990a). Analogous to the epidermis, a similar relationship between monocytes, veiled cells and IDC in the intestine has been proposed (Sminia et al., 1983). Additional arguments for a monocytic origin of dendrocytes come from the observation that functional dendrocytes can be obtained from cultured blood monocytes (Peters et al., 1987). Furthermore, under some specific experimental conditions, IDC have been shown to become phagocytic (Duijvestijn et al., 1982a; Fossum et al., 1984).

In conclusion, we demonstrated that mononuclear phagocytes present at distinct microanatomical sites in the spleen exhibit distinctive phenotypes. This compartmentalization of dendrocyte and macrophage subpopulations within the spleen is most likely critical for their function. Furthermore, the phenotypical overlap between dendrocytes and macrophages is indicative of the close relationship between these cell types.

Acknowledgments. The authors thank Mr. Tar van Os for preparing the figures and Mrs. Cary Meijerink-Clerkx for typing the manuscript. Drs. Siamon Gordon, Clemens Sorg, Mark Jutila and Georg Kraal are greatly acknowledged for the donation of hybridoma cells or antibodies.

9. DISTRIBUTION OF MONOCYTE/MACROPHAGE MARKERS IN THE NON-LYMPHOID REGIONS OF THE GASTRO-INTESTINAL TRACT

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Submitted for publication.

SUMMARY

In this study we investigated the distribution of macrophage antigens in the murine gastro-intestinal tract. It was observed that macrophage-like cells were abundant throughout the tract. Based on morphological, phenotypical and enzyme histochemical criteria they could be subdivided into three subpopulations. The first population consisted of small elongated spindle-shaped cells that expressed the markers T-200 (common leukocyte antigen), Ia and often Mac-1, and exhibited nonspecific esterase activity. Part of these cells additionally expressed the antigens F4/80, FcRII and ER-HR3. Cells of the second population possessed the same phenotypical characteristics but they were larger, more rounded, and additionally exhibited a strong acid phosphatase activity. The third population consisted of large cells with a comparable phenotype as the previous one. However, they lacked the Ia antigen. It was observed that the three cell types were not equally distributed over the gastro-intestinal tract. Type 1 cells were predominate in the proximal parts, whereas type 2 cells were the predominate cells in the middle parts. The presence of type 3 cells was restricted to the caecum. Furthermore, it was noted that some of the macrophage-specific antigens were also expressed by other cell types such as epithelial, fibroblastoid, and nerve cells.

INTRODUCTION

Macrophages originate from hemopoietic stem cells in the bone marrow and differentiate via the monocytic lineage. Together with their precursor cells they constitute the mononuclear phagocyte system. Cells of this system are phagocytic and display marked heterogeneity in morphology, enzyme contents, surface marker expression and functions (Van Furth, 1982). Apart from their accessory function in the immune response, macrophages perform important local functions by producing a large variety of secretory products and interacting with many different cells and molecules in their environment (Takemura and Werb, 1984; Gordon et al., 1988a). Most of these properties are well-defined for monocytes and macrophages derived from the blood, the bone marrow and the peritoneal cavity, but little is known about the phenotype and function of other tissue macrophages, including the intestinal macrophages.

The macrophage content of different murine tissues was recently determined by Lee et al. (1985) in a quantitative absorption immunoassay using the macrophage-

specific antibody F4/80. From this study it was obvious that the gastro-intestinal macrophage population is one of the largest macrophage populations of the body. Isolation and characterization of intestinal cells from the villous parts of the colon revealed that about 20% of all nucleated cells consist of macrophages (Golder and Doe, 1983). They stain positively for the enzymes acid phosphatase, nonspecific esterase and ATP-ase, and are able to phagocytize. The latter property may be of significant importance for their function in the gastro-intestinal tract. Since the intestine is one of the first tissues that intimately contacts with food and microbial antigens, intestinal macrophages are thought to play an important role in the first line host defense. Indeed, it has been demonstrated that macrophages in vivo (Wells et al., 1987) and in vitro (Beeken et al., 1987) are able to phagocytize bacteria and that they produce antibacterial enzymes such as lysozyme (LeFevre, 1979; Spencer et al., 1986). Furthermore, they are involved in T cell proliferation and viability (Gibson et al., 1985) and it has been postulated that macrophages support epithelial integrity (Hume et al., 1984a).

In addition to functional studies, a detailed morphological characterization of macrophages may contribute to a better understanding of their relationship with other cells. The availability of a wide range of monoclonal antibodies allows the in situ examination of macrophage-like cells (MLC) at the light microscopic level by means of immunohistochemical staining techniques. Histological studies demonstrate that MLC are present in all layers of the gastro-intestinal tract such as the epithelium (Dillon and MacDonald, 1984), the lamina propria and the submucosa (Hume et al., 1984; Witmer and Steinman, 1984), and the muscularis externa (Mikkelsen et al., 1985). Furthermore, there are indications that phenotypical differences occur among macrophages located in the different parts of the gastro-intestinal tract (Selby et al., 1983; Sminia et al., 1986).

The present paper reports on a study that was performed to investigate the phenotypical heterogeneity of MLC located at specific anatomical sites within the various tissue layers of the murine gastro-intestinal tract in more detail.

MATERIAL AND METHODS

Mice. Adult male (CBA/Rij x C57BL/Rij)F1 mice were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands, and kept under conventional conditions.

Tissue preparation. The gastro-intestinal tract was removed under Avertin anaesthesia and was collected in 5 ml of the tissue preservative Histocon. Segments of approximately 5 mm were removed from the oesophagus (about 2 cm proximal the stomach), the fundal, corpal and the pyloric parts of the stomach, the duodenum (2 cm distal the pylorus), the jejunum (2 cm from the ligament of Treitz), the terminal ileum (2 cm proximal the caecum), the middle part of the caecum, and the colon (2 cm distal the caecum). After approximately 1 hr, the tissue segments were washed with Histocon, embedded in Tissue-tek on a specimen stub, and frozen on solid carbon dioxide. Five micron frozen sections were cut on a cryostat (Leitz model 1720) and collected on microscope slides precoated with a solution containing 0.1% gelatin and 0.01% chromium potassium sulphate.

Monoclonal antibodies. In this study we utilized a number of monoclonal antibodies directed against monocytes/macrophages and other leucocytes. Used antibodies were 30-G12 (T-200 antigen; Ledbetter et al., 1979), 59-AD2.2 (Thy-1 antigen; Ledbetter and Herzenberg, 1979; Basch and Berman, 1982), M5/114 (Ia antigen; Bhattacharya et al., 1981), M1/70 (Mac-1 antigen; Springer et al., 1979), M3/38 (Mac-2 antigen; Ho and Springer, 1982; Flotte et al., 1983), M3/84 (Mac-3 antigen; Ho and Springer, 1983; Flotte et al., 1983), F4/80 (F4/80 antigen; Hirsch et al., 1981), 2.4G2 (FcRII; Unkeless, 1979) and ER-HR3 (ER-HR3 antigen; De Jong et al., 1987). The tissue distribution and specificity of these antibodies are described in the references.

Pararosanilin fixation. Tissue sections were air-dried and fixed for 2 min with a solution of hexazotized pararosanilin prepared as described by de Jong et al. (1990c). In short, 0.5 ml of a 4% (w/v) pararosanilin/HCl solution was added to 0.5 ml 4% (w/v) $NaNO_2$ in distilled water. This mixture was added to 12.5 ml of distilled water. After fixation the slides were placed in phosphate buffered saline, pH 7.8 (PBS) supplemented with 0.02% (w/v) gelatin and 0.05% (v/v) Tween-20 (PBT).

Immunohistochemical staining. The sections were overlaid with 70 μl of the first-step monoclonal antibody and incubated for 1 hr at room temperature. During incubation the sections were kept in moist chambers to prevent air drying. After rinsing the sections with PBT, they were overlaid with 70 μl of a horseradish-coupled rabbit-anti-rat Ig solution (Dakopatts, Copenhagen, Denmark) and incubated for 1 hr at room temperature. Sections were rinsed with PBT and the enzyme activity was visualized by incubation with a diaminobenzidine (Sigma, St. Louis, MO, USA) solution (1 mg/ml PBS supplemented with 20 μl 1% (v/v) H_2O_2). After rinsing with tap water, sections

were counterstained with hematoxylin, dehydrated, embedded in Entellan mounting medium (Merck, Darmstadt, FRG) and cover-slipped.

Enzyme histochemistry. Acid phosphatase and nonspecific esterase activities were demonstrated as described by Burnstone (1962) with naphthol-AS-BI phosphate (Sigma) and α-naphthyl acetate (Sigma) as substrates, respectively. In both procedures hexazotized pararosanilin was used as diazonium salt.

RESULTS

General description

Macrophage-like cells (MLC) were present in all investigated parts of the gastrointestinal tract. Their number was relatively low in the more proximal parts of the tract but increased towards the caecum. MLC were especially abundant in the lamina propria, the submucosa and the muscularis externa. Furthermore, sparse MLC were located in between the epithelial cells. Like MLC, the incidence of cells expressing the enzymes nonspecific esterase (NSE) or acid phosphatase (AcP) also increased towards the distal parts of the tract. However, the number NSE-positive cells exceeded that of AcP-positive cells.

Epithelium

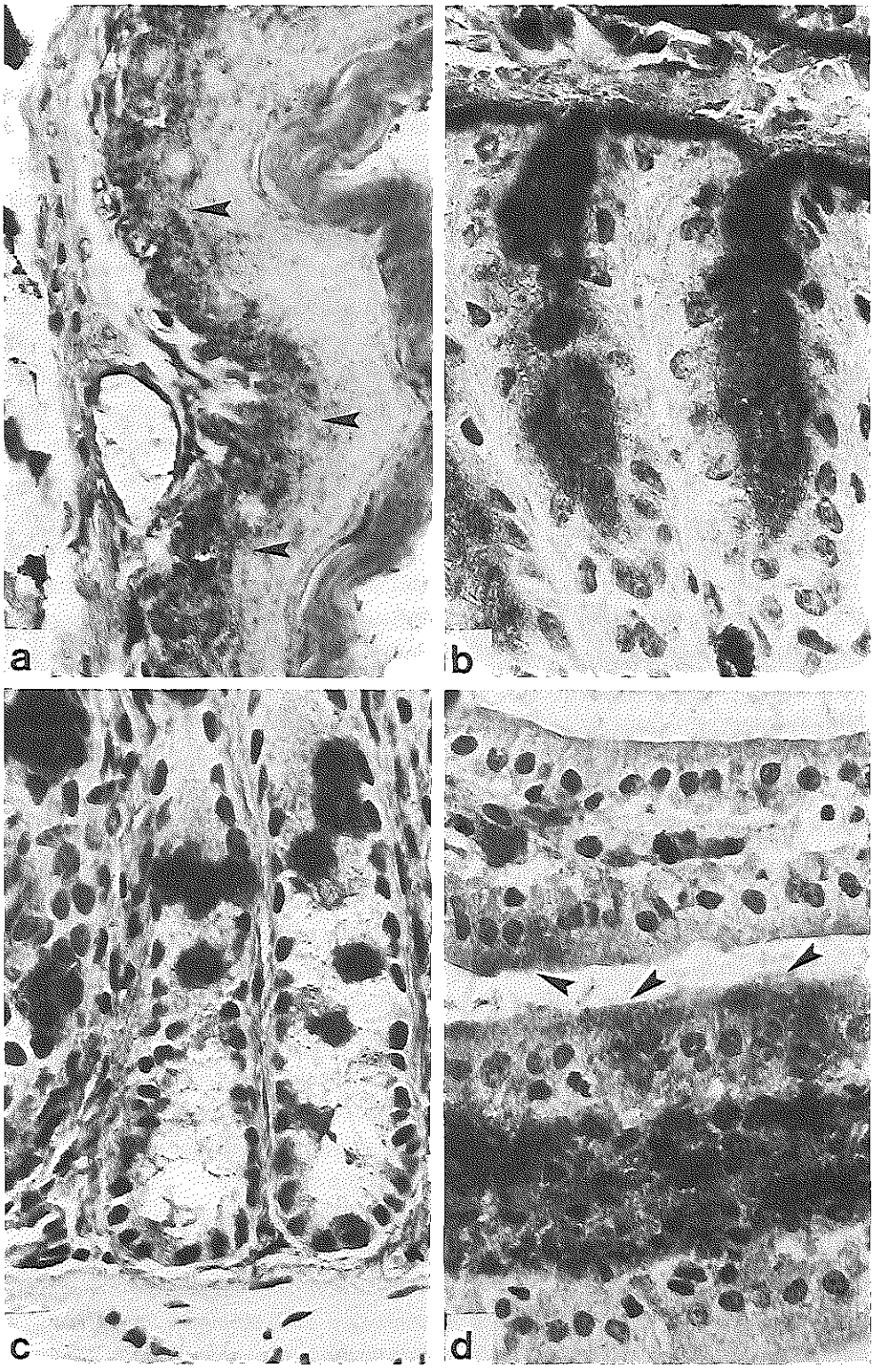
The antigens Mac-2 and Mac-3 were expressed by most intestinal epithelial cells. Within the oesophagus (Fig. 9.1a) and the fundus the reactivity of both antigens was restricted to the lowest cell layer of the epithelium. However, in other parts of the tract the distribution of Mac-2 and Mac-3 did not coincide. Mac-3 was expressed by all epithelial cells and its expression decreased towards the colon. Cells expressing this antigen exhibited a stronger reactivity at their apical pole. In contrast to the Mac-3 antigen, the expression of the Mac-2 antigen by epithelial cells was much more variable. Within the corpus and the pylorus Mac-2 reactivity was confined to the mucus of apically located epithelial cells on the rugae (Fig. 9.1b) and to the gastric glands, respectively. Mac-2 expression in other parts of the tract was also restricted to the mucus of the goblet cells (Fig. 9.1c) and the brush border. The staining intensity for this antigen increased towards the upper parts of the villi. Another marker expressed by many epithelial cells was the Ia antigen. Within the duodenum and the jejunum individual cells or small groups of epithelial cells were positive for this antigen (Fig. 9.1d). Towards the ileum all epithelial cells became Ia-positive. However, the epithelium of

the caecum and colon lacked the Ia antigen. Throughout the gastro-intestinal tract the localization of MLC was suggestive of their migration from the lamina propria to the epithelium (Fig. 9.1e). Most of these migrating MLC expressed the T-200 antigen. A subpopulation of these cells additionally stained with both mAb M5/114 (anti-Ia) and F4/80. Occasionally, ER-HR3 (oesophagus and fundus) or Thy-1-positive cells (jejunum and duodenum) were observed in between the epithelium. The latter antigen was also observed on the basement membrane of the epithelial cells of the fundus but not the other parts of the tract (Fig. 9.1f). Many epithelial cells exhibited strong NSE and AcP activity. NSE activity was observed in all investigated parts of the tract whereas AcP was found to be restricted to epithelial cells present in parts distal of the stomach. Moreover, NSE activity was distributed intracellularly throughout the cells wheras AcP activity was confined to the apical regions of the cells and the brush border.

Lamina propria

Especially in the proximal parts of the gastro-intestinal tract, i.e. the oesophagus and the stomach, the lamina propria was well developed. Positive cells were observed (1) 'free' in the connective tissue and (2) against the epithelium (Fig. 9.2a), (3) adjacent to the endothelium of blood vessels, and (4) the muscularis mucosae (Fig. 9.2b). All MLC in the proximal part of the tract were small and elongated and expressed the T-200 antigen. Moreover, most cells expressed the Ia and Mac-1 antigens. A minor subpopulation additionally possessed the ER-HR3, the F4/80 and the FcRII antigens. No relation was observed between the phenotype and location of the MLC. In addition to these populations, T-200-positive mast cells were observed. Within the lamina propria of the gastric corpus and pylorus MLC were mainly located in the basal regions of the rugae. They all expressed the T-200 antigen and most of them additionally expressed the Ia, F4/80 and FcRII antigens. However, relatively few cells (approximately 10% of the T-200-positive population) possessed the Mac-1 antigen. The latter cells were often located against the muscularis mucosae (Fig. 9.2b). In addition to these MLC, infiltrates of T-200, Thy-1-positive cells were frequently observed in the proximal parts of the tract. Some of the MLC in these parts exhibited NSE activity but a relationship between this activity and marker expression was not observed. AcP-positive cells were not observed in these parts of the tract.

Within the middle parts of the tract, i.e. the duodenum, the jejunum, and the ileum, the precise location of positively staining cells was difficult to determine because the lamina propria in these parts was very thin. Generally, identical populations of MLC were observed as described for the oesophagus and stomach. In addition, a population of large cells was observed mainly located in the upper parts of the villi.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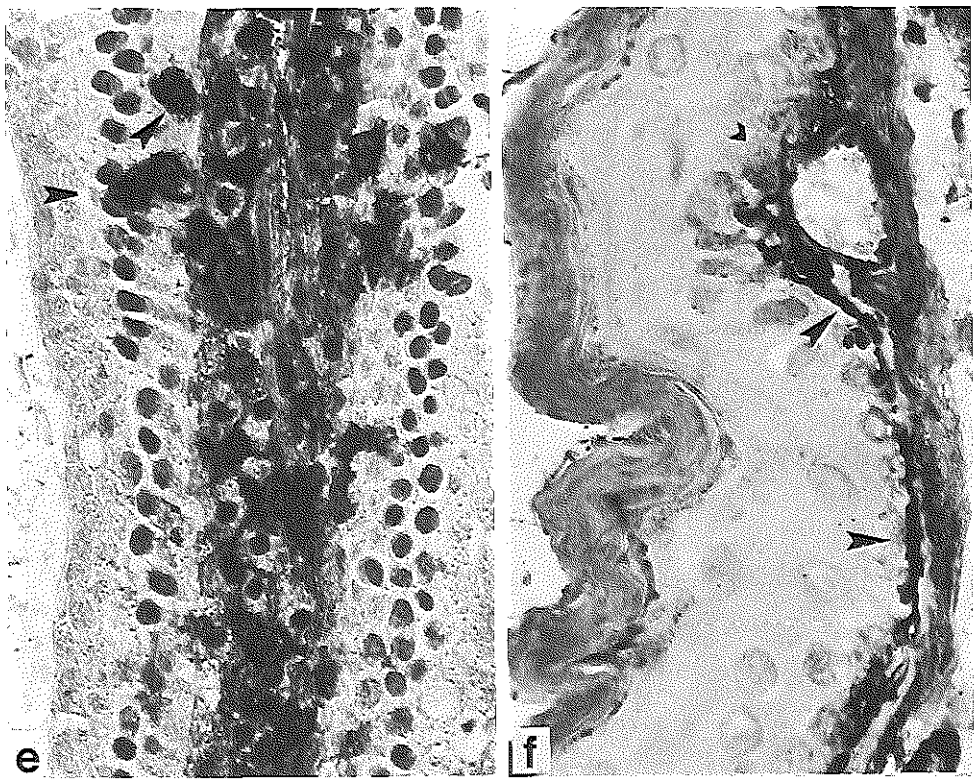
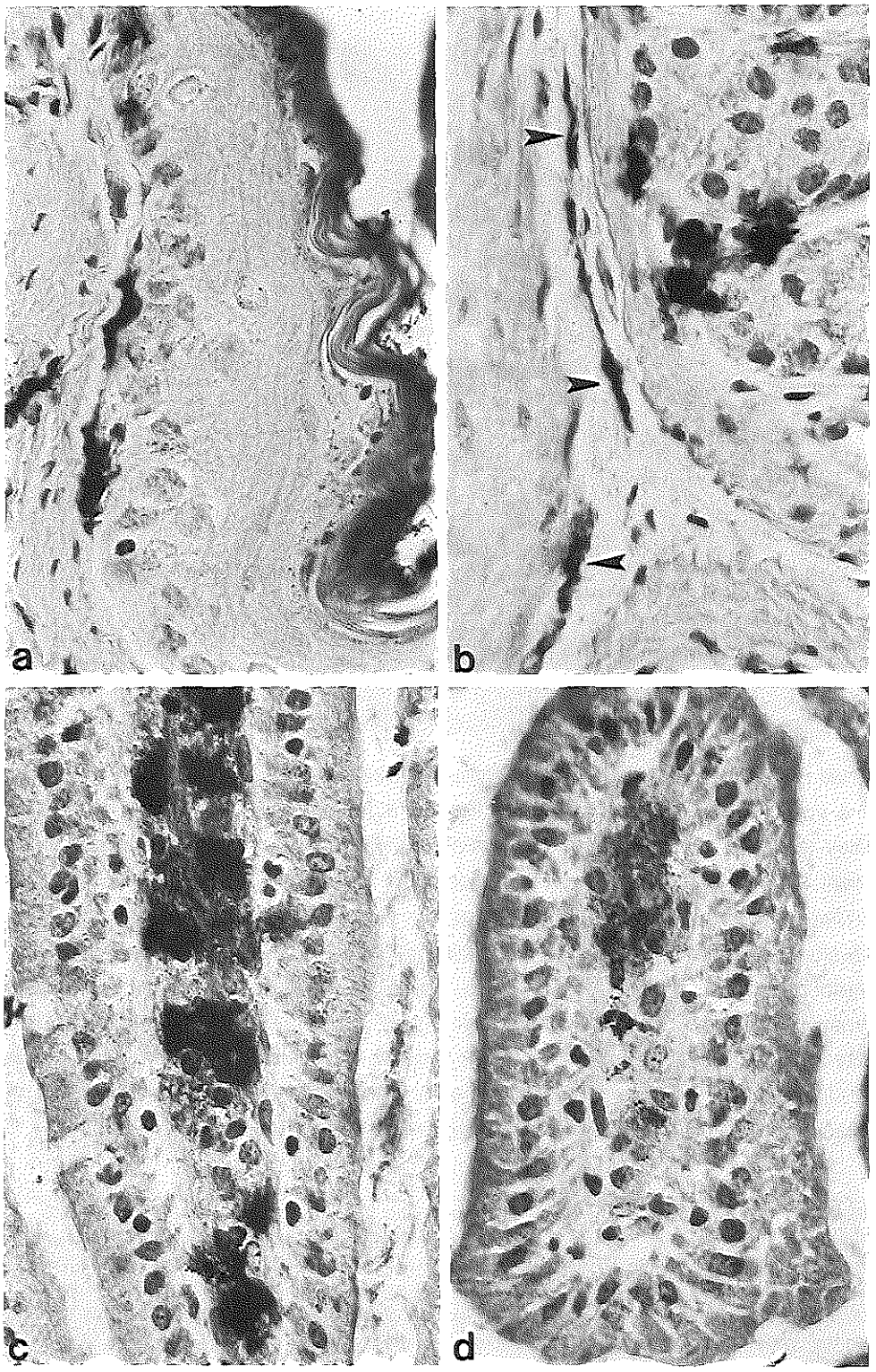


Their number increased fom the duodenum towards the caecum. The cells expressed the antigens T-200, Ia, Mac-1 (Fig. 9.2c), F4/80, FcRII and ER-HR3 (Fig. 9.2d), and they exhibited NSE and AcP activity. Within the lamina propria of the caecum MLC were abundant. They were located especially in the lower parts of the crypts adjacent to the muscularis mucosae (Fig. 9.2e) and they expressed all aforementioned antigens. Besides MLC, other positively staining cell types were observed in the lamina propria. Amongst others, a population of T-200, Thy-1-positive cells was present. This latter antigen was also observed on fibroblastoid cells and their surrounding connective tissue fibers. Also the Mac-2 and Mac-3 antigens were expressed by many cell types and their distribution was rather nonspecific. Most striking was the presence of the Mac-2 antigen on a cell layer located just beneath the epithelium (Fig. 9.2f).

Figure 9.1. Distribution of macrophage antigens on the intestinal epithelium. Magnification x650. (a) expression of the Mac-3 antigen (arrows) by the lowest cell layer of the oesophagal epithelium; (b) expression of the Mac-2 antigen by the epithelial cells of the corpus; (c) expression of the Mac-2 antigen by goblet cells of the colon. (d) expression of the Ia antigen by epithelial cells of the jejunum; (e) T-200-positive cells present in between the epithelial cells of the jejunum; (f) presence of the Thy-1 antigen on the basement membrane of oesophagal epithelial cells.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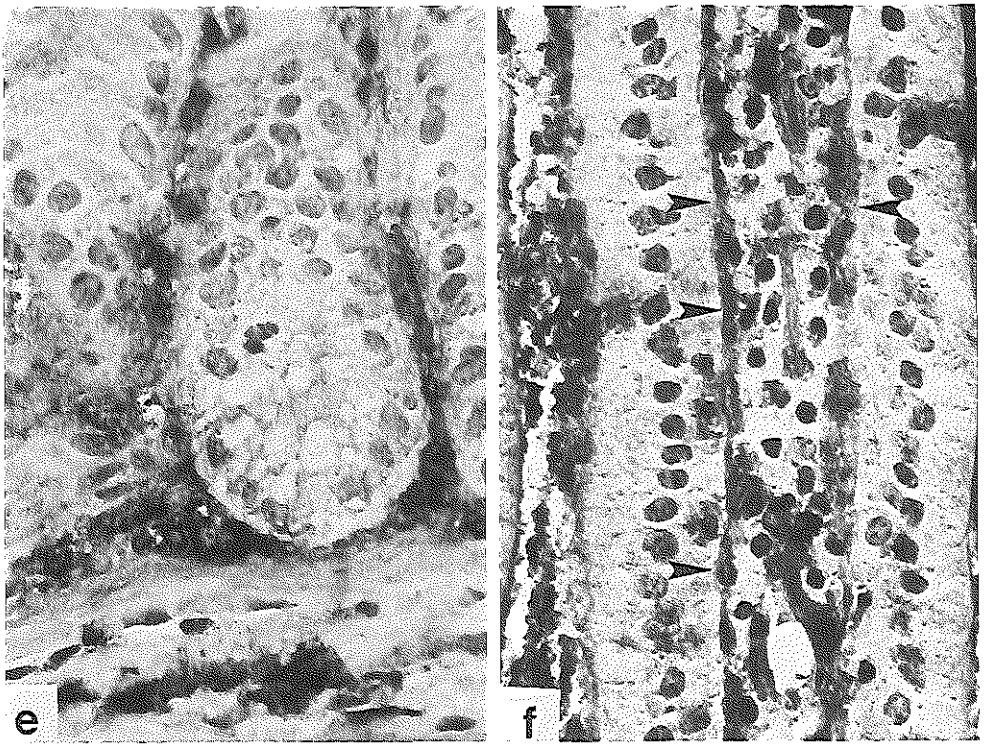
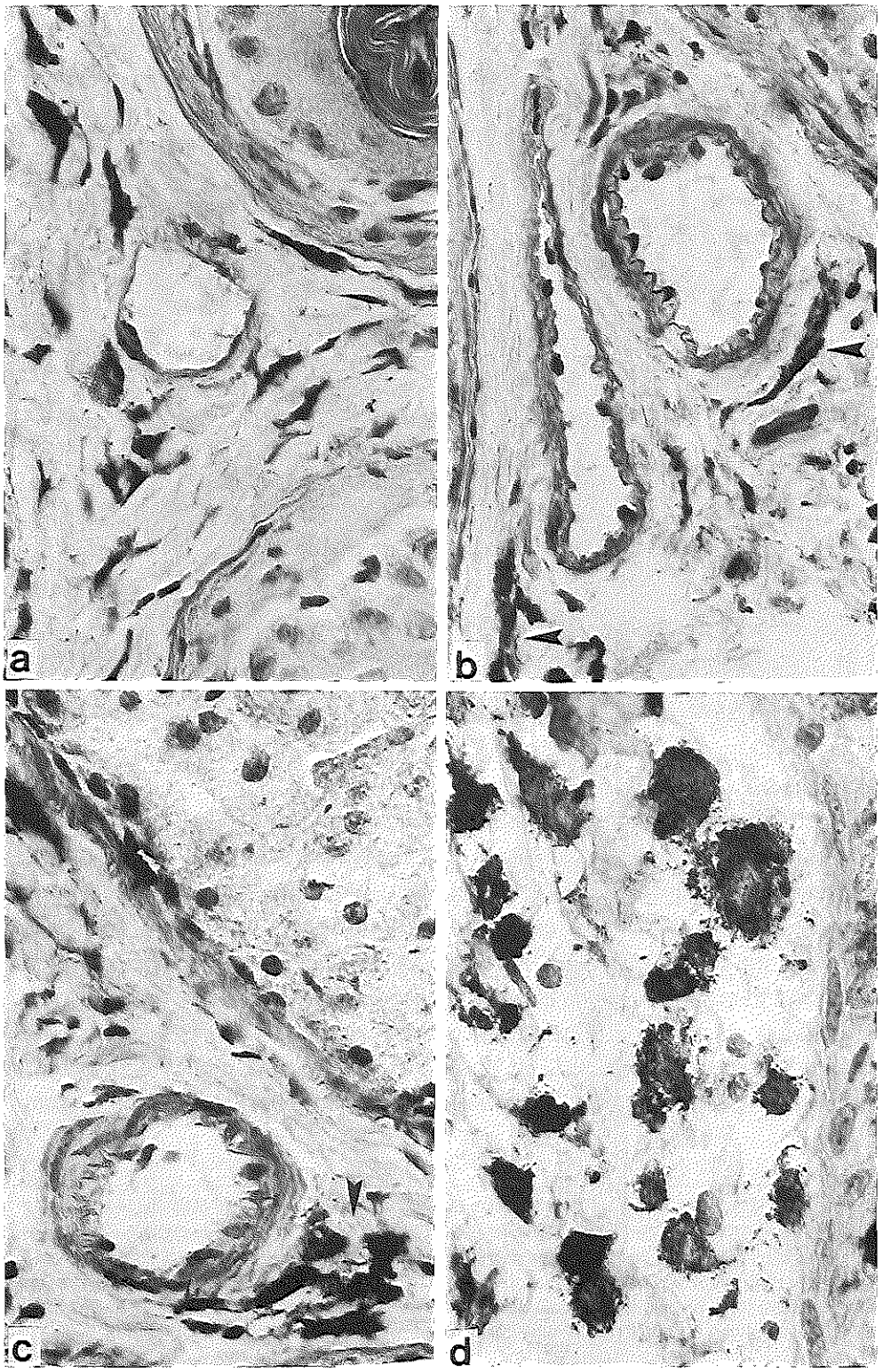


Submucosa

Most MLC of the submucosa could morphologically and phenotypically not be distinguished from the small elongated cells in the lamina propria. Nearly all cells possessed the antigens T-200 (Fig. 9.3a), Mac-1 (Fig. 9.3b) and Ia (Fig. 9.3c) and relatively few cells additionally expressed the F4/80 antigen, the FcRII and, less frequently, the ER-HR3 antigen. The cells were more or less randomly distributed throughout the submucosa and no specific association with certain tissue structures or cell types wasobserved. The largest part of the cells stained positively for the enzyme NSE but lack the enzyme AcP. Many MLC of the corpus and pylorus possessed the FcRII. Furthermore, it was striking that the submucosa of the caecum

Figure 9.2. Expression of macrophage antigens by cells in the lamina propria. Magnification x650. (a) expression of the Ia antigen by subepithelial cells in the lamina propria of the oesophagus; (b) Mac-1 expression by cells in the lamina propria of the pylorus; (c) expression of the Mac-1 antigen by cells in the lamina propria of the ileum. (d) expression of the ER-HR3 antigen by cells in the lamina propria of the jejunum; (e) expression of the FcRII by cells in the lamina propria of the caecum; (f) expression of the Mac-2 antigen by subepithelial cells in the lamina propria of the jejeunum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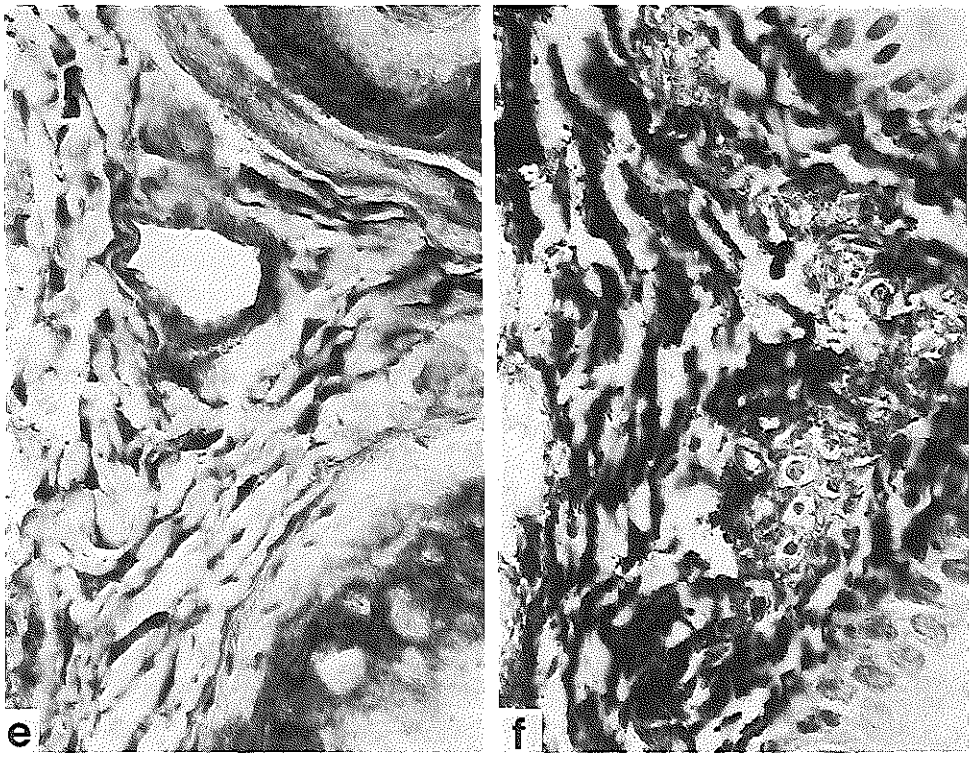
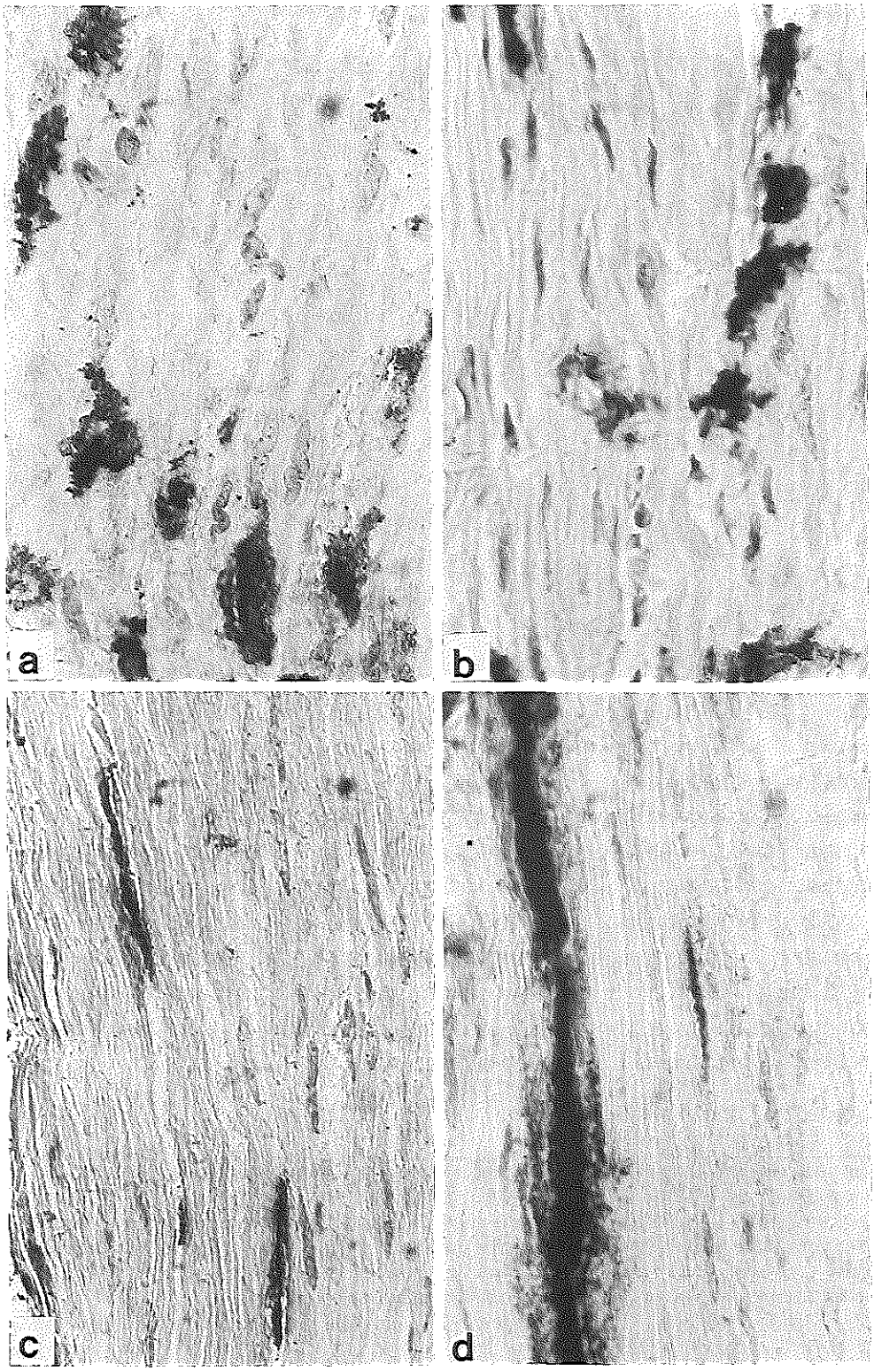


contained an additional population of large cells (Fig. 9.3d). These cells lacked the Ia antigen, but possessed the antigens T-200, F4/80, Mac-1, Mac-3, FcRII and ER-HR3 and exhibited a strong NSE and weak AcP activity. In addition to MLC, a submucosal population of T-200-positive mast cells was present. Furthermore, within the corpus and pylorus of the stomach many T-200, Thy-1-positive lymphocytes were observed. In the submucosa of other parts this cell type occurred less frequently. Expression of Thy-1 and Mac-2 in the submucosa was not only restricted to cells. Within the oesophagus and lower parts of the intestine connective tissue fibers also stained positively for both markers (Figs. 9.3e and 9.3f).

Figure 9.3. Expression of macrophage antigens by cells in the submucosa. Magnification x650. (a) expresssion of the T-200 antigen by cells in the submucosa of the corpus; (b) expression of the Mac-1 antigen by cells in the submucosa of the corpus; (c) expression of the Ia antigen by cells in the submucosa of the pylorus; (d) Mac-1 expression by cells in the submucosa of the caecum; (e) Mac-2 expression by cells and connective tissue fibers in the submucosa of the fundus; (f) Thy-1 antigen present on connective tissue fibers in the submucosa of the oesophagus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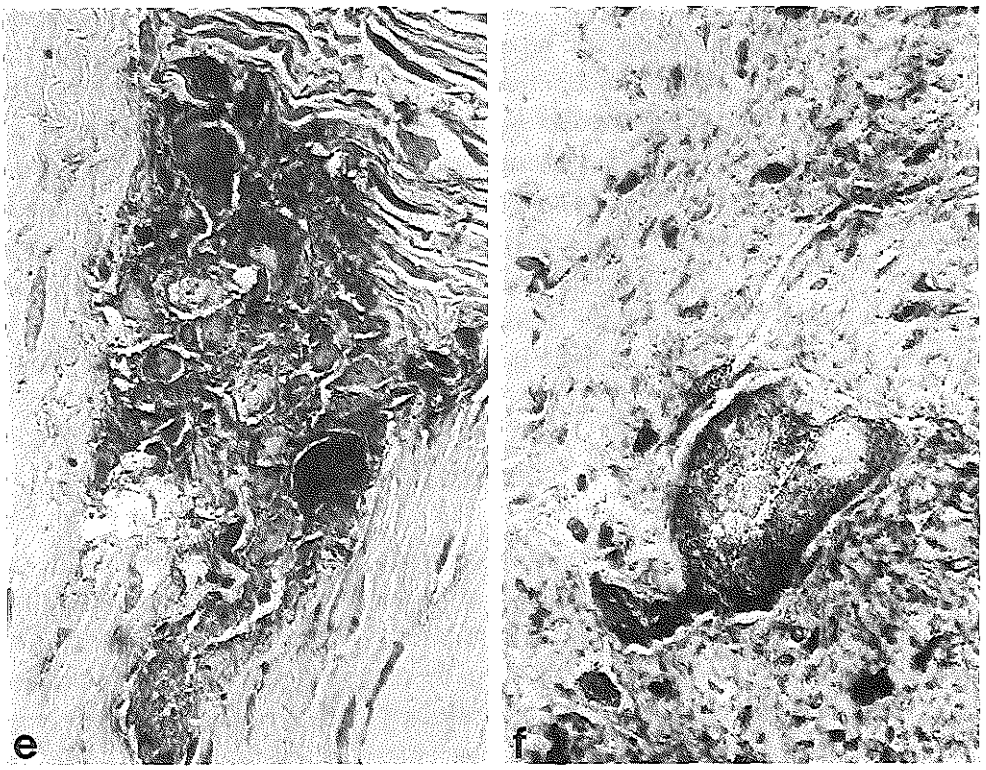

Muscularis externa

MLC were abundant in the muscularis externa. They were especially present in the connective tissue between the circular and longitudinal muscle layers at the level of the plexus of Auerbach. But also in the well developed circular muscle of the stomach and at the serosal level MLC occurred. Most cells expressed the markers T-200 (Fig. 9.4a) and Ia (Fig. 9.4b) and showed a strong NSE activity. Dependent on which part of the tract was investigated, a variable number of these cells additionally expressed the antigens Mac-1, Mac-3, F4/80, 2.4G2 and ER-HR3. All MLC present in the muscularis externa of the proximal parts of the tract expressed Mac-1. The relative number of Mac-1-positive cells slowly decreased till approximately 50% of the MLC in the pylorus

Figure 9.4. Expression of macrophage-specific antigens on cells present in the muscularis externa. Magnification x650. (a) T-200-positive cells in the muscularis externa of the corpus; (b) Ia-positive cells in the muscularis externa of the corpus; (c) Mac-1-positive cells present in the muscularis externa of the pylorus; (d) Thy-1 antigen present on connective tissue fibers of the muscularis externa; (e) Mac-3 expression by nerve cells present in the plexus of Auerbach; (f) Thy-1 expression by nerve cells present in the plexus of Auerbach

and stabilized at this level. Only within the caecum around 80% of the MLC expressed Mac-1. In contrast, Mac-3 showed an opposite distribution. Less than half of the T-200, Ia-positive cell population additionally expressed the F/80 antigen and FcRII, and cells with the latter phenotype were present mainly in the more distal parts of the tract. Only few cells in the muscularis externa expressed the ER-HR3 antigen. Besides these MLC also T-200-positive mast cells were observed frequently between the circular and longitudinal muscle layers. Occasionally these cells were present within the circular muscle layer. The Thy-1 antigen was present on some fibers of the connective tissue (Fig. 9.4d) between the two muscle layers. Furthermore, the Mac-3 (Fig. 9.4e) and Thy-1 (Fig. 9.4f) antigens were expressed by the nerve cells of the plexus of Auerbach.

DISCUSSION

In this study we utilized a number of anti-macrophage monoclonal antibodies (mAb) to characterize the macrophage-like cells (MLC) present in the various parts of the murine gastro-intestinal tract. It was obvious that MLC were present in all investigated parts. They were abundant especially in the middle and distal parts of the tract and their number reached a maximum in the caecum. MLC likely originate from hemopoietic stem cells and therefore possess the common leukocyte antigen (T-200). The distribution of this antigen was used to localize the total population of MLC. Based on morphological, phenotypical and enzyme histochemical criteria various subpopulations could be defined. Morphologically, three cell types were recognized, namely (a) small spindle-shaped cells (type 1); (b) larger, more rounded cells (type 2); and (c) very large round cells (type 3).

Type 1 cells were abundantly present in all layers and in all investigated parts of the tract (Figs. 9.1-9.4). They stained positively for the enzyme nonspecific esterase (NSE) but lacked the enzyme acid phosphatase (AcP). The majority of the cells expressed the antigens Ia and Mac-1. These characteristics fit well with those desribed for dendrocytes (Austyn, 1987), the in vivo representatives of the classical dendritic cells described by Steinman and Cohn (1973). A minority of type 1 cells additionally expressed the antigens F4/80, ER-HR3 and FcRII. Cells of these subpopulations phenotypically and morphologically share many characteristics with Langerhans cells, the dendrocytes in the epidermis of the skin. Langerhans cells are well-known to express antigens F4/80 (Hume et al., 1984), FcRII (Austyn, 1987) and ER-HR3 (de Jong et al., 1990a), and to exhibit NSE activity (Austyn, 1987). Thus, type 1 cells resemble Langerhans cells in all their characteristics.

Type 2 cells were absent from the oesophagus and the stomach. They were located mainly in the lamina propria (Fig. 9.2c,d) of the duodenum, jejunum, ileum and the colon. Their number increased from the dudoenum towards the ileum and they were less frequently observed in the colon. The cells contained large nuclei with clearly visible nucleoli and exhibited NSE and strong AcP activity. In addition to the T-200 antigen the cells expressed the antigens Mac-1, Ia, F4/80, FcRII and ER-HR3. The morphology and strong AcP-positivity of the cells suggest that they represent macrophages. Also their localization in the upper parts of the villi correlates well with that described for macrophages (Le Fevre et al., 1979).

The large type 3 cells were exclusively observed in the submucosa of the caecum (Fig. 9.3d). The cells were irregular in shape and their plasma membrane formed many extensions. Furthermore, they possessed a large nucleus with prominent nucleoli. They lacked the Ia antigen but expressed the antigens T-200, Mac-1, Mac-3, F4/80, FcRII and ER-HR3, and exhibited a weak NSE and AcP activity. Since the caecum contains a lot of immunoreactive tissue these cells may represent immature or activated macrophages. The lack of MHC class II antigens and the low enzyme activity in the cells favors the former hypothesis.

Interestingly, we observed MLC in the muscularis externa of all investigated parts of the tract. The presence of such cells in the muscularis externa of the small intestine has already been described by Mikkelsen et al.(1985). Like these authors, we observed MLC in the subserosal layer, between the circular and longitudinal layers and within the circular muscle layer. Recently, it has been demonstrated that MLC in the muscularis externa express the antigens F4/80, Mac-1 and Ia. Furthermore, they do not exhibit lysozyme activity. This enzyme appears to be a reliable marker for macrophages (Way et al. 1980). Based on these findings it has been speculated that these MLC form a specialized class of cells comparable with Langerhans cells (Mikkelsen et al., 1988). In our study we demonstrated that all cells in the muscularis externa expressed the T-200 and Ia antigens (Figs. 9.4a and b). However, other antigens were expressed less frequently and their expression depended on which part of the gastro-intestinal was investigated. Moreover, all MLC in the muscularis externa exhibited a strong NSE activity but lacked AcP activity. Within our classification these MLC must be classified as type 1 cells and our data support the observations of Mikkelsen et al. (1988) that these cells have many chararteristics in common with Langerhans cells.

Some of the macrophage antigens were not restricted to MLC. For example, the markers Mac-2, Mac-3 and Ia were also expressed by epithelial cells present in the various parts of the tract. Comparable results have been obtained by other investigators (Flotte et al., 1983; Barclay and Mason, 1982; Mayrhofer et al., 1983). Striking

was the observation that the Mac-2 and Mac-3 antigens were only expressed by the lowest layer of the stratified squamous epithelium of the oesophagus and the fundus (Fig. 9.1a). Furthermore, the Mac-3 antigen was often present on structures in the connective tissue of the lamina propria and submucosa. The Thy-1 antigen was also present on fibers of the connective tissue. Especially the connective tissue of the oesophagus and the stomach were strongly positive for this antigen, which hampered a good determination of the cellular distribution in these regions. In man, it has been well established that Thy-1 is present in the fibro-muscular stroma of distinct organs (Rettig et al., 1988).

From our study it is obvious that the MLC in the gastro-intestinal tract are heterogeneous. Besides the three main cell types described a few cells do not fit within any of these descriptions. For example, sparse spindle-shaped cells were observed that in addition to the characteristics described for cell type 1 also exhibited acid phosphatase activity. These populations were very small and double staining techniques were required to further characterize these cells. Our results demonstrate that the distinct MLC populations are not randomly distributed among the various parts of the digestive tract. These results are in good agreement with data obtained in man (Selby et al., 1983) and rat (Sminia and Jeurissen, 1987).

The function of the MLC populations in the gastro-intestinal tract remains speculative. Isolated intestinal MLC manifest most functions of other mononuclear phagocytes. They are able to phagocytize (Golder and Doe, 1983; Mikkelsen et al., 1985) and secrete many immunoregulatory products (LeFevre et al., 1979). Moreover, most MLC constitutively express the Ia antigen suggesting that they are involved in the immune response (Unanue, 1984). Interestingly, it has been observed that there is a close similarity in the distribution of F4/80-positive macrophages and the bradykinin receptors in the lamina propria of the small intestine (Hume et al., 1984a). This may be indicative of a role of macrophages in the response to this mediator. Furthermore, the disposal of indigestible material by migration of loaded macrophages into the epithelium and their release in the lumen may provide a potential excretion mechanism (LeFevre et al., 1979).

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10. DISTRIBUTION OF MACROPHAGE MARKERS IN THE MURINE KIDNEY

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Submitted for publication

SUMMARY

In the present study we investigated the distribution of macrophage antigens in the murine kidney. The majority of the investigated markers was expressed by macrophage-like cells present in the interstitium of the renal cortex and medulla. Roughly, two populations of macrophage-like cells could be recognized. The first population consisted of small elongated cells that possessed the antigens T-200, Ia, F4/80, BM8, and FcRII. They lacked acid phosphatase and nonspecific esterase activity. The cells were present in the cortex and the outer medulla but were especially abundant in the latter region. The second population consisted of large cells that possessed the T-200 and ER-HR3 antigen. Additionally, they exhibited a strong nonspecific esterase and weak acid phosphatase activity. These cells were restricted mainly to the outer medulla although they occasionally occurred the inner medulla. In addition to these two populations, a small population of 'circulating' monocytes was present in and near the blood vessels of the cortical and medullary interstitium, and within the glomeruli.
Expression of macrophage markers appeared to be not always restricted to macrophage-like cells. For example, the antigens Mac-2, Mac-3 and MOMA-2 were present on the epithelial cells of the cortical and medullary tubuli, while Thy-1 occurred on connective tissue fibers. Cells expressing the dendritic cell antigens NLDC-145 or MIDC-8 were not observed in the kidney.

INTRODUCTION

Mononuclear phagocytes of the kidney are thought to play an important role in renal pathology. They are involved in diseases like glomerulonephritis, tubulo-interstitial nephropathies and allograft rejection (Harry et al., 1982; Laohapand et al., 1983; Appel and Kunis, 1983). It is still a matter of debate whether the macrophages involved in these processes are derived from 'passenger' monocytes or reside in the kidney for a longer period of time. Information concerning the resident and transient macrophage population(s) of the kidney is rather incomplete and controversial.

Various investigators used the distribution of MHC class II antigens as a measure for the distribution of interstitial monocytes/macrophages and dendritic cells (Hart and Fabre, 1981; Steiniger et al., 1984). More recent studies discuss the immunohistochemical evidence for the presence of such cells. It has been suggested that their detection was based on the staining of artificial structures, like for example compressed or obliquely sectioned capillaries (Alpers and Beckstead, 1985). However, in

a quantitative study with the pan-macrophage marker F4/80 (Austyn and Gordon, 1981) it has been demonstrated that the murine kidneys contain a considerable part of the body's total macrophage population (Lee et al., 1985). F4/80-positive cells are shown to be present throughout the interstitium of the renal cortex and medulla and were particularly abundant amongst the medullary tubules. Within the cortex they have been found in close contact with the distal and proximal tubules and Bowman's capsule (Hume and Gordon, 1983). Moreover, it has been suggested that F4/80-positive cells may be identical to the type 1 renomedullary interstitial cells (Hume and Gordon, 1983). If so, macrophages may perform important functions in the homeostasis and physiology of the kidney (Bohman et al., 1988; Pitcock, 1988). Better knowledge of the localization and phenotype of the renal macrophage population(s) may give more insight into their distinct functions.

Recently, we developed a new fixation method for frozen tissue sections. With this method we obtained a good preservation of murine tissues without the loss of antigenicity when analyzed in an immunohistochemical study employing a panel of monoclonal antibodies directed against monocytes/macrophages (De Jong et al., 1990c). We utilized this procedure to characterize and to investigate the distribution of renal macrophage subpopulations in more detail.

MATERIALS AND METHODS

Tissue preparation. Renal tissue was obtained from male (CBA/Rij x C57BL/Rij)F1 mice. Mice were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands and were kept under conventional conditions. Hemisected kidneys were embedded in Tissue-tek II (Miles Laboratories, Inc., Naperville, MO., USA) on a specimen stub, and frozen on solid carbon dioxide. Frozen sections with a thickness of 5 μm were cut on a Leitz cryostat and collected on precoated microscopic slides. Precoating was performed with a solution of 0.1% gelatin and 0.01% chromium potassium sulphate. The slides were stored at -20°C or air dried and used immediately.

Immunohistochemical staining. Sections were fixed with a hexazotized pararosanilin solution for 2 min (De Jong et al., 1990c). In short, 0.4 ml of a 4% (w/v) pararosanilin in 2M HCl solution was added to 0.4 ml of a 4% (w/v) $NaNO_2$ in destilled water solution. After fixation, slides were rinsed with phosphate-buffered saline, pH 7.8 (PBS) supplemented with 0.02% (w/v) gelatin and 0.05% (v/v) Tween-20 (PBT). Immunostaining was performed in moist chambers to prevent section drying. Sections were

Table 10.1
Specificities of the monoclonal antibodies (Mab) used for antigen detection

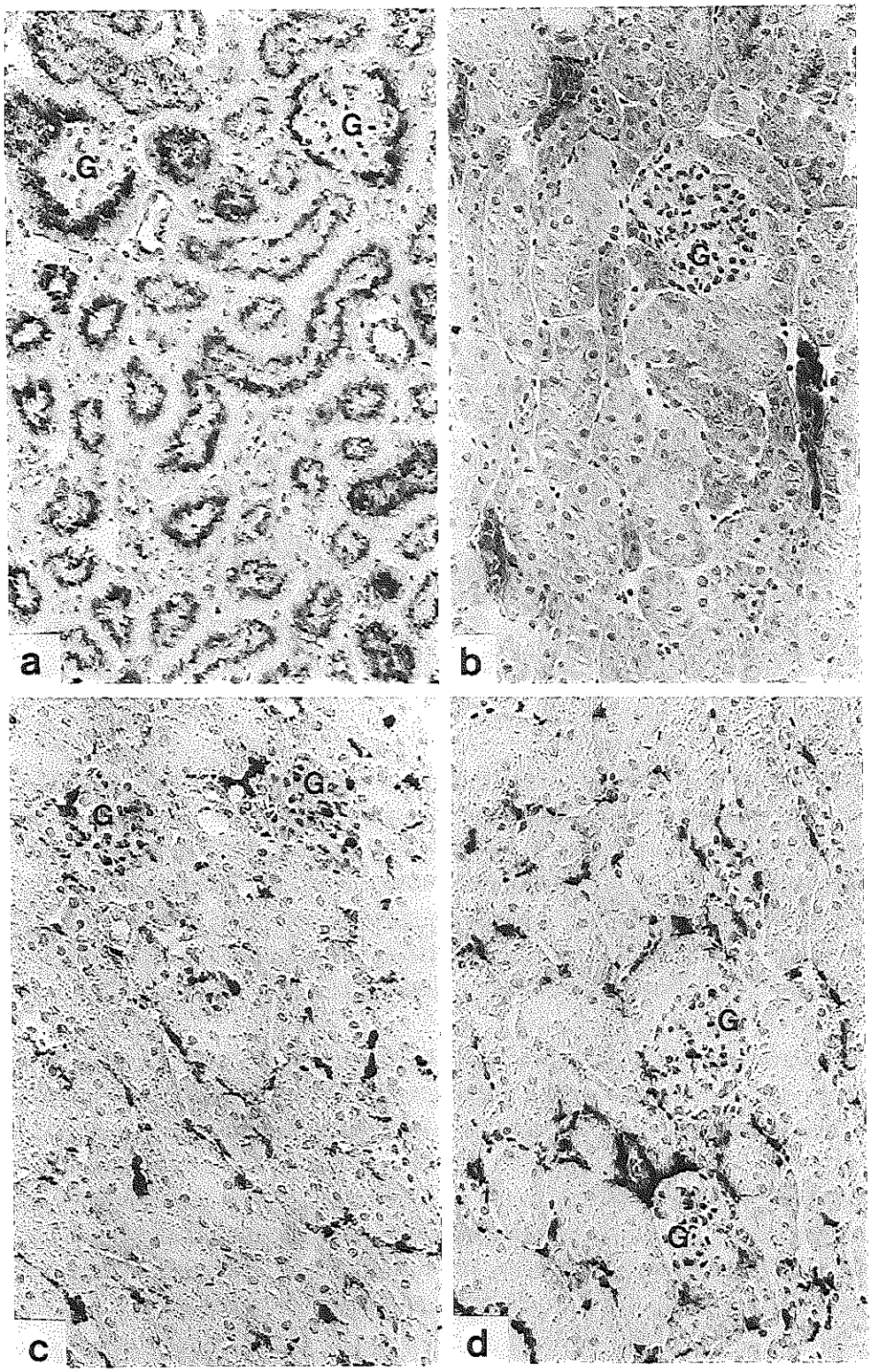
Mab	Antigen	Specificity	References
30-G12	T-200	all leukocytes	Ledbetter et al., 1979
M5/114	H-2,I-A,I-E	MHC class II	Bhattacharya et al., 1981
F4/80	F4/80 Ag	Mϕ	Hirsch et al., 1981
M1/70	Mac-1, CR3	non-fixed Mϕ, granulocytes, NK cells	Springer et al., 1979
M3/38	Mac-2	Mϕ,dendritic cells epithelium	Ho et al., 1982 Flotte et al., 1983
M3/84	Mac-3	Mϕ, dendritic cells, epi- and endo-thelium	Flotte et al., 1983 Ho et al., 1983
2.4G2	FcRII	Mϕ, PMN, B cells	Unkeless, 1979
ER-HR3	ER-HR3 Ag	Mϕ	De Jong et al., 1987
59-AD2.2	Thy-1	T cells, stem cells, myelocytic progenitors	Basch et al., 1982 Ledbetter et al., 1979
BM8	BM8 Ag	Mϕ	Malorny et al., 1986
MOMA-2	MOMA-2 Ag	Mϕ	Kraal et al., 1987
MIDC-8	MIDC-8 Ag	dendrocytes	Breel et al., 1987
NLDC-145	NLDC-45 Ag	dendrocytes	Kraal et al., 1986 Breel et al., 1987

MHC, major histcompatibility complex; Mϕ, macrophage; NK, natural killer; PMN, polymorphonuclear granulocytes

successively overlaid with 70 μl of the first step monoclonal antibody, rinsed with PBT, and overlaid with a horseradish peroxidase-conjugated rabbit-anti-rat second antibody (Dakopatts, Copenhagen, Denmark). Antibody incubations were performed for 1 hr at room temperature. Hereafter, sections were rinsed with PBT and the peroxidase activity was visualized with 0.1% diaminobenzidine (Sigma, St. Louis, MO, USA) solution in 0.05% (v/v) H_2O_2. Sections were counterstained with hematoxylin, dehydrated in alcohol and embedded in Entellan mouting medium (Merck, Darmstadt, FRG).

Monoclonal antibodies. Hybridoma culture supernatant or optimally diluted purified antibody was applied as source of first-step antibody. The monoclonal antibodies together with their specificity and distribution are depicted in Table 10.1.

Enzyme histochemistry. Acid phosphatase and nonspecific esterase activities were demonstrated as described by Burnstone (1962) with Naphthol-AS-BI phosphate (Sigma, USA) and α-naphthyl acetate (Sigma, USA) as substrates, respectively. Hexazotized pararosanilin was used as a diazonium salt in both procedures.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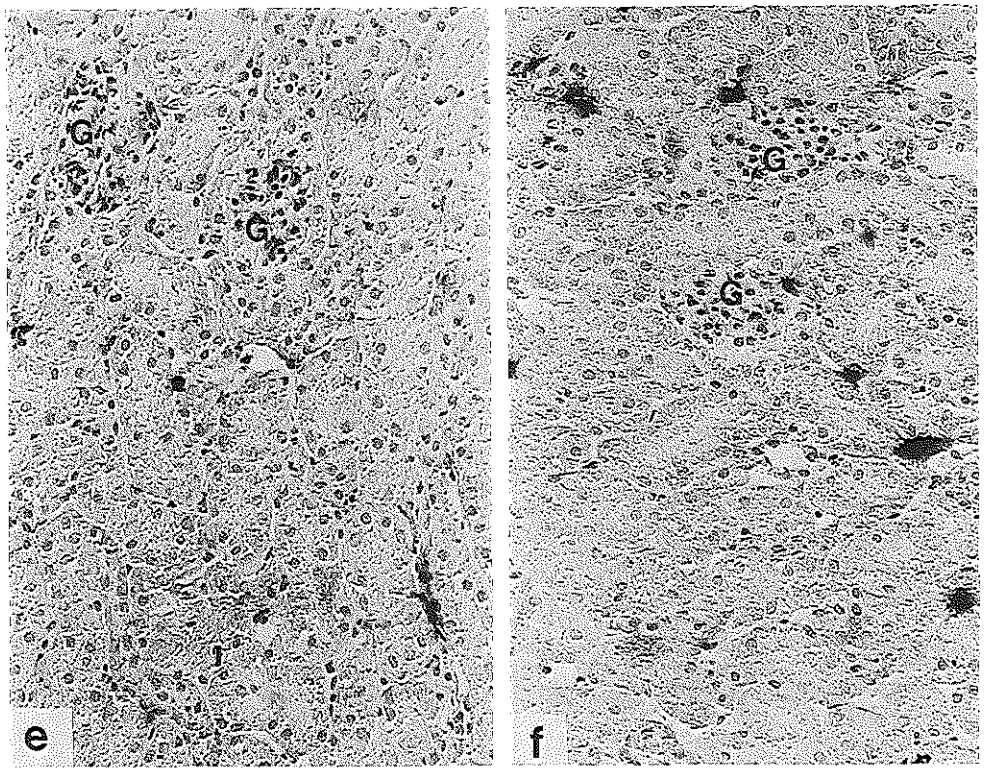


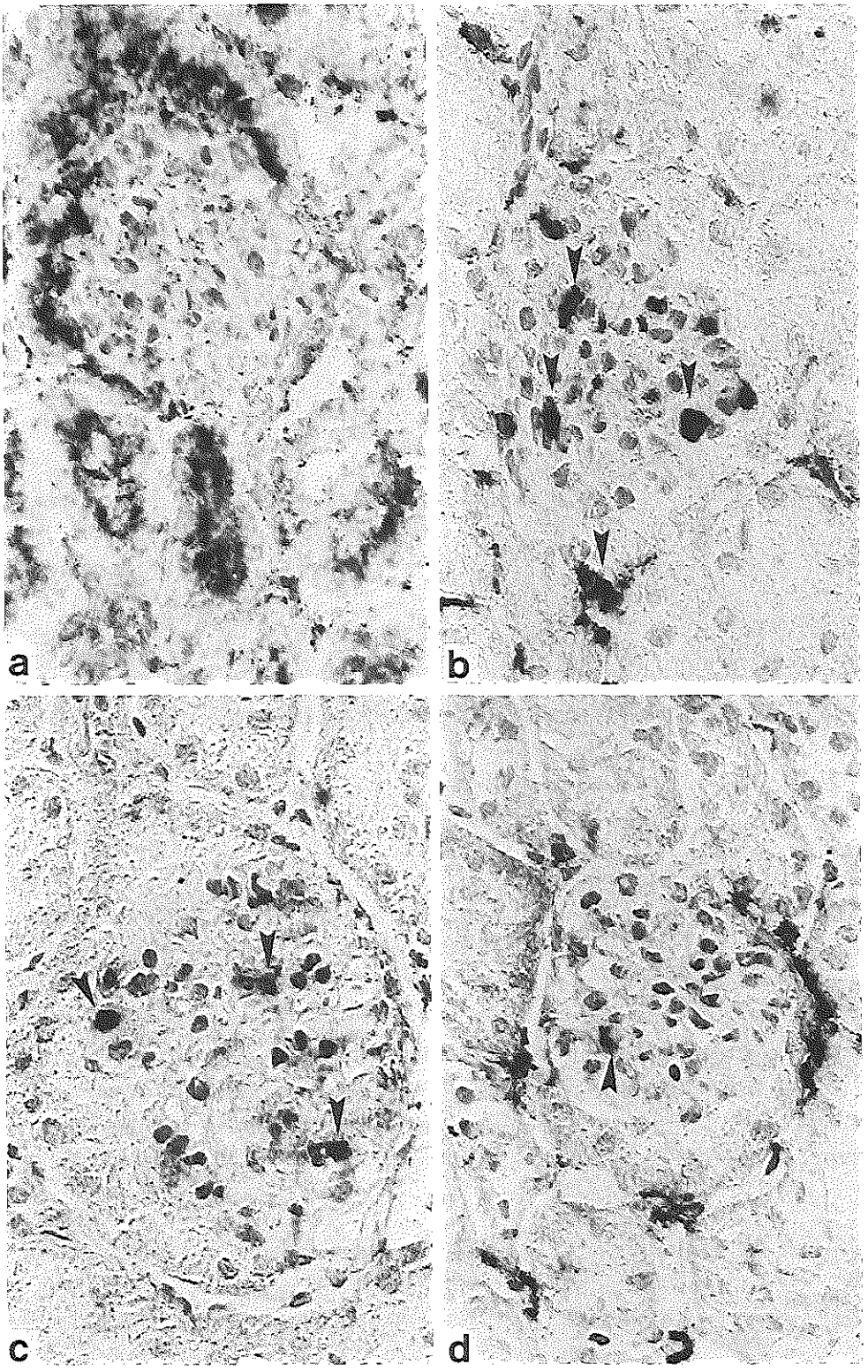
RESULTS

For reasons of clarity, in this results section both the cortex and medulla will be subdivided in a tubulous and an interstitial region. The glomeruli are considered separately as constituents of the cortex.

Distribution of macrophage antigens in the renal cortex

Few of the macrophage antigens were expressed by the tubulous epithelium. MOMA-2 (Fig. 10.1a) and Mac-3 were present on all tubules whereas Mac-2 expression was restricted to some of the collecting tubules (Fig. 10.1b). Intracellularly,

Figure 10.1. Distribution of monocyte/macrophage antigens in the renal cortex. (a) MOMA-2; (b) Mac-2; (c) T-200; (d) Ia; (e) Mac-1; and (f) Thy-1. Magnification x250
G, glomeruli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the majority of the cells exhibited stronger MOMA-2 and Mac-3 reactivity at their apical pole. In contrast, the intracellular Mac-2 reactivity was stronger at the distal part of the cell.

Within the interstitium of the renal cortex many macrophage-like cells were present. The majority of these cells was small and elongated. They expressed the T-200 (Fig. 10.1c), Ia (Fig. 10.1d), Mac-3, F4/80 and BM8 antigens, and also possessed the FcRII. However, they lacked nonspecific esterase and acid phosphatase activity. The cells were observed occasionally in close association with the capsule of Bowman forming a rim around it (Figs. 10.1d, 10.2d). A minority of the T-200-positive cells in the cortex expressed the Mac-1 (Fig. 10.1e) or Thy-1 (Fig. 10.1f) antigen. Based on morphological criteria most of these cells were judged as monocytes/granulocytes or T cells, respectively. Thy-1-positive cells could be observed in the connective tissue around the blood vessels.

Large parts of the glomeruli stained positively for Mac-3 (Fig. 10.2a) and MOMA-2. However, only few cells present in the glomeruli were reactive with other anti-macrophage mAb. The latter cells all expressed the T-200 antigen. Additionally, some cells expressed Thy-1. Others expressed the Mac-1 and/or Ia antigens. Microscopic analysis revealed that these cells were circulating lymphocytes, monocytes and granulocytes.

ER-HR3, MIDC8 and NLDC-145-positive cells were not observed in the renal cortex.

Distribution of macrophage antigens in the renal medulla

The macrophage marker expression in the outer medulla was nearly identical to that of the cortex. All tubules were reactive with mAb Mac-3 and MOMA-2 and only part of the collecting tubules stained positively for Mac-2.

Macrophage-like cells in the medullary interstitium were abundant and their number exceeded that of the cortical interstitial macrophage-like cells. Phenotypically, two populations could be recognized. The first population had identical morphological and phenotypical characteristics as their cortical counterparts. They stained positively for mAb 30-G12, M5/114 (Fig. 10.3a), F4/80 (Fig. 10.3b), 2.4G2 (anti-FcRII) and BM8, and lacked nonspecific esterase and acid phosphatase activity. The second population consisted T-200, ER-HR3-positive cells. These cells were larger than the cells of the aforementioned population and their distribution was more clustered (Fig. 10.3c). The cells showed a moderate nonspecific esterase and weak acid phosphatase activity.

Figure 10.2. Distribution of monocyte/macrophage antigens associated with the glomeruli. (a) Mac-3; (b) T-200; (c) Mac-1; and (d) Ia. Magnification x650

Three other T-200-positive cell types were scattered throughout the outer medulla. They occurred less frequently than the other populations and also expressed the Mac-1 or Thy-1 (Fig. 10.3d) antigen. They were judged as monocytes/granulocytes and T cells, respectively.

The expression of macrophage markers gradually decreased towards the inner medulla. Few tubuli stained positively for Mac-3 and in the interstitium sparse T-200, ER-HR3-positive cells were observed.

MIDC8 or NLDC-145-positive cells were not observed in the medulla.

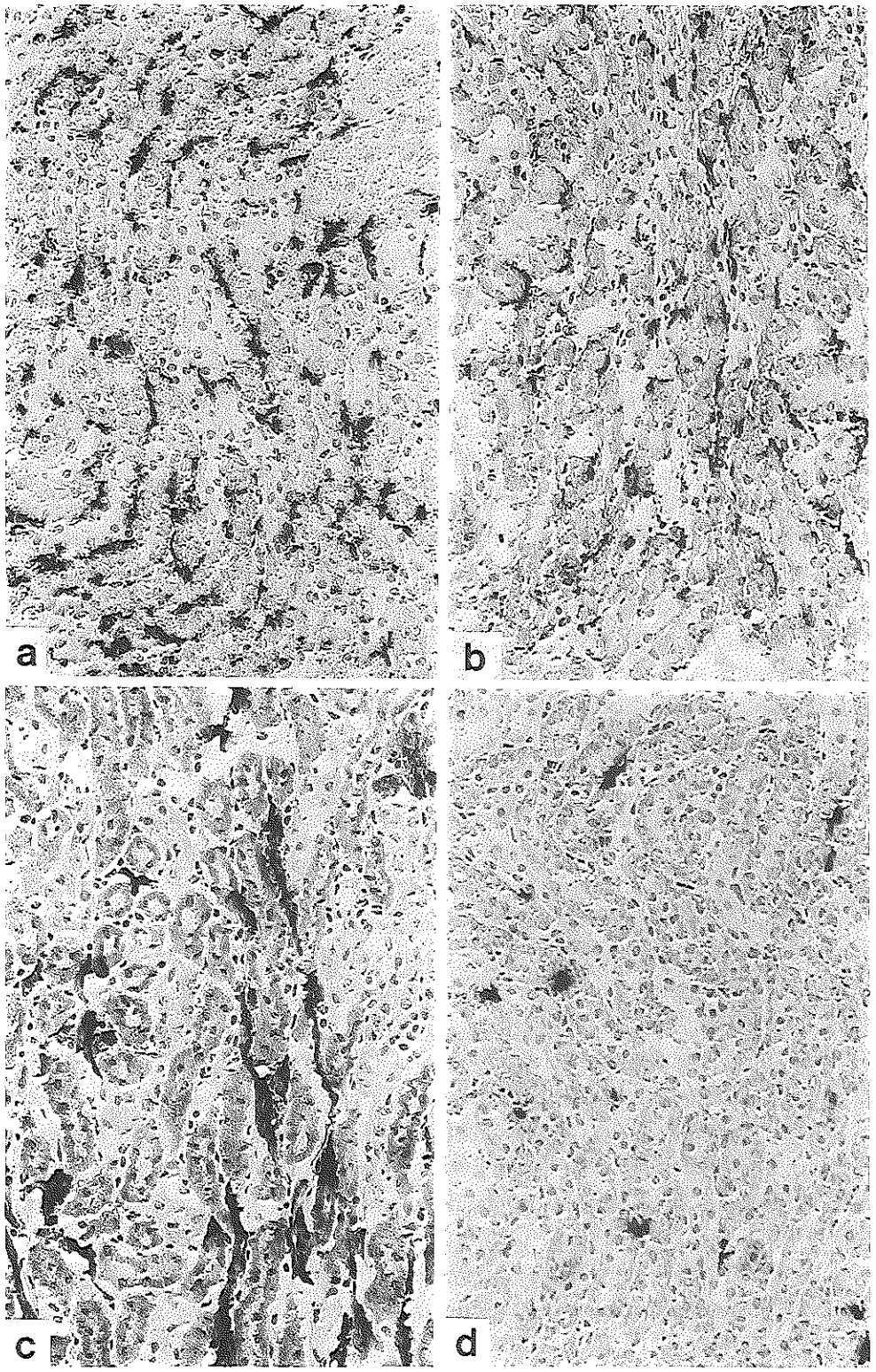
DISCUSSION

Knowledge on the presence of macrophages within the kidney is of great interest because these cells are thought to be involved in distinct nephropathies (Harry et al., 1982, 1984; Laohapand et al., 1983, Appel and Kunis, 1983). However, in the last few years the presence of resident macrophages in the kidney has been a matter of considerable debate. It has been demonstrated that Ia-positive cells are abundant in rat (Hart and Fabre, 1981) and murine (Benson et al., 1985) kidneys. Additionally, part of these cells express the pan-macrophage marker F4/80 (Hume and Gordon, 1983). However, the presence of a population of resident macrophages has been denied by others (Alpers and Beckstead, 1985). The present study was performed (a) to investigate whether or not resident macrophages are present in the kidney and (b) to obtain information about the localization and phenotype of these cells.

Our study clearly demonstrates that a population of resident macrophages is present in all compartments of the murine kidney. They particularly are abundant in the interstitium of the outer medulla. This distribution correlates well with the described distribution of MHC class II antigens in rats (Hart and Fabre, 1981; Steiniger et al., 1984). Here, we demonstrate that most of these cells additionally express the antigens T-200, F4/80, BM8, Mac-3 and FcRII. These observations, together with morphological characteristics obtained at high power magnification exclude the possibility of artificial staining of vascular structures as suggested by Alpers and Beckstead (1985).

Roughly, the resident population of macrophage-like cells in the renal interstitium could be subdivided into two subpopulations. The first and largest subpopulation of

Figure 10.3. Distribution of monocyte/macrophage antigens in the renal outer medulla. (a) Ia; (b) F4/80; (c) ER-HR3; and (d) Thy-1. Magnification x250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macrophage-like cells share many characteristics with the dendritic cell described by Steinman and Cohn (1973). They are small and elongated. Their nuclei possess dense chromatin and they exhibit weak acid phosphatase and lack nonspecific esterase activity in their cytoplasm. Furthermore, they strongly express MHC class II antigens and possess the common leukocyte antigen. In contrast, the occurrence of the antigens F4/80, BM8 and the FcRII are typical characteristics of macrophages (Austyn and Gordon, 1981; Malorny et al., 1986; Unkeless, 1979). Interestingly, it has been demonstrated that the dendritic cells of the skin, the Langerhans cells, possess F4/80 (Hume et al., 1984), BM8 (Malorny et al., 1986) and FcRII (Austyn, 1987). Thus, most macrophage-like cells of the kidney greatly overlap in their marker expression with the Langerhans cell. However, renal macrophages lack the dendritic cell markers NLDC-45 and MIDC-8 that are present on Langerhans cells (Kraal et al., 1986; Breel et al., 1987).

The second and smallest population of macrophage-like cells was restricted mainly to the outer medulla of the kidney. Cells of this population were larger than the cells of the formerly described population. Sometimes the cells were well spread and contained a large nucleus with evident nucleoli. They expressed T-200 and the ER-HR3 antigen but lacked the Ia antigen. Additionally, they exhibited a strong nonspecific esterase and a weak acid phosphatase activity. The differences in size and Ia expression suggest that the descerned populations resemble the type 1 and 2 interstitial cells, respectively, as described by Bohman (Bohman, 1983; Bohman et al., 1988).

In addition to these populations a few other populations of T-200-positive cells were observed. As judged by high power magnification they resemble Mac-1-positive granulocytes and monocytes or Thy-1-positive T cells. Occasionally, some of the monocytes express class II antigens (M5/114) and FcRII receptors (2.4G2). All three populations of cells were also observed in the glomeruli. They mostly occurred in the vasculature of the glomerulus and are therefore considered as "passenger" cells. However, in the rat a small resident population of Ia-positive cells has been observed (Schreiner et al., 1981; Gurner et al., 1987) and it cannot be excluded that a similar population is present in the murine glomeruli.

Not all markers were restricted to macrophage-like cells. Mac-3 and MOMA-2 were present on the epithelium of all tubuli whereas Mac-2 expression was restricted to some collecting tubules. The distribution of Mac-2 and Mac-3 is in good agreement with an earlier study (Flotte et al., 1983). Remarkable was the presence of Thy-1 on connective tissue fibers in the medullary rays of the cortex and around the larger blood vessels. Marker expression was restricted to specific sites, which indicates that Thy-

1 is not a "common" connective tissue marker. The staining of stromal regions with mAb against Thy-1 was also demonstrated in man (Rettig et al., 1988).

Little is known about the functions of renal macrophages. It is thought that they are involved as antigen-processing and antigen-presenting cells in immunologically mediated tubulo-interstitial nephropathies and allograft rejection (Gelfand et al., 1979; Harry et al., 1984). Moreover, if macrophages represent the renomedullary type 2 interstitial cells as proposed by Hume and Gordon (1983), they may perform an important anti-hypertensive function (Pitcock, 1988). Of special interest are the ER-HR3-positive cells in the medullary interstitium. It has been demonstrated that ER-HR3-positive cells likely are involved in the erythropoiesis (De Jong et al., 1987; De Jong et al., 1990a). In the bone-marrow, spleen and fetal liver they are surrounded by a rim of erythroblasts. Furthermore, during phenylhydrazine-induced extramedullary erythropoiesis ER-HR3-positive cells appear in the adult liver. The observations that splenic and hepatic macrophages are able to produce erythropoietin under hypoxic conditions (Rich, 1987) and that the erythropoietin-producing cells are located in the renal medullary interstitium (Schuster et al., 1987) make the ER-HR3-positive cells important candidates for this hemopoietic function.

ACKNOWLEDGMENTS

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11. A MONOCLONAL ANTIBODY (ER-HR3) AGAINST MURINE MACROPHAGES: I. ONTOGENY, DISTRIBUTION AND ENZYME HISTOCHEMICAL CHARACTERIZATION OF ER-HR3-POSITIVE CELLS

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Submitted for publication.

SUMMARY

In this study we introduce a new macrophage-specific antibody, ER-HR3. ER-HR3-positive cells were characterized and their distribution in the fetal and adult mouse was investigated. It is demonstrated that ER-HR3-positive cells have the electronmicroscopic and enzyme histochemical characteristics of macrophages. Additionally, they are able to phagocytize. It is concluded that the ER-HR3 antigen is expressed by a majority of the blood monocytes and is present on a subpopulation of resident macrophages in multiple organs. ER-HR3-positive cells were abundant in the bone marrow, the splenic red pulp, the mesenteric lymphoid paracortex and the interfollicular areas of the Peyer's patch. Relatively few ER-HR3-positive cells were observed in the thymic cortex and the connective tissues of the gastro-intestinal tract, the dermis and the renal medulla. Moreover, epidermal Langerhans cells expressed the antigen. No cross-reactivity with other cell types was observed.

INTRODUCTION

It is well accepted that tissue macrophages originate from the pluripotent hemopoietic stem cells via the monocytic lineage. Promonocytes in the bone marrow differentiate to blood monocytes which migrate into the various organs and tissues (van Furth, 1980). Within these tissues, macrophages perform many functions, including phagocytosis and pinocytosis (Steinman et al., 1983), secretion of regulatory products (Takemura and Werb, 1984), antigen presentation to lymphocytes (Unanue, 1984), and the killing of intra and extracellular micro-organisms (Babior, 1983). To obtain more insight in this functional diversity, it is important to distinguish the different macrophage subsets. Immunohistochemistry, using monoclonal antibodies (mAb) to identify cell-specific surface molecules, is ideally suited for this purpose. Various mAb against antigens on cells of the monocyte/macrophage series are available. They vary in their specificity and sometimes recognize other cells than macrophages, e.g. neutrophils, lymphocytes and endothelial cells (Flotte et al., 1983). Some mAb, e.g. the pan-macrophage marker F4/80 (Austyn and Gordon, 1981) are claimed to react with all cells of the mononuclear phagocyte system including the more immature cells (Hume et al., 1985). Others, like BM8 (Malorny et al., 1986) or A1-3 (Ewans et al., 1986; Hancock et al., 1986) exclusively recognize certain subpopulations such as resident or activated macrophages. Knowledge concerning the precise tissue distribution of a marker may reveal information about its possible function. For example, the ER-TR9 antigen is exclusively present on macrophages in the marginal

zone of the splenic follicles and the medulla of lymph nodes. In close association with these cells a subpopulation of specific B lymphocytes occurs. This distribution is suggestive of an interaction between these two cell types and an involvement of the ER-TR9 antigen in the immune response (van Vliet et al., 1985).

In this study we introduce a macrophage-specific mAb, ER-HR3, formerly described as a mAb against reticular bone marrow cells (Piersma et al., 1985). ER-HR3-positive cells are characterized and their distribution in fetal and adult mice is investigated.

MATERIALS AND METHODS

Production of monoclonal antibody. The ER-HR3 antibody was produced in our laboratory as described in detail elsewhere (Piersma et al., 1985). In short, adherent bone marrow cells cultured for 4 weeks were used to immunize Louvain rats intraperitoneally at days 0, 30, 60, 61 and 62. At day 64 spleen cells were fused to P3-X63-Ag8.563 myeloma cells and hybridoma clones were grown in α-medium (α-modification of Dulbecco's minimal essential medium) containing HAT. Selected clones were cultured in α-medium supplemented with 10% (v/v) fetal calf serum (FCS).

Production and purification of ascites

Ascites was produced by a combination of the methods described by Weissman et al. (1985) and Gillette (1987). Male or female BALB/c mice were intraperitoneally injected with 0.2 ml incomplete Freund's adjuvant (Difco Laboratories, Detroit, USA) and intramuscularly with 3 mg hydrocortisone acetate (Hydro-Adreson^R, Organon, Oss, The Netherlands) four and three days before hybridoma cell injection, respectively. Two days later they were sublethally irradiated with a dose of 500 rad total body gamma irradiation (^{137}Cs gamma source, Atomic Energy of Canada Ltd., Ottawa, Canada). The next day mice were intraperitoneally injected with 7×10^6 hybridoma cells. Within two weeks all injected mice produced ascites. The ascites was purified according to Neoh et al. (1986). Ascites of mice was harvested and centrifuged (10 min, 500 x g, room temperature). For delipidation, the supernate was mixed in a 1:1 ratio with 0.1 M phosphate buffer, pH 8.0, (PB) and 150 mg silica powder per 10 ml diluted ascites was added. The suspension was incubated at room temperature for one hour under shaking. Subsequently, the fluid was clearified by centrifugation (20 min, 2000 x g, room temperature).

Purification of the monoclonal antibody

The monoclonal antibody was purified from the ascites fluid or culture medium with protein A affinity chromatography followed by ion exchange chromatography on a mono-Q column within a FPLC system (Pharmacia, Uppsala, Sweden). The Sepharose protein A column (Pharmacia) was pre-equilibrated with a 0.1 M PB, and successively layered with purified ascites and 0.1 M PB. Bound immunoglobulins were eluted with a 0.1 M glycine/HCl buffer, pH 2.5. To avoid protein damage the eluate was immediately neutralized with a 0.1 M Tris/HCl buffer, pH 8.5. The eluate was dialysed for 48 hr against a 14 mM PB and concentrated with an filtration unit (Amicon, Danvers, MA, USA). One ml aliquots of this solution were sampled on a mono Q anion exchanger and eluted with a 14 mM PB. Bound protein was removed by a PB gradient of increasing ionic strength ranging from 0.014 M to 0.17 M. Protein peaks were collected and checked for purity by SDS-PAGE (Laemmli, 1970).

Mice

Adult male (CBA/Rij), female (C57BL/Rij) and (CBA/Rij x C57BL/Rij) F1 (BCBA) mice of both sexes were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands, and kept under conventional conditions. To obtain BCBA embryos and neonatal mice, C57BL/Rij females were mated with CBA/Rij males. Four females and one male were kept together for four hours, whereafter the females were removed and scored for the appearance of a vaginal plug. Positive-scored females were separated from the male. The day of removal was estimated as day 0 of gestation. We used animals of the following ages: fetuses of 8 days of gestation upto birth, neonatal mice of 0, 1, 3, 5, 7 and 11 days after birth, juvenile mice of 4 weeks and adult mice older than 10 weeks.

Tissue preparation

Mice were anaesthetized with 0.5 ml 2.5% (w/v) Avertin intraperitoneally and the spleen, thymus, lymph nodes, gastro-intestinal tract, testis, lung, pancreas and liver were removed. The tissues were embedded in Tissue tek II (Miles Laboratories, Inc., Napelville, USA) on a specimen stub and frozen on solid carbon dioxide. Fetuses were decapitated and embedded in total. Frozen sections of 5 μm were cut on a cryostat (Leitz, Germany; model 1720) and collected on microscope slides precoated with a solution containing 0.1% gelatin and 0.01% chromium potassium sulphate. The sections were air-dried and fixed for 2 min with a solution of hexazotized pararosanilin prepared as described by de Jong et al. (1990c). In short, one volume 4% (w/v) pararosanilin in 2 M HCl was added to 4% (w/v) $NaNO_2$ in distilled water. Immediately

after mixing, 0.8 ml of this solution was added to 10 ml of distilled water. After fixation the slides were placed in phosphate buffered saline, pH 7.8 (PBS) supplemented with 0.02% (w/v) gelatin and 0.05% (v/v) Tween-20 (PBT).

Staining method

The sections were overlaid with 70 µl of the first stage antibody and incubated for 1 h at room temperature. During incubations the sections were kept in moist chambers to prevent air-drying. After rinsing the sections with PBT they were overlaid with 70 µl of optimally diluted horseradish peroxidase-conjugated rabbit-anti-rat Ig (Dakopatts, Copenhagen, Denmark) and incubated for 1 h at room temperature. Normal mouse serum was added at an end concentration of 2% (v/v) to avoid background staining. Sections were rinsed again with PBT and the peroxidase activity was visualized by incubation with a diaminobenzidine (Sigma, St.Louis, MO, USA) solution (1 mg/ml PBS supplemented with 20 µl 1% (v/v) H_2O_2). The sections were counterstained for 10 min with hematoxylin, rinsed with tap water, dehydrated, and embedded in Entellan mounting medium (Merck, Darmstadt, Germany).

Indirect immunogold labelling for electron microscopy

This method was performed as described by Willemsen et al. (1986). In short, adult male BCBA mice were perfused with 1% acrolein and 0.4% glutaraldehyde (AGA) in 0.1 M PB under Avertin anaesthesia. The spleen was removed and cut in 2 x 2 mm blocks. After post-fixation for 60 min in AGA at 4°C, the blocks were rinsed twice in 0.1 M PB and subsequently soaked for 2 x 5 min in 2% and 10% (w/v) gelatin/0.1 M PB at 37°C. The gelatin-embedded tissue was solidified on ice, and the gelatin was cross-linked by postfixation in cold AGA. The blocks were stored in 1% paraformaldehyde in 0.1 M PB containing 1 M sucrose at 4°C. Ultracryotomy was carried out using an LKB Nova ultramicrotome equipped with the Cryo Nova (LKB, Bromma, Sweden).

Immunostaining was performed as described by Geuze et al. (1981) with the following minor modifications. Prior to incubation with the first antibody, the sections were incubated for 10 min with sodium borohydride (2 mg/ml PB) to reduce residual aldehyde groups of the fixative. Binding of ER-HR3 mAb was visualized by using goat-anti-rat Ig coupled to 10 nm colloid gold (GAR 10, Janssen Pharmaceutics, Beerse, Belgium). Sections were counterstained with uranyl acetate and embedded in 1.5% methylcellulose. Control sections were treated with GAR 10 only. Background labelling appeared to be negligible.

Enzyme cytochemistry

Acid phosphatase and nonspecific esterase activity were demonstrated according

to the method of Burnstone (1962) with Naphthol-AS-BI (Sigma, St. Louis, USA) and α-naphtyl butyrate phosphate (Sigma) as substrates, respectively. In both procedures hexazotized pararosanilin was used as diazonium salt.

Carbon ingestion

To investigate the phagocytic properties of ER-HR3-positive cells, mice were injected intravenously with 0.5 ml of a 1:600 dilution of India ink (Pelikan 17 Negro) in PBS. Half an hour and 1 h after injection mice were sacrificed and their spleens were dissected.

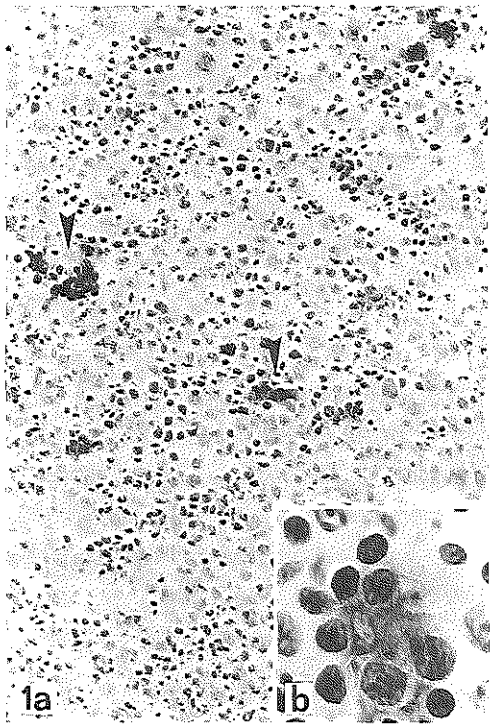
Blood leukocyte isolation and staining

The blood of BCBA mice was collected in heparinized tubes. One ml blood was mixed with 4 ml of cold lysis buffer containing 17 mM Tris/HCl and 0.14 M NH_4Cl. Erythrocytes were lysed by incubation for 10 min at 4°C. After centrifugation (250 x g, 10 min, 4°C) the cells were washed twice with medium. Cells were fixed with 0.05% glutaraldehyde (Polyscience, Warrington, USA) for 5 min and further labelled using the immuno-ß-galactosidase assay as described by Leenen et al. (1987).

RESULTS

Ontogeny of ER-HR3-positive cells

Hemopoietic activity was observed for the first time at day 9 of fetal development in the blood islands of the yolk sac. However, none of these cells expressed the ER-HR3 antigen. The first ER-HR3-positive cells were observed in the fetal liver at day 13 of fetal life and they subsequently appeared in the mesenchymal loose connective tissue (day 15), the synovial cavities (day 16) and the spleen (day 17). In the liver the number of positive cells increased until day 16, whereafter it stabilized. These cells possessed a stellate morphology and branched between surrounding immature hemopoietic cells (Fig. 11.1) being part of so-called hemopoietic islands. Relatively few of these hemopoietic islands contained ER-HR3-positive cells. In the spleen the ER-HR3-positive cells expressed the same morphological characteristics as in the liver and again blood islands containing positive cells were observed (Fig. 11.2). The number of positive cells in the spleen gradually increased until birth. In contrast with the cells described in liver and spleen, ER-HR3-positive cells in the loose connective tissue (Fig. 11.3) and the synovial cavities (Fig. 11.4) showed a more rounded morphology. These cells contained a relatively large nucleus with loose chromatin. During fetal life no positivity was observed in other organs.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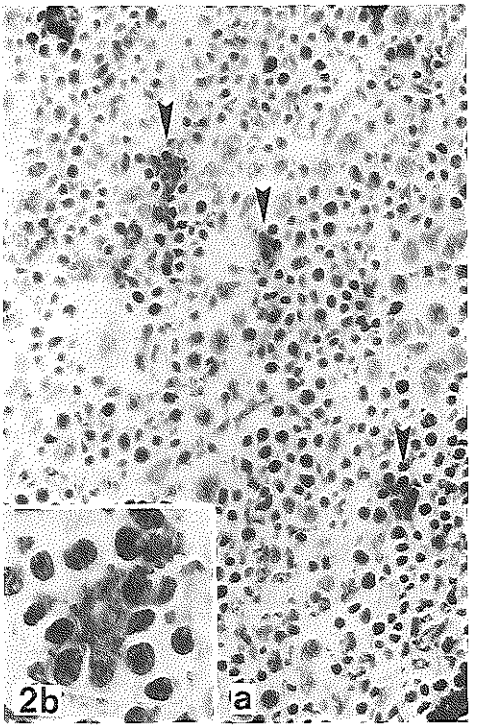
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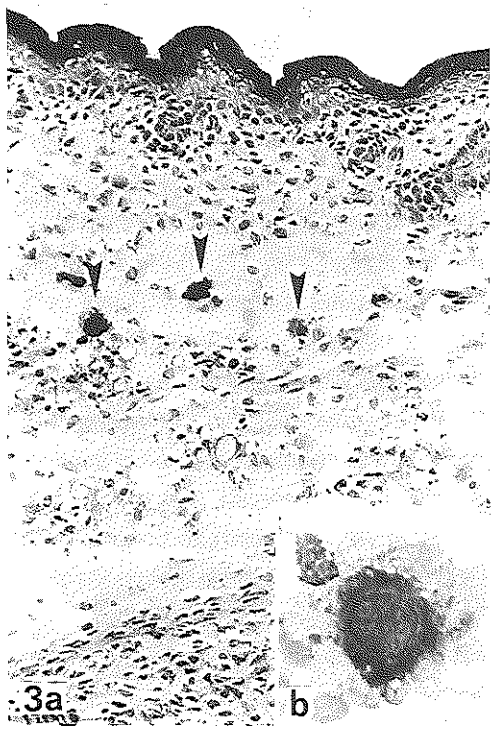
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Distribution of ER-HR3-positive cells in juvenile and adult mice

Around birth, positive cells appeared in the bone marrow, the lymph nodes, the thymus and in the gastro-intestinal tract. In contrast, the number of positive cells in the liver decreased and they disappeared around day 18 together with the hemopoietic activity in this organ. In the spleen the number of positive cells further increased, and with the onset of lymphopoietic activity in this organ, positivity was confined to the red pulp (Fig. 11.5). A gradient of positive cells was established with the highest density of cells beneath the splenic capsule and the lowest in the centre of the spleen. Within the bone marrow positive cells were distributed at random and a specific clustering of positive cells could not be observed. Without exception the ER-HR3-positive cells in the bone marrow were surrounded by hemopoietic progenitor cells, likely of the erythropoietic lineage (Fig. 11.6). The appearance of positive cells in the thymus was restricted to the thymic cortex and no positive cells were observed in the medulla. Their number increased from birth until day 14, whereafter it sharply declined. In the adult thymus only a few positive cells were observed in the thymic cortex (Fig. 11.7). The distribution of the ER-HR3 antigen in lymph nodes was of particular interest. After birth sparse cells were observed and their number slightly increased in the following weeks. The number of positive cells in the different lymph nodes varied. The branchial, inguinal, axillary and para-aortic lymph nodes contained sparse cells in the paracortex. In contrast, the mesenteric lymph node contained many ER-HR3-positive cells (Fig. 11.8). These cells were mainly present in the paracortex, although some large cells were observed in the medulla of the lymph node.

In addition to the cells of the lympho-hemopoietic organs, epidermal Langerhans cells and some cells in the subcutaneous connective tissue possess the ER-HR3 antigen (Fig. 11.9). The latter cells had a very dendritic morphology. Furthermore, ER-HR3-positive cells occurred in the gastro-intestinal tract and the kidney. The number of ER-HR3-positive cells in the gastro-intestinal tract increased from birth to the adult stage and a specific distribution pattern developed. Positivity increased from the

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Figure 11.1 (a) Distribution of ER-HR3-positive cells (arrows) in the liver of a 16-day-old fetus. Magnification x160. (b) Higher magnification (x650) of a hemopoietic island. Hemopoietic cells are clustered around an ER-HR3-positive central macrophage

Figure 11.2. (a) Distribution of ER-HR-3-positive cells (arrows) in the spleen of a 17-day-old fetus. Magnification x250. (b) Hemopoietic cells clustered around an ER-HR-3-positive macrophage. Magnification x650

Figure 11.3. (a) Distribution of ER-HR-3-positive cells (arrows) in the embryonic subcutaneous loose connective tissue of an 18-day-old fetus. Magnification x160. (b) High power magnification (x650) of an ER-HR3-positive cell

Figure 11.4. ER-HR3-positive cells (arrows) in a skeletal joint. Magnification x50

stomach towards the distal part of the terminal ileum and the caecum and decreased again towards the colon. In the proximal part of the tract elongated positive cells were observed beneath the epithelium and larger, more rounded cells were observed in the connective tissue in tops of the villi (Fig. 11.10). The presence of ER-HR3-positive cells in the Peyer's patches was confined to the interfollicular T cell areas (Fig. 11.11). In ontogenetic development, the kidney was the last organ in which ER-HR3-positive cells appeared. Around the tenth day of adult life the first positive cells were observed in the interstitium of the inner medulla of the kidney. Their number increased during the following week and at that time also positive cells appeared the outer medulla. No cells occurred in the cortex of the kidney. Positive cells were lining the collecting tubules and formed long radial strands around the pelvis (Fig. 11.12). In the adult animals no positivity was observed in the liver, lung, pancreas, brain and testis.

Electronmicroscopic characteristics

Morphological characterization of ER-HR3-positive cells in the spleen was obtained using an immunogold electronmicroscopic technique. The dendritic appearance of the positive cells was confirmed in this study. Positive gold-labelled cells possessed a chromatin loose nucleus with prominent nucleoli. They often contained effete cells and extruded nuclei from erythroid cells. In addition, their cytoplasm contained many mitochondria, lysosomes and rod-shaped inclusions (Figs. 11.13 and 11.14). ER-HR3-positive cells were labelled with gold particles in varying density. Some cells were labelled heavily whereas others contained relatively few particles. Noteworthy, gold particles were abundant on cytoplasmic membranous structures and on the plasma membrane (Fig. 11.14a).

Peripheral blood leukocyte cell suspensions

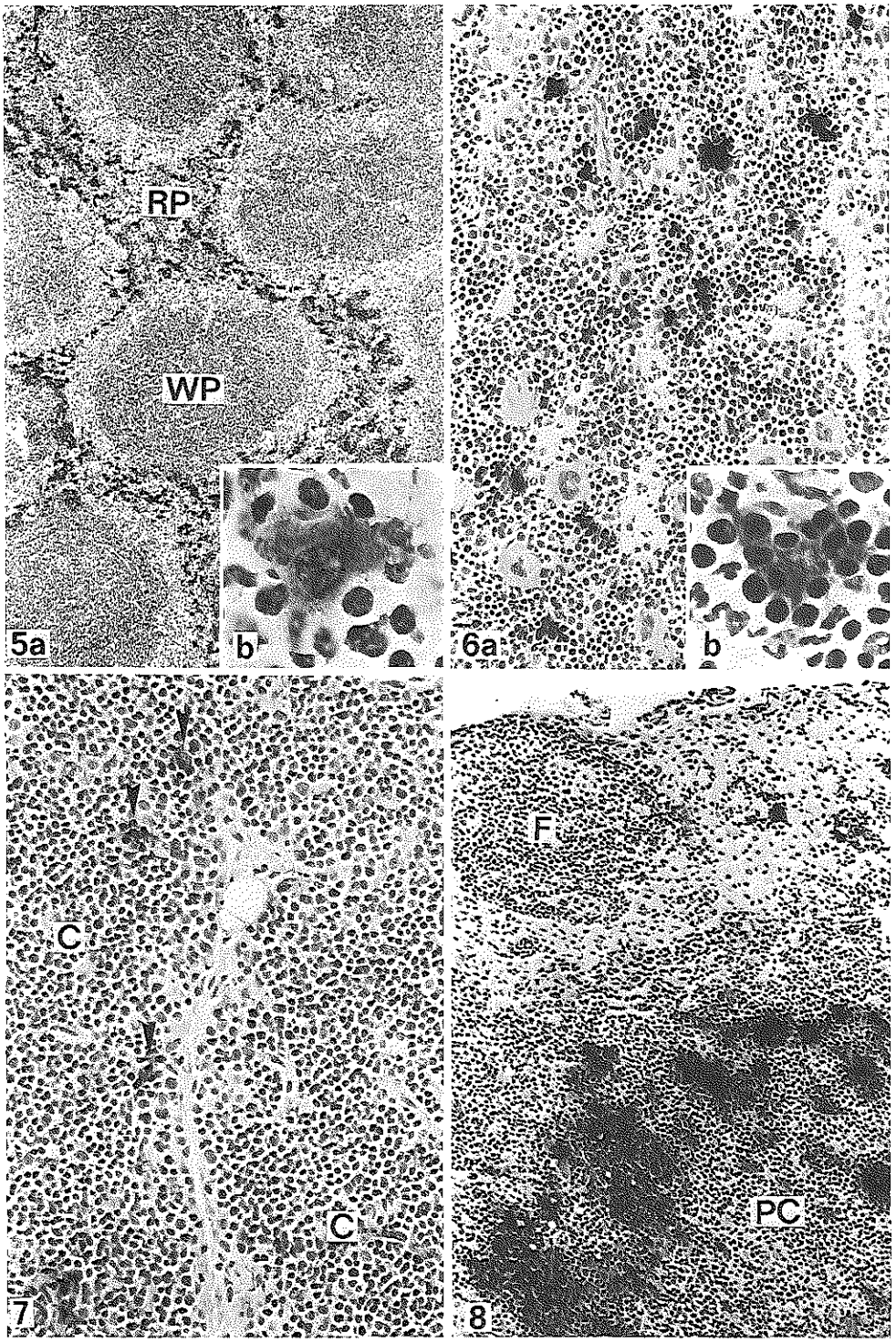
Erythrocytes from peripheral blood were lysed with lysis-buffer and only nucleated cells were recovered. After immunostaining with ER-HR3 or F4/80, a marker restricted to the monocytic lineage, cells were stained with May Grünwald Giemsa stain and

Figure 11.5. (a) Distribution of ER-HR3-positive cells in the murine spleen. RP, red pulp; WP, white pulp. Magnification x35. (b) ER-HR3-positive cells surrounded by hemopoietic cells. Magnification x650

Figure 11.6. (a) Distribution of ER-HR3-positive cells in the murine bone marrow. Magnification x130. (b) ER-HR3-positive cells surrounded by hemapoietic cells. Magnification x450

Figure 11.7. ER-HR3-positive cells (arrows) in the thymic cortex. C, cortex. Magnification x200

Figure 11.8. ER-HR3-positive cells in the mesenteric lymph node.
F, follicle; PC, paracortex. Magnification x125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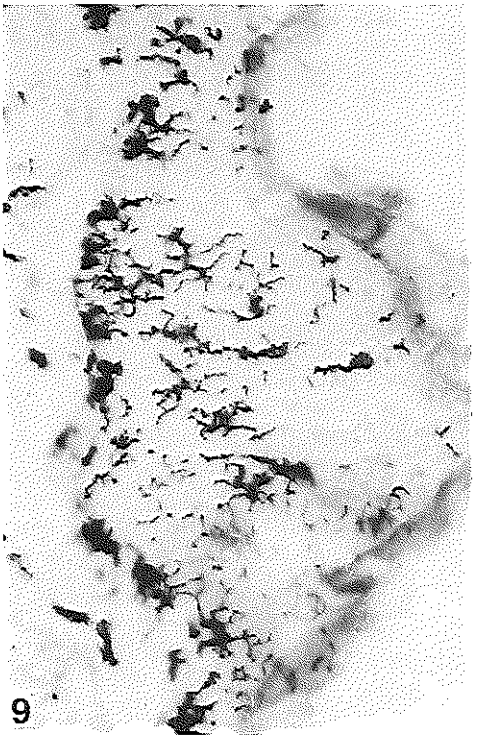
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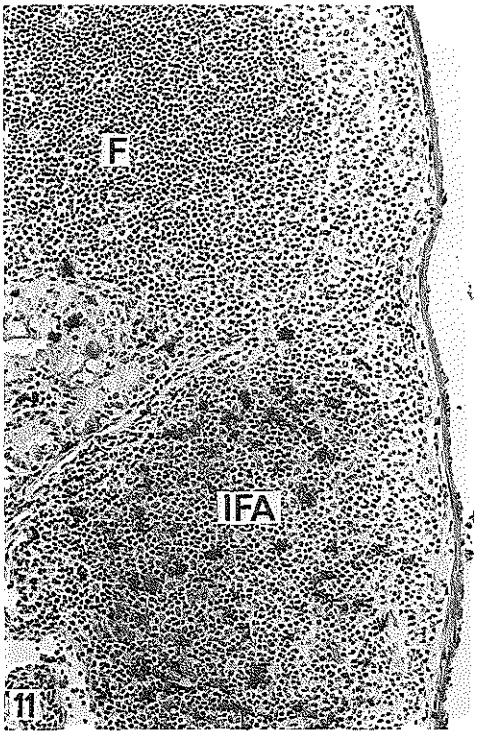

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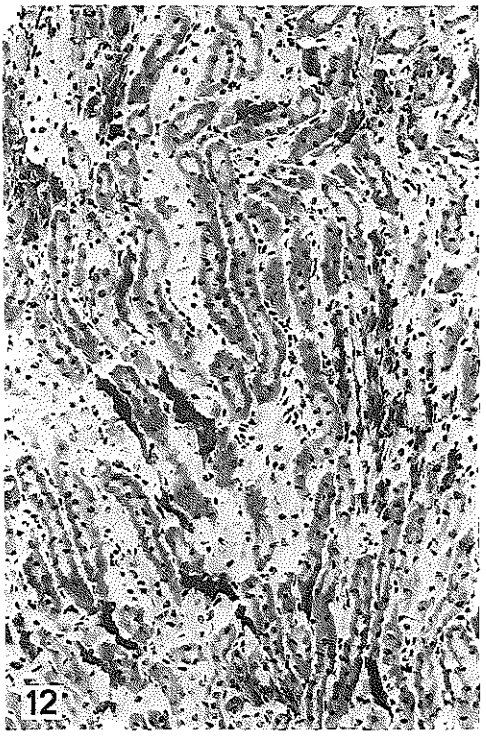
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identified. From these experiments the percentage of ER-HR3-positive monocytes was estimated. This positive subpopulation encloses 70% of the total monocyte population in the blood. No other leukocytes were found to react with ER-HR3.

Enzyme histochemical characterization and phagocytosis

Enzyme histochemical characterization of ER-HR3-positive cells was performed either simultaneously with immuno-histochemical staining on the same section, or on serial sections following immuno-histochemical staining. It was demonstrated that all ER-HR3-positive cells were positive for the enzyme acid phosphatase (not shown). The expression of the enzyme nonspecific esterase was not uniform. Some ER-HR3-positive cells stained negative, others weak or strongly positive.

The phagocytosis of carbon particles after an intravenous injection was investigated in the spleen. Half an hour after the carbon injection phagocytosis in the spleen was restricted to the cells adjacent to and present within the marginal zone. The majority of these cells did not express the ER-HR3 antigen. Only a minority phagocytized carbon and simultaneously expressed the ER-HR3 antigen. Furthermore, we observed some ER-HR3-positive cells without carbon. One hour after carbon injection all ER-HR3-positive cells contained carbon particles.

DISCUSSION

In this study we describe the ontogeny and distribution of cells containing the ER-HR3 antigen. There are a number of observations which indicate that expression of the antigen is restricted to a subpopulation of monocytes and macrophages. Positive cells appeared to possess a variable morphology. This morphology could be rounded, elongated or dendritic as observed in the mesenchymal loose connective tissue of fetuses (Fig. 11.3), the connective tissue of the gut and kidney (Figs. 11.10 and 11.12) and the epidermis (Fig. 11.9), respectively. As judged by our electron

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Figure 11.9. ER-HR3-positive Langerhans cells in the epidermis of the murine ear (no counterstain applied). Magnification x320

Figure 11.10. ER-HR3-positive cells (arrows) located apically in a villus of the ileum. Magnification x650

Figure 11.11. Distribution of ER-HR3-positive cells in the Peyer's patch.
F, follicle; IFA, intra follicular area. Magnification x125

Figure 11.12. ER-HR3-positive cells present in the interstitium of the renal outer medulla. Magnification x125

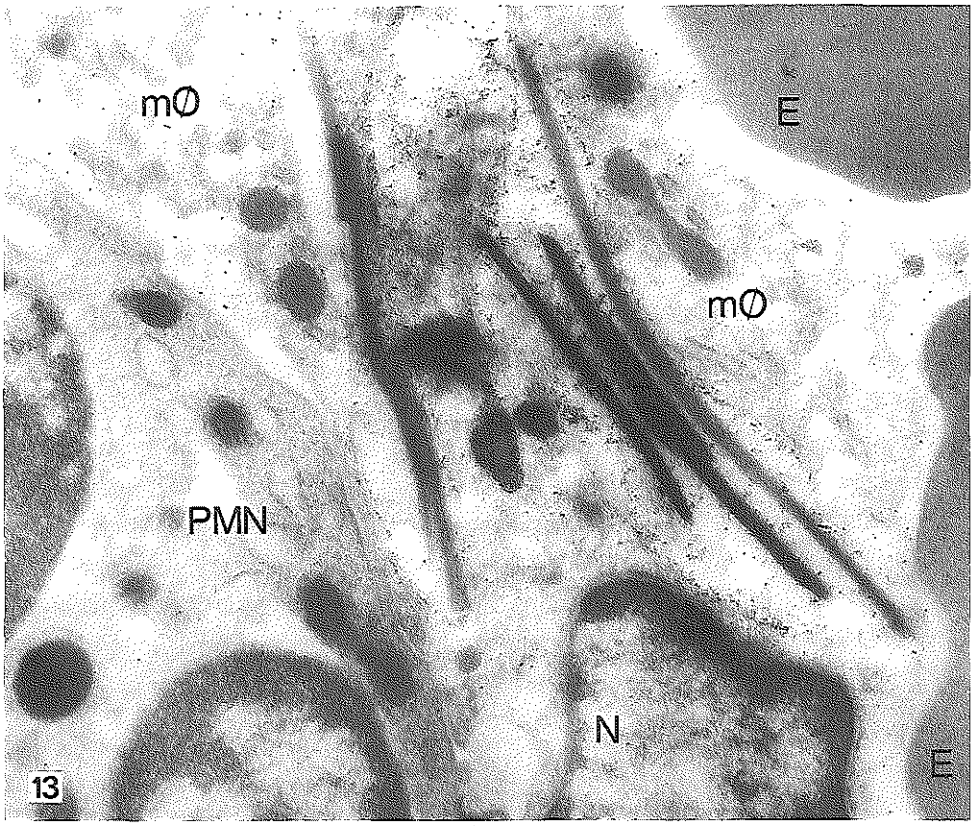

Figure 11.13. ER-HR3-positive macrophage (mϕ) in the murine spleen. E, erythrocyte; PMN, polymorphonuclear neutrophilic granulocyte; N, nucleus. Magnification x35.000

microscopic investigation, positive cells in the spleen contained effete erythrocytes, expelled normoblast nuclei, lysosomes and rod-shaped inclusions (Figs. 11.13 and 14) typical for murine macrophages (Berman, 1967). Furthermore, most ER-HR3-positive cells were able to phagocytize colloidal carbon particles and stained positively for the enzymes nonspecific esterase and acid phosphatase. These markers can be used as reliable criteria for macrophages (van Furth, 1980).

With exception of the pan-macrophage marker F4/80, little information is available on the distribution of macrophage markers in fetal tissues. F4/80 is firstly evident on cells in the yolk sac on day 9 of fetal life (Gordon, 1986), two days after the onset on fetal hemopoiesis (Moore and Metcalf, 1970). From day 11 on, the antigen appears succesively in the fetal liver, the spleen and the bone marrow concomitant with the shift of hemopoietic activity from one site to another. Like F4/80-positive cells, the ER-HR3-positive cells in the hemopoietic organs had a stellate appearance and were often found in close association with erythroid progenitor cells. In other tissues the infiltration occurs in association with vascularization, being demonstrable in brain, kidney and

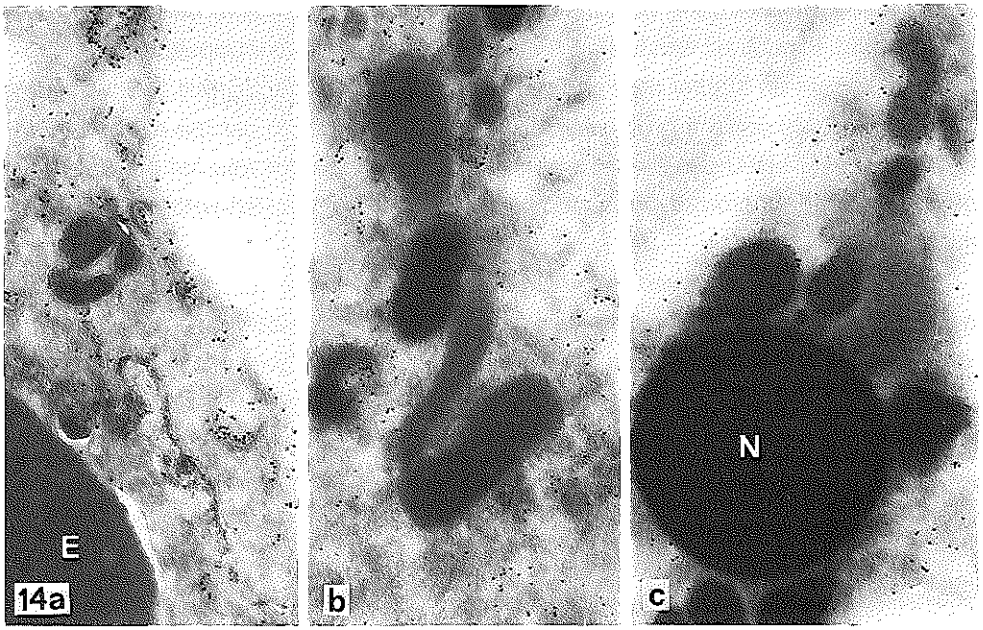

Figure 11.14. Electron microscopic characteristics of ER-HR3-positive cells showing (a) membrane-associated expression of the ER-HR3-antigen, (b) mitochondria, and (c) expelled normoblast nuclei and lysosomal structures in the cytoplasm. E, erythrocyte; N, normoblast nucleus. Magnification x60.000

small intestine by day 15/16 of gestation (Hume and Gordon, 1985). Although F4/80 and ER-HR3 antigens partly overlap in their fetal distribution some clear differences were observed. For example, no ER-HR3-positive cells were present in the yolk sac. Moreover, the appearance of ER-HR3-positive cells in the various tissues always occurrs in a later stage of development than that of F4/80-positive cells. The onset of ER-HR3 expression in fetal liver (day 13), spleen (day 17) and bone marrow (day 1 after birth) correlated closely with the onset of adult type hemoglobin (Hb-A) production in these organs as described by Metcalf and Moore (1970). Additionally, the decrease of the antigen in the liver correlated with the disappearance of the erythropoiesis in this organ. These observations are suggestive of a role of the ER-HR3 antigen in adult erythropoiesis.

The distribution of ER-HR3 in the adult animal differed from other macrophage markers. Like in fetal tissues, immunostaining of serial sections with ER-HR3 and F4/80 revealed that both markers partly overlap in their distribution. However, the number of ER-HR3-positive cells never exceeded the number of F4/80-positive cells. Furthermore, organs such as liver, pancreas, lung and brain contained many F4/80-positive cells, but lack ER-HR3-positive cells. Another macrophage marker, exclusively present on mature resident macrophages, is BM8 (Malorny et al., 1986). In many ways the distri-

bution pattern of this marker resembles that of ER-HR3 but also here clear differences can be noted. In contrast with BM8, ER-HR3 was present on a subpopulation of monocytes but absent on Kupffer cells and cells in the medulla of the thymus. In the spleen ER-HR3 and BM8-positive cells were restricted to the red pulp but the decrement in the number of ER-HR3-positive cells towards the splenic centre was not observed for the latter cells. Furthermore, the various organs contained less ER-HR3-positive cells than BM8-positive cells.

Interestingly, we observed many ER-HR3-positive cells in the paracortex of the mesenteric lymph node, whereas lymph nodes at other locations were negative or contained /only few positive cells. Furthermore, ER-HR3-positive cells were observed in the interfollicular areas of the Peyer's patches. The large numbers of positive cells in these T cell regions may suggest that macrophages instead of interdigitating cells play an important role in antigen presentation in gut-associated lymphoid tissues as has been postulated by Sminia et al. (1983). However, the ER-HR3 antigen probably is not involved in this process since ER-HR3 was unable to inhibit the antigen-specific T cell proliferation (de Jong et al., 1990b).

The role of the ER-HR3 antigen at other sites also remains speculative. ER-HR3-positive cells of the kidney likely resemble type 1 renal medullary macrophages as described by Bohman (1983). These cells are thought to perform important functions in the physiology of the kidney (Bohman et al., 1988; Pitcock et al., 1988). However, the close association between ER-HR3-positive cells and the tubulous epithelium may suggest a function of the antigen in the maintenance of the epithelial integrity. It is well known that macrophages are able to secrete mediators that influence the epithelium (Hume et al., 1984a). The presence of the ER-HR3 antigen on Langerhans cells and its occurence on cells in the intestinal lamina propia and subdermis support this view.

Our results indicate that ER-HR3 has an unique distribution pattern not known from other macrophage markers. The occurrence of ER-HR3-positive cells in the hemopoietic organs during fetal and juvenile life correlates well with the onset of adult-type hemoglobulin production and is suggestive of a role of the ER-HR3 antigen in this process. Involvement in the regulation of the immune response or the support of epithelial integrity may be other functions of ER-HR3-positive macrophages.

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LOCALIZATION AND PHENOTYPICAL CHARACTERIZATION OF MURINE MACROPHAGES

J.P. DE JONG

12. A MONOCLONAL ANTIBODY (ER-HR3) AGAINST MURINE MACROPHAGES: II. BIOCHEMICAL AND FUNCTIONAL ASPECTS OF THE ER-HR3 ANTIGEN

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Submitted for publication.

SUMMARY

In this study we describe the purification and intracellular distribution of an antigen present on a subpopulation of murine macrophages and recognized by monoclonal antibody ER-HR3. Using the ER-HR3 antibody as an immobilizing ligand, two proteins were isolated. Under non-reducing conditions their molecular mass was estimated as 69 kD and 55 kD. Under reducing conditions their molecular mass was estimated as 76 kD and 67 kD, respectively. Intracellularly, these proteins occurred in close association with membranous structures as was demonstrated with gold-labelled protein A in an electron-microscopic study of the ER-HR3-positive cell line AP284. Part of the antigen was present in vesicles. To gain more insight into the possible function of the ER-HR3 antigen its tissue distribution was investigated under distinct experimental conditions. In BCG-infected mice ER-HR3-positive cells were observed in many, but not all, granulomata of the spleen, the lung and the liver. The ER-HR3 reactivity in these mice clearly differred from other anti-macrophage monoclonal antibodies such as F4/80, M5/114 and M1/70. Furthermore, it was demonstrated that phenylhydrazine-induced extramedullary erythropoiesis in the liver is accompanied by the ER-HR3 expression on a subpopulation of macrophages. Finally, we demonstrated that the addition of monoclonal antibody ER-HR3 to an antigen-specific T cell proliferation assay did not inhibit T cell proliferation.

INTRODUCTION

Macrophages are a heterogeneous cell population that are widely distributed throughout the body (Dougherty and McBride, 1984). They express a variety of surface antigens such as receptors and immunoregulatory molecules (Wright and Silverstein, 1986; Gordon et al., 1988a). The development of the hybridoma technique made it possible to produce monoclonal antibodies (mAb) against these antigens. These mAb can be used to characterize macrophages, to investigate their tissue distribution, and to classify them into distinct subpopulations. Examples of such mAb are F4/80 (Austyn and Gordon, 1981), BM8 (Malorny et al., 1986) and MOMA-2 (Kraal et al., 1987). Other mAb may be useful tools to provide information about the differentiation or activation state of macrophages. Examples are AcM.1 (Taniyama and Watanabe, 1982), MM9 and WE15 (Taniyama and Tokunaga, 1983), 7/4 (Hirsch and Gordon, 1983), and ER-MP20 (Leenen et al., 1990b). Moreover, mAb against well-defined antigens can be used to elucidate the specific functions of these antigens. An example of this category is mAb M18/2 (Springer et al., 1984). This mAb is directed against the β-unit of a class

of antigens referred to as leukocyte adhesion molecules. The use of M18/2 in a number of functional studies has revealed that these molecules are important mediators in processes like chemotaxis, aggregation, adhesion and migration (Anderson et al., 1986; Springer and Anderson, 1986).

Recently, we described the distribution of a new mAb (ER-HR3) against a subpopulation of macrophages in the fetal and adult mouse (De Jong et al., 1990a). In this study we present the results of a number of experiments to obtain more information about the function of the antigen recognized by this mAb. We purified the ER-HR3 antigen, determined its molecular mass and investigated its intracellular distribution. Moreover, we assessed its expression under experimental conditions in which macrophages might adapt their antigen expression in correlation with altered functions. Such adaptive changes may occur after injection with phenylhydrazine or sublethal doses of pathogenic organisms such as Bacillus Calmette Guerin (BCG). Additionally, we investigated the antigen expression on a number of macrophage cell lines and on macrophages in bone marrow cultures in order to substantiate the possible relationship between ER-HR3 antigen expression and macrophage differentiation.

MATERIALS AND METHODS

Mice. Male and female (CBA/Rij x C57BL/Rij) F1 (BCBA) mice were obtained from the Radiobiological Institute TNO (Rijswijk, The Netherlands) and kept under conventional conditions.

Cell lines. The classification and original references of the macrophage cell lines used are depicted in Table 12.1. The cells were cultured in RPMI1640 medium supplemented with 10% fetal calf serum (FCS) and antibiotics. The cell line AP284-D4 was isolated in our laboratory from the spleen of a BCBA mouse.

Immobilization of ER-HR3 to protein A-Sepharose. The ER-HR3 antibody was purified and immobilized as described by De Jong et al. (1990a) and Schneider et al. (1982), respectively. Briefly, purified antibody was mixed with protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) in 0.1 M borate buffer, pH 8.2, and incubated for 30 min at room temperature under gentle shaking. Beads were successively washed with borate buffer and 0.2 M triethanolamine, pH 8.2. Hereafter, they were resuspended in 20 ml of 50 mM dimethyl pimelimidate dihydrochloride (Pierce, Rockford, IL, USA) freshly made up in 0.2 M triethanolamine with the pH re-adjusted to 8.2. The mixture

was agitated gently at room temperature for 1 h. The reaction was stopped by centrifuging the beads (500 x g for 1 min) and resuspending them in an equal volume of 50 mM ethanolamine, pH 8.2. After 5 min the crosslinked beads were washed three times with borate buffer, pH 8.2, supplemented with 0.02% sodium azide.

Table 12.1
Cell lines and references

Cell line	Cell type	Reference
M1	myeloblast	Ichikawa, 1969
RMB-1	myelocyte	De Both et al., 1981
RMB-3	myelocyte	De Both et al., 1981
WEHI-3B	immature macrophage	Warner et al., 1969
WEHI-3	immature macrophage	Warner et al., 1969
Pu5-1.8	macrophage	Ralph et al., 1974
J774-1.6	macrophage	Ralph et al., 1975
P388D1	macrophage	Koren et al., 1975
RAW264.7	macrophage	Raschke et al., 1978
RAW309Cr.1	macrophage	Raschke et al., 1978
WR19M.1	macrophage	Raschke et al., 1978
AP284	macrophage	Klasen et al., 1988

Isolation and characterization of the ER-HR3 antigen. Cell suspensions of the spleen and terminal ileum were obtained by cutting these organs into pieces and pushing them through a mesh sieve. After centrifugation (200 x g, 15 min, 4°C) the cell pellet was dissolved in a lysis buffer of 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF, stock: 2 mg PMSF/ml dimethyl sulfoxide (DMSO), 0.5 µl in 1 ml lysis buffer), 1 µg/ml leupeptin (Sigma, St. Louis, MO, USA) and 1 µg/ml pepstatin (Sigma) in phosphate buffered saline (PBS). The cellular fragments were further homogenized in a Dounce homogenizer by 20-30 fast strokes with a tight fitting pestle. The homogenate was centrifuged (1500 x g, 30 min, 4°C) and the supernatant was diluted by addition of an equal volume of 0.1 M phosphate buffer, pH 8.0. Cellular proteins binding to protein A-Sepharose were removed by preclearance of the solution on a protein A-Sepharose column. Hereafter, the eluate was layered onto an ER-HR3-Sepharose column. Bound proteins were removed with a 0.1 M glycine/HCl buffer, pH 2.5. The eluate was neutralized with a 0.1 M Tris/HCl buffer (pH 8.5), concentrated in an Amicon filtration unit and processed for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). The separated proteins were electrophoretically transferred to nitrocellulose (Towbin et al., 1979) and immunologically detected as described below (see **spot-test**).

Spot-test. Aliquots of 1 µl of the affinity-purified cellular proteins and control solutions were spotted directly on nitrocellulose sheets. Sheets were successively incubated for 15 min in PBS, pH 7.8, supplemented with 0.02% (w/v) gelatin and 0.05% (v/v) Tween-20 (PBT), for 1 h with ER-HR3, rinsed with PBT, and finally incubated for 1 h with a 1:40 diluted solution of a peroxidase-conjugated rabbit-anti-rat antibody (Dakopatts, Copenhagen, Denmark). They were washed twice with PBT whereafter the enzyme activity was visualized with diaminobenzidine. All steps were performed at room temperature (De Jong et al., 1990a).

Immunogold labelling. Cells of the cell line AP284 were collected by centrifugation and fixed for 1 h at 4°C in 0.1 M phosphate buffer (PB), pH 7.3, containing 1% acrolein and 0.4% glutaraldehyde. Cells were rinsed twice with 0.1 M PB, resuspended in 10% (w/v) gelatin in 0.1 M PB at 37°C, and centrifuged. The pellet was solidified on ice and processed as described previously (de Jong et al., 1990a).

BCG infection of mice. Mice were intravenously injected with 6×10^6 viable Bacillus Galmette Cuerin (BCG, strain 601A), suspended in PBS, pH 7.4. Twenty-one days after infection the spleen, lung and liver were removed and processed for immunostaining according to de Jong et al. (1990a).

ELISA. The expression of the ER-HR3 antigen by macrophage cell lines was quantitatively assessed by means of a micro-ELISA system (Leenen et al., 1990). In short, cells were coated to Terasaki trays with 0.05% glutaraldehyde, incubated for 1 h with ER-HR3, rinsed with PBT, incubated for 1 h with optimally diluted ß-galactosidase-coupled anti-rat Ig, rinsed again, and finally incubated for 1 h at 37 °C with the fluorogenic substrate 4-methylumbelliferyl galactopyranoside. The amount of generated fluorescent product was then determined with a scanning microfluorometer. The fluorescent product generated in a Terasaki tray saturated with purified rat Ig was used as a positive internal control. Antigen expression is given in arbitrary units (AU), implying that for 100 AU a fluorescence signal was obtained that equalled this positive control.

Bone marrow cultures. Macrophages were cultured from murine bone marrow under the following conditions. Bone marrow was flushed from the femurs with a 23-gauge needle and collected in 6 ml of α-medium containing 5% FCS. The cells were centrifuged and resuspended in α-medium supplemented with 10% FCS and 20% L-cell conditioned medium, the latter as source of M-CSF. After the cell concentration was adjusted to 5×10^4 cells/ml medium, one ml aliquots were plated in 24 well plates

(Costar, Cambridge, MA, USA) on Lux polystyrene coverslips (Lux, Naperville, IL, USA) and cultured at 37°C in a humidified atmosphere consisting of 5% CO_2 in air. Both the non-adherent and adherent cell fractions were collected every two days until day 12 of culture and the cells were immunostained as described by Leenen et al.(1987).

Antigen presentation. The antigen presentation assay was performed in vitro as described by Klasen et al. (1988). In short, a mBSA-specific T cell clone was cultured for three days in serum-free medium with or without antigen together with irradiated macrophages. Proliferation of the T cells was measured by ^{3}H-thymidine incorporation during the last 8 or 18 hrs of culture. The effect of the ER-HR3 antibody in this assay was investigated by addition of increasing amounts of the mAb to the culture medium.

Phenylhydrazine treatment. Mice were intraperitoneally injected with 1 mg phenylhydrazine dissolved in PBS (PHZ; Merck, Darmstadt, FRG) for four subsequent days. Three days after the last injection some mice were intravenously injected with carbon particles 30 min before the removal of the spleens and livers. These organs were removed, sectioned and immunostained with the ER-HR3 monoclonal antibody. Immunostaining and carbon injection were performed as previously described (de Jong et al, 1990a).

RESULTS

Purification of the ER-HR3 antigen

From a previous study in which we determined the tissue distribution of ER-HR3, it was obvious that the spleen and the terminal ileum were sites at which ER-HR3-positive cells were abundantly present (de Jong et al., 1990a). In order to determine the molecular mass of the antigen recognized by ER-HR3 we used tissue from these organs to prepare cellular lysates. Additionally, a cellular lysate was obtained from the macrophage cell line AP284 that expressed relatively high amounts of the antigen. Furthermore, the culture supernatant of this cell line was collected and concentrated. After affinity purification, samples were analyzed by SDS-PAGE under reducing and non-reducing conditions. Under non-reducing conditions two single protein bands of approximately 69 and 55 kD were observed (Fig. 12.1, lane 1). Surprisingly, under reducing conditions two larger protein bands of approximately 76 kD and 67 kD were observed (Fig. 12.1, lane 2). Only the largest of these proteins was present in the culture supernatant of the cell line AP284 (not shown). After electrophoretical transfer

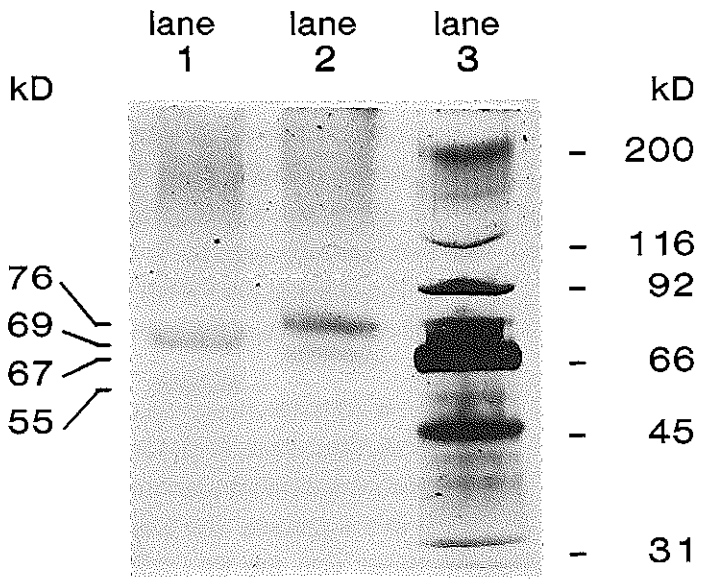


Figure 12.1. SDS-PAGE pattern of a cellular lysate from the spleen after affinity chromatography with the ligand ER-HR3. Lane 1, non-reduced; lane 2, reduced; lane 3, marker proteins

of the proteins to nitrocellulose and successive immunostaining with ER-HR3, no protein band could be detected irrespective of whether the proteins were reduced or not. However, direct spotting of the unreduced purified proteins on nitrocellulose and successive immunostaining revealed positive spots (Fig. 12.2).

Intracellular distribution of the ER-HR3 antigen

The intracellular distribution of the antigen was investigated by immunogold labelling of the cell line AP284. Cells of this line express a number of macrophage-specific markers. Furthermore, it has been shown that they were able to phagocytose carbon and stain positively for the enzymes acid phosphatase and non-specific esterase (Klasen et al., 1988). Figure 12.3 shows the intracellular distribution of the ER-HR3 antigen in this cell line. From these photographs it is obvious that part of the antigen is present on the outer cell membrane (Fig. 12.3a). Moreover, most of the antigen present in the cytoplasm is also membrane-associated (Fig. 12.3a). Another part is stored in vesicles (Figs. 12.3b and 12.3c).

The ER-HR3 antigen as macrophage differentiation marker

The question whether the antigen recognized by ER-HR3 was a differentiation marker was approached in two manners. Firstly, we determined the number of positive cells in aging M-CSF-stimulated bone marrow cultures. It was observed that at the initiation of the culture 50% of the total cell population possessed the ER-HR3 antigen. In time, their number increased and at day 10 of culture all cells expressed the marker (Fig. 12.4). Secondly, we investigated the expression of the antigen on distinct macrophage lines. These lines can be ordered in a linear maturation sequence depending on the antigens they express (Leenen et al., 1986b). It was demonstrated that the ER-HR3 antigen is expressed by many of these cell lines. In general, there is a tendency that lines with a more mature phenotype show a higher expression of the antigen (Fig. 12.5). Hence, in both models expression of the ER-HR3 antigen appears to correlate with macrophage maturity.

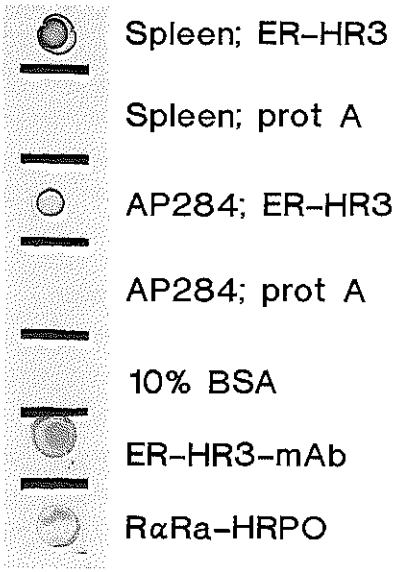


Figure 12.2. Spot test of protein A and ER-HR3 affinity-purified cellular lysates from the spleen and the cell line AP284. Positive spots were observed only in protein fractions obtained after ER-HR3 affinity chromatography (Spleen; ER-HR3 and AP284; ER-HR3). Aspecific binding of ER-HR3 to protein A-affinity purified proteins was not observed (Spleen; Prot. A and AP284; Prot. A). BSA was used as a negative control. ER-HR3 and the horseradish peroxidase-conjugated rabbit-anti-rat immunoglobulin (RαRa-HRPO) were used as positive controls

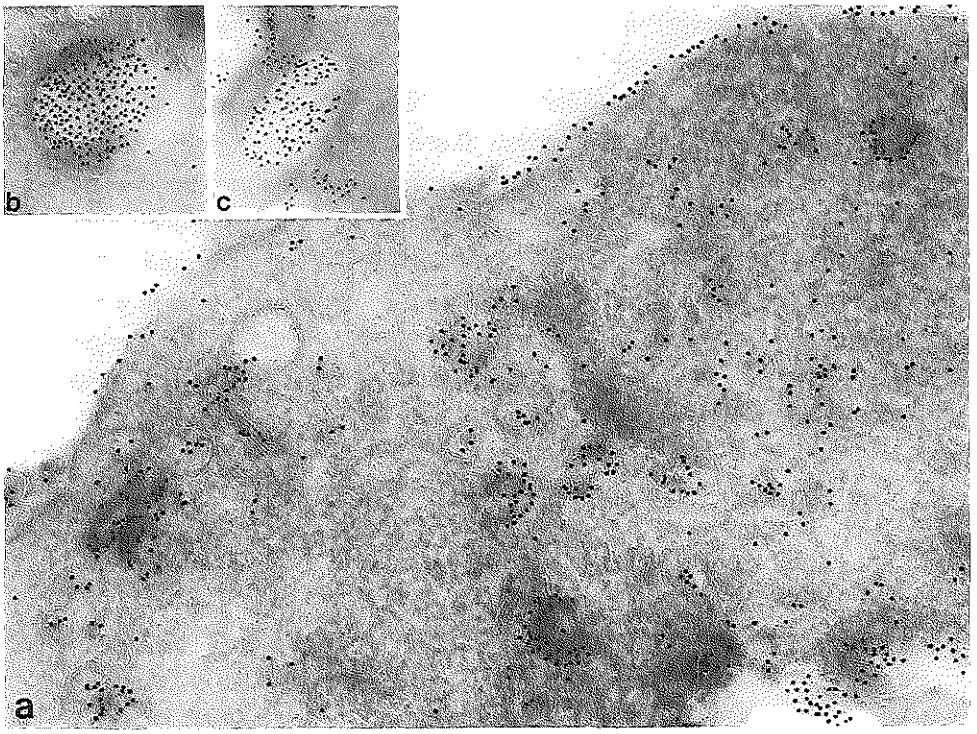


Figure 12.3. Intracellular distribution of the ER-HR3 antigen in the cell line AP284. Gold particles are present (a) on the plasma membrane and cytoplasmic membranous structures or (b,c) within vesicles. Magnification x 60.000

Expression of the ER-HR3 antigen in BCG-infected mice

At day 21 of BCG infection we investigated the ER-HR3 antigen expression by cells in the lung, liver and spleen. For comparison we also judged the expression of the CR3 (Mac-1; mAb M1/70), MHC class II antigens (Ia; mAb M5/114) and F4/80 antigen. Control lungs of uninfected mice contained only few cells positive for F4/80, M1/70 and M5/114. ER-HR3-positive cells were not present in the lungs. Within BCG-infected lung granulomata were mainly located near to the bronchi and large arteries. M5/114 staining was not restricted to these sites but was observed throughout the lung (Fig. 12.6a). Within the granulomata most cells were heavily stained by M1/70 (Fig. 12.6c). Macrophages within the lesions showed a weak expression of F4/80 (Fig. 12.6b). ER-HR3 antigen expression was restricted to cells present in the granulomata around the arteries but not the bronchi (Fig. 12.6d).

In control livers most, if not all, Kupffer cells stained with monoclonal antibody F4/80. Approximately 10 per cent of these cells were Ia-positive. The ER-HR3 and

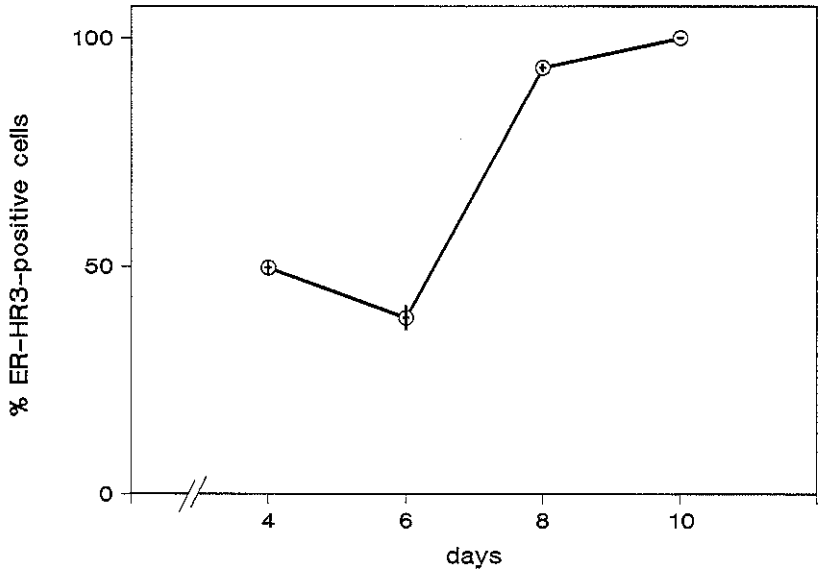


Figure 12.4. ER-HR3 and F4/80 antigen expression on M-CSF-stimulated bone marrow cells. Data are presented as percentage of the total cell population

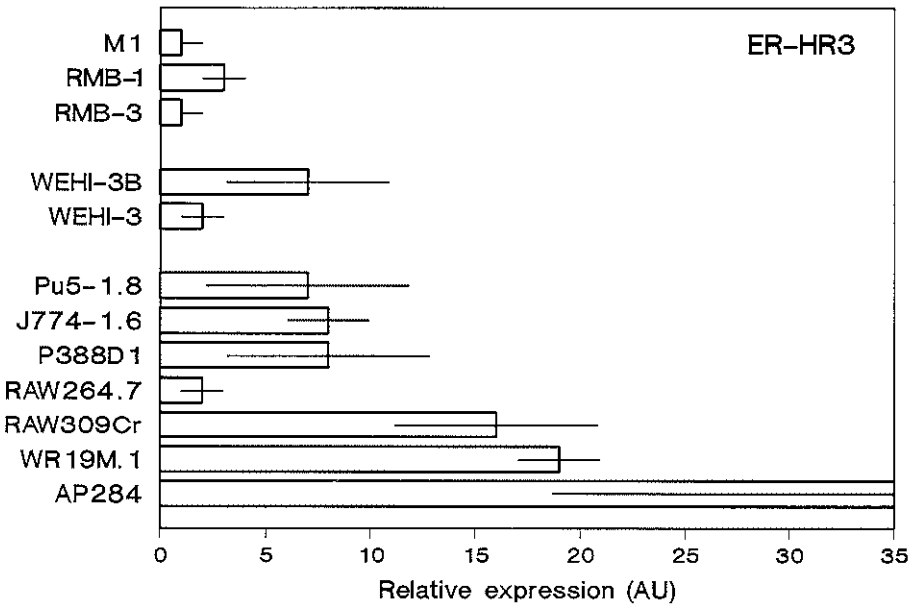


Figure 12.5. ER-HR3 antigen expression by macrophage cell lines. These are arranged in order of maturation according to Leenen et al.(1986b). M1 examplifies the most immature and AP284 the most mature cell line

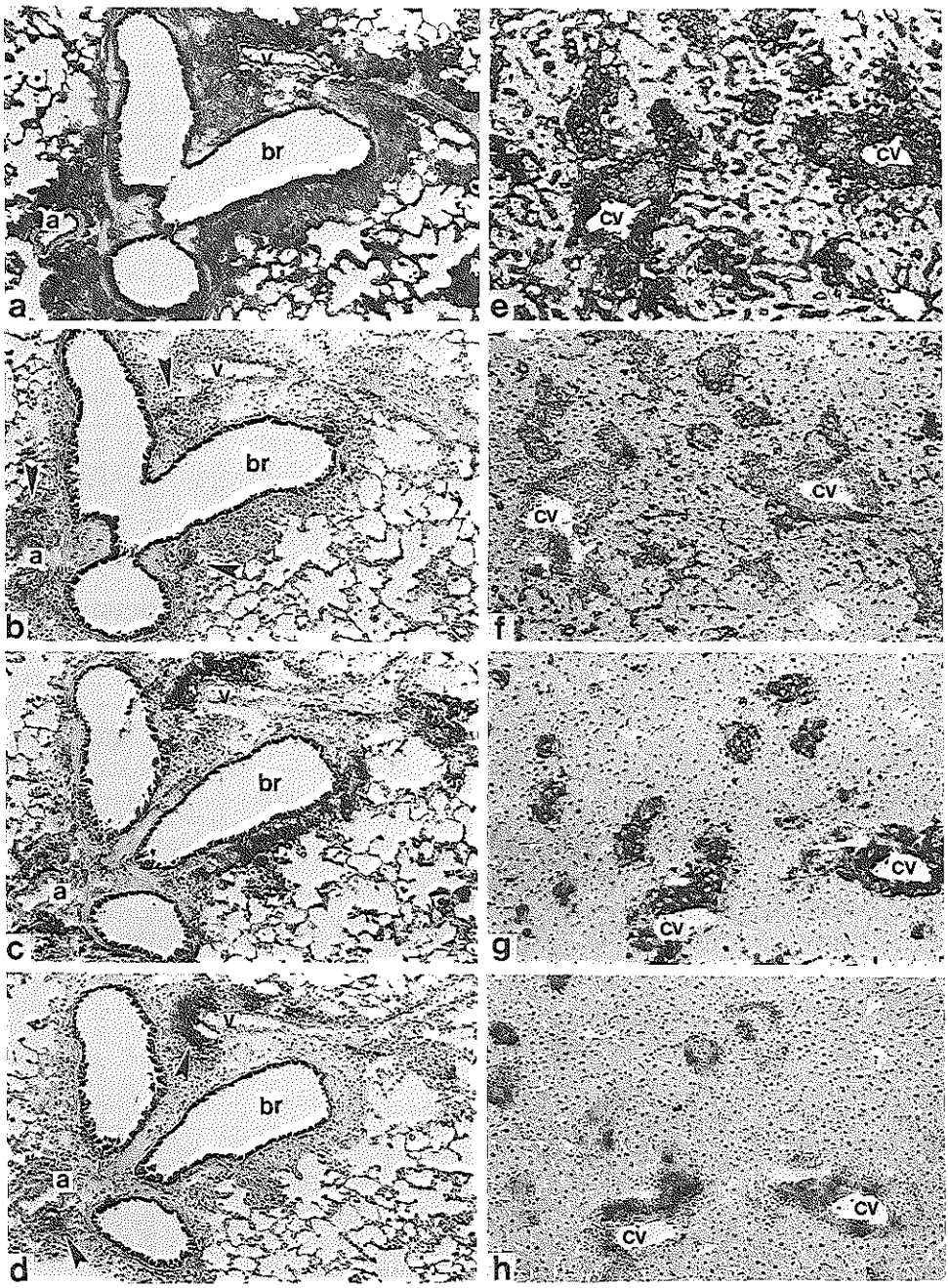


Figure 12.6. Distribution of macrophage antigens in the lung (a-d) and spleen (e-h) of BCG-infected mice. Antigens investigated were Ia (a,e); F4/80 (b,f); Mac-1 (c,g); and ER-HR3 (d,h). Arrows (Figs. b, d) indicate sites of positivity. Magnification x 60.
a, artery; br, bronchiolus; c, central vein

Mac-1 antigens were not expressed by cells in the liver. In BCG-infected livers (Figs. 12.6e,f,g,h) the Ia antigen was expressed by many cells, i.e. the sinusoid lining cells, Kupffer cells and by most cells present in the granulomata (Fig. 12.5e). The F4/80 antigen was present on macrophages in the granulomata and on Kupffer cells (Fig. 12.6f). Expression of the F4/80 antigen on the latter cell type was strongly increased when compared to uninfected livers. The markers Mac-1 and ER-HR3 were mainly confined to the granulomata (Figs. 12.6g and 12.6h). ER-HR3 staining was less intense than M1/70. In control spleens F4/80 and ER-HR3-positive cells were confined to the red pulp. M1/70 strongly stained the granulocytes and weakly some large macrophages in this region. Moreover, the antigen appeared to be expressed by cells in the marginal zone. Ia expression was restricted to cells in the follicle centre and to some cells in the red pulp. In BCG-infected spleens the architecture was disrupted by the formation of granulomata. Especially the region surrounding the marginal zone was affected. Infiltrated cells weakly expressed the antigens recognized by ER-HR3 and F4/80. Both, the Ia and the Mac-1 antigen were strongly expressed on cells throughout the spleen.

Expression of the ER-HR3 antigen in PHZ-treated mice

Mice injected with PHZ are known to develop a hemolytic anemia. They show splenomegaly and extramedullary erythropoiesis in the liver. In PHZ-treated mice the splenic structure was disrupted due to a strong enlargement of the red pulp. The increase in erythropoietic activity was well visualized by the presence of many erythroblasts. Moreover, there was a large increase in the number of ER-HR3-positive cells (Fig. 12.7a) in the spleen. Positive cells appeared to be larger compared with those in control mice. They often were surrounded by erythroid precursors (Fig. 12.7b). The most striking difference between control and PHZ-treated mice with respect to the ER-HR3 antigen distribution was the appearance of ER-HR3-positive cells in the liver (Fig. 12.7c). They were mainly present in the liver sinusoids. Occasionally they were surrounded by a number of erythroblasts (Fig. 12.7c, insert). Injection of carbon after the PHZ-treatment resulted in carbon staining of all ER-HR3-positive cells of the liver, including the central macrophages surrounded by erythroblasts (Fig. 12.7d).

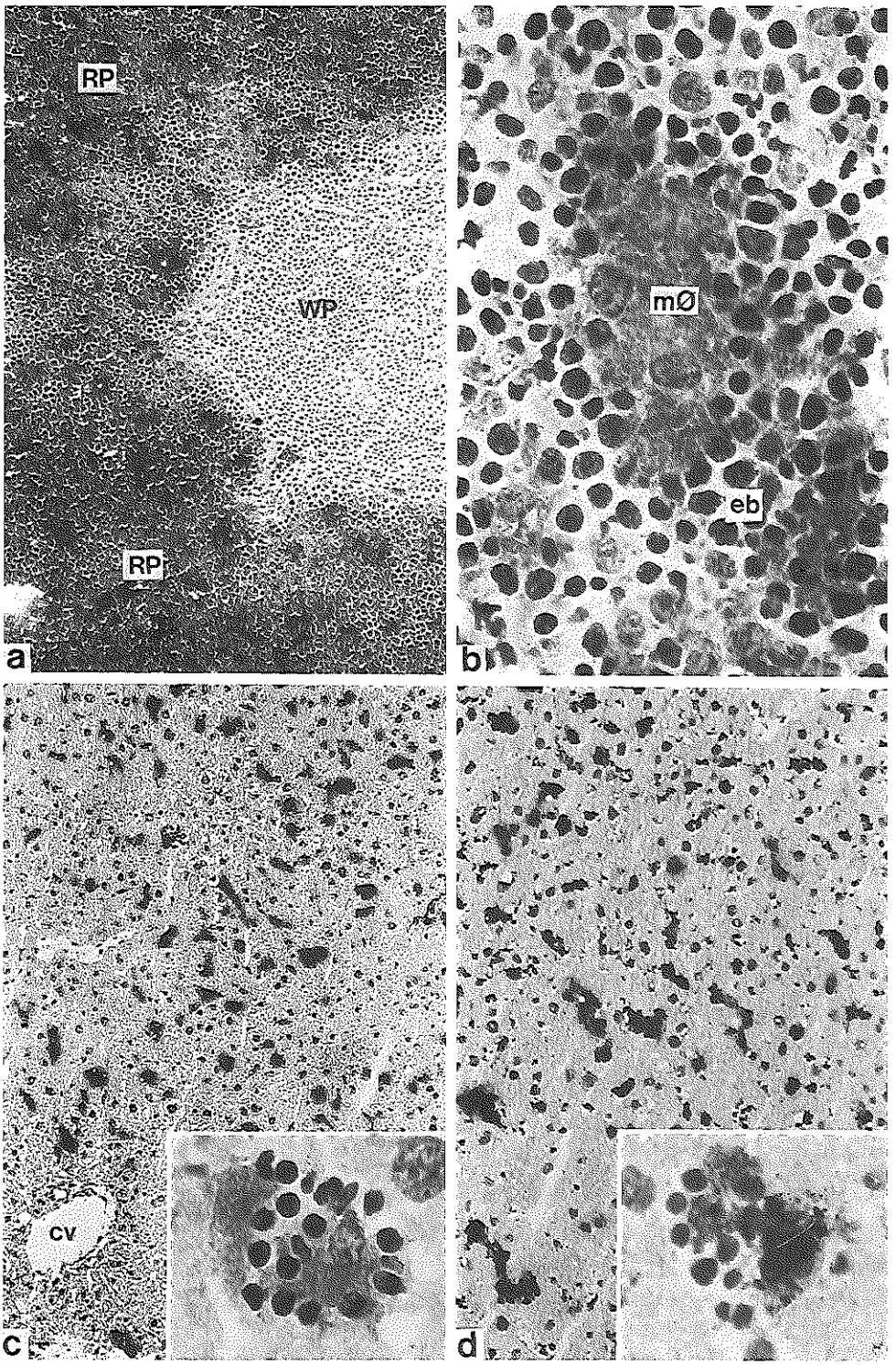
Antigen presentation

It was observed that ER-HR3 antibody had no effect on mBSA presentation by the ER-HR3-positive cell line AP284 to mBSA primed T cells (data not shown). However, mAb M5/114 (anti-Ia), used as a positive control, greatly diminished antigen-specific T cell proliferation.

DISCUSSION

In a previous paper we reported on the distribution of the antigen recognized by antibody ER-HR3 that is directed against a subpopulation of macrophages located predominantly in lympho-hemopoietic organs (de Jong et al., 1990a). Now we report on the purification of the antigen recognized by ER-HR3 and its expression under different experimental conditions. The monoclonal antibody recognized two distinct proteins with molecular weights of 69 kD and 55 kD (Fig. 12.1). The observation that their apparent molecular masses increase under reducing conditions was somewhat surprising. The presence of intramolecular disulfide bridges may explain this result. Disconnection of the disulfide bridges results in unfolding of the protein, which likely interferes with its electrophoretic mobility. Since both proteins share this property they may be related in structure. Two possible explanations are (1) that one of the two proteins is the precursor of the other; and (2) that one protein is present in two different forms, for instance, a membrane-associated and a secreted form. The latter phenomenon has also been observed for IL-1 (Kurt-Jones et al., 1985). The observation that the cell line AP284 secretes the 69 kD protein only supports the second explanation. Expression of the ER-HR3 antigen by this cell line (Figs. 12.3 and 12.5) makes this the valuable tool for investigating the precise intracellular distribution of the antigen. Immunogold labelling was observed at three sites, i.e. on the outer cell membrane, on cytoplasmic membranous structures and in vesicles (Fig. 12.3). Thus, part of the antigen is membrane-associated and another part is stored in vesicles. Since the largest protein was isolated from the culture supernatant of the AP284 cell line, likely, this protein is present in the vesicles. Membrane-associated occurrence of the antigen correlated well with observations from our in vivo study (de Jong et al., 1990a). Macrophages in situ lacked the antigen-loaded vesicles as observed in AP284 cells. This suggests that the secretion rate of the antigen under in vivo conditions is much higher, a condition which prevents the storage of the protein in the vesicles. A high secretion rate followed by re-internalization of the antigen by AP284 cells may be an alternative explanation.

Figure 12.7. Distribution of ER-HR3-positive cells in the spleen (a,b) and liver (c,d) of phenylhydrazine-treated mice. (a) ER-HR3-positive cells are abundant in the red pulp. Magnification x 125.
(b) High power magnification (x 650) of erythrocyte-loaded ER-HR3-positive macrophages (mϕ) surrounded by erythroblasts. (c) Distribution of ER-HR3-positive cells in liver. Magnification x 140. Insert: high power magnification (x 650) of ER-HR3-positive cell surrounded by erythroblasts.
(d) Distribution of carbon-loaded cells in the liver. Small spots indicate Kupffer cells, large spots indicate carbon loaded ER-HR3-positive cells. Magnification x 140 Insert: high power magnification (x 650) of a carbon loaded ER-HR3-positive cell surrounded by erythroblasts.
cv, central vein; eb, erythroblasts; rp, red pulp; wp, white pulp


RP
WP
RP
a
mØ
eb
b
cv
c
d

The data from our differentiation studies indicated that the antigen recognized by ER-HR3 probably is expressed by the more mature cells of the mononuclear phagocyte system. The number of ER-HR3-positive cells in cultures of CSF-1-stimulated bone marrow cells increased gradually in time (Fig. 12.4). Additionally, when distinct macrophage cell lines are ordered in a differentiation sequence (Leenen et al., 1986b; Klaasen et al., 1988), it was demonstrated that the antigen was predominantly present on cell lines with a more mature phenotype (Fig. 12.5). Presumably, expression of the antigen starts on monocytes since part of them is stained positively by the ER-HR3 antibody (de Jong et al., 1990a).

To obtain more information about the functional properties of the antigen we investigated its expression under different experimental conditions, i.e. BCG infection and PHZ treatment. Organs of BCG-infected mice were removed at day 21. Around this day the number and size of granulomata in the various organs reached maximal values (Nibbering et al., 1987c). The localization and expression of the distinct macrophage antigens under these conditions differred markedly. Both the number of positive cells and the expression of the Ia (Fig. 12.6e) and F4/80 (Fig. 12.6f) antigens were greatly increased. Moreover, Ia and F4/80-positive cells were present throughout the liver. In contrast, Mac-1 (Fig. 12.6g) and ER-HR3 antigen (Fig. 12.6h) expression was confined to cells present in granulomata. Like in control mice, the Kupffer cells and other sinusoid-lining cells did not stain for these antigens. Therefore, it is likely that macrophages expressing these markers are recruited from other sites caused by the BCG infection. Such recruitment of Mac-1-positive cells has also been described after infection of mice with Plasmodium yoelii or Listeria monocytogenes (Gordon et al., 1988a). The observation that the localization of ER-HR3-positive cells in the lungs of BCG-infected mice was restricted to granulomata around the arteries and not around the bronchi (Fig. 12.6d) favours the hypothesis that only the migratory macrophages express this marker. Macrophages in granulomata around bronchi that express Mac-1 but not ER-HR3 (Fig. 12.6c) could be derived from the lung's own resident macrophage population. Other studies with infectious agents indicate that the expression of MHC class II antigens is correlated with macrophage activation (Ezekowitz et al., 1981; Adams and Hamilton, 1987). In our study we demonstrated that ER-HR3 expression and Ia expression did not co-exist as was demonstrated by their distinct distribution patterns. Therefore, it is likely that the ER-HR3 antigen is not involved in the process of activation.

ER-HR3 antigen expression was also investigated after PHZ injection. It has been demonstrated that repeated injections of PHZ cause a hemolytic anemia in adult mice that is accompanied by extramedullary erythropoiesis in the liver (Ploemacher and van Soest, 1977). Interestingly, after PHZ-treatment many cells in the liver were stained

positively by ER-HR3 (Fig. 12.7c). They occurred in the liver sinusoids and venes. Some of them were surrounded by a rim of erythroblasts (Fig. 12.7c, insert) and were considered as central macrophages. Carbon injection after the PHZ treatment resulted in carbon labelling of all ER-HR3-positive cells, including the central macrophages (Fig. 12.7d). Although an influx of macrophages from other sites can not be excluded, this result suggests that the ER-HR3 antigen after PHZ-treatment is present on a subpopulation of Kupffer cells. The observation that Kupffer cells under anoxic conditions were able to produce erythropoietin (Rich et al., 1987) together with our observation that ER-HR3-positive Kupffer cells were surrounded by erythroblasts argues for a role of the ER-HR3 antigen in erythropoiesis. Moreover, it has been demonstrated that the antigen is expressed by renal medullary interstitium cells (de Jong et al., 1990a). Under physiological conditions erythropoietin is produced at this site (Schuster et al., 1987). Other indications for the presumed function of the ER-HR3 antigen in erythropoiesis are the occurrence of erythropoietic islands with ER-HR3-positive central macrophages in the fetal and adult spleen as well as the fetal liver and adult bone marrow (de Jong et al., 1990a). Nevertheless, ER-HR3-positive cells also occurred at other locations and it cannot be excluded that the antigen is involved in other processes as well. For example, the presence of ER-HR3-positive cells in the T cell-associated regions of lymph nodes may be indicative of a function in the immune response. Our observation that ER-HR3 was unable to inhibit antigen-specific T cell proliferation makes it unlikely that the ER-HR3 epitope is directly involved in antigen presentation.

Acknowledgements. The authors thank Prof.Dr. O. Vos and Dr. P.J.M. Leenen for their critical review of the manuscript, Mr. Tar van Os for printing the photographs and Mrs. Cary Meijerinck-Clerkx for typing the manuscript.

13. ERYTHROPOIESIS AND MACROPHAGE SUBSETS IN MEDULLARY AND EXTRAMEDULLARY SITES

J.P. de Jong, P.G.J. Nikkels, A.H. Piersma and R.E. Ploemacher

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SUMMARY

Morphological and functional evidence exists that macrophages support in vivo erythropoiesis. Using cultured primary adherent cells from murine bone marrow as antigenic source, we have prepared a hybridoma cell line secreting a monoclonal antibody (mAb) to reticular cells of bone marrow and non-lymphoid domains in the spleen. The mAb (ER-HR3) also binds to some elongated cells in the subcutaneous tissue and to intertubular areas of the renal medulla. In addition, the ER-HR3 antigen is expressed on some reticular cells in the capsular sinuses and paracortex of lymph nodes and in the lamina propria of the ileum and colon, with increasing expression towards the distal ileum. The expression of the ER-HR3 antigen in non-lymphoid domains of hemopoietic organs is proposed to be associated with adult type hemoglobin (Hb) erythropoiesis as evidenced by (1) the absence in the yolk sac, (2) the exact correlation with the presence of adult type Hb erythropoiesis, but not granulopoiesis, in fetal and neonatal liver and spleen and postnatal bone marrow and spleen, and (3) the correlation with phenylhydrazine-induced hepatic erythropoiesis in the adult liver.

Following injection of highly purified ER-HR3 into neonatal mice, a transient decrease of hemopoietic progenitor cells, but not of day-7 and day-12 CFU-S, was observed in the bone marrow, resulting in a moderate macrocytic anemia. Injection of ER-HR3 also limited the erythropoietin-induced ^{59}Fe incorporation in the bone marrow of adult hypertransfused mice. The data obtained sofar, strongly suggest that the ER-HR3 antigen is exclusively expressed by a macrophage subset.

INTRODUCTION

There is evidence that macrophages play a supportive role in in vivo erythropoiesis. Erythroblasts are aggregated around so-called "central" macrophages in bone marrow (Bessis and Breton-Gorius, 1962), spleen (Orlic et al., 1965) and yolk sac (Sorenson, 1961), and presumably in fetal liver (Zamboni, 1965; Naito and Wisse, 1977). The adherence of the erythroid cell clusters to this macrophage seems to be relatively strong as evidenced by the fact that erythroid islets can be isolated from bone marrow (Le Charpentier and Prenant, 1975; Macario et al., 1981; Crocker and Gordon, 1985). Indeed, central macrophages may branch between the adjacent erythroblasts with very thin sheath-like and thread-like protrusions (Bessis and Breton-Gorius, 1962; Ploemacher et al., 1977a; Crocker and Gordon, 1985). Different stages of erythroblasts have been observed to surround the macrophage with the most differentiated cells at

the outside (Le Charpentier and Prenant, 1975). It seems probable that also early erythroid precursor cells may be located in the vicinity of these macrophages (Orlic et al., 1965; Ben-Ishay and Yoffey, 1974; Ploemacher, 1979). In long-term bone marrow cultures, in which erythroid activity had been greatly augmented in the presence of anemic mouse serum and erythropoietin, erythroblasts were also specifically located on top of flattened macrophages (Allen and Dexter, 1982).

The function of macrophages in erythropoiesis is as yet not fully understood. They have a surveillance function as evidenced by their phagocytosis of effete erythroblasts and expelled normoblast nuclei (Cavallin-Stahl et al., 1974). Furthermore, macrophages may facilitate interaction between cells of the developing erythroid clone, while the macrophage capability to recognize cell membrane epitopes (Shortman et al., 1970; Rosenstreich et al., 1976) may serve as a mechanism which regulates cell traffic in the hemopoietic cords.

Macrophages have been described to be able to produce a number of regulatory substances and hemopoietic growth factors, including erythropoietin (Rich et al., 1982; Rich, 1986a, b) and activities that determine the cell cycle state of day-8 CFU-S (Wright et al., 1982). Suggestive evidence has been found for a mediatory role of bone marrow macrophages in the modulation of early erythropoiesis via the production of burst feeder activity by bone marrow stromal cells (Ploemacher et al., 1979; Rich, 1986b).

The present paper is concerned with the composition of ectopic erythroid clusters in anemic mice. In addition, the immunohistochemical localization of the ER-HR3 antigen, and its possible role in erythropoiesis is reported.

MATERIALS AND METHODS

Mice. Conventional male (C57BL/Rij x CBA/Rij)F1 mice, 14-20 weeks old, were used. They were purchased from the Laboratory Animals Centre of the Erasmus University, Rotterdam, and the REPGO TNO Institute, Rijswijk, The Netherlands.

Colony assays. Hemopoietic stem cells (CFU-S) were quantitated according to Till and McCulloch (1961). Hemopoietic progenitor cells (CFU-GM, BFU-E and CFU-Mix) were quantitated in semi-solid cultures as previously described (de Jong et al., 1984).

Light and electronmicroscopy. Details have been published previously (Ploemacher et al., 1977a, b).

Morphology of ectopic erythropoiesis

In order to investigate whether macrophages are also associated with erythroid clusters in ectopic locations, we injected adult mice with four daily i.p. injections of 1 mg phenylhydrazine (PHZ). This treatment induced a severe transient hemolytic anemia with hematocrit values down to 28 (control 48). As judged by light microscopy, affected red blood cells and their debris were cleared from the circulation mainly by macrophages in the liver, spleen and bone marrow. The anemia caused a marked increase of erythropoiesis as measured by ^{59}Fe-incorporation in the spleen up to 10 times the control level at 7 days after the first PHZ injection. In the bone marrow 40 to 50% of all nucleated cells were erythroblasts from day 3 through day 7, which was nearly twice as much as in untreated mice. Intrasinusoidal erythropoiesis in the marrow was evident for more than a week. In the liver erythroid cells were found to be present in central veins and sinusoids, close to the central veins.

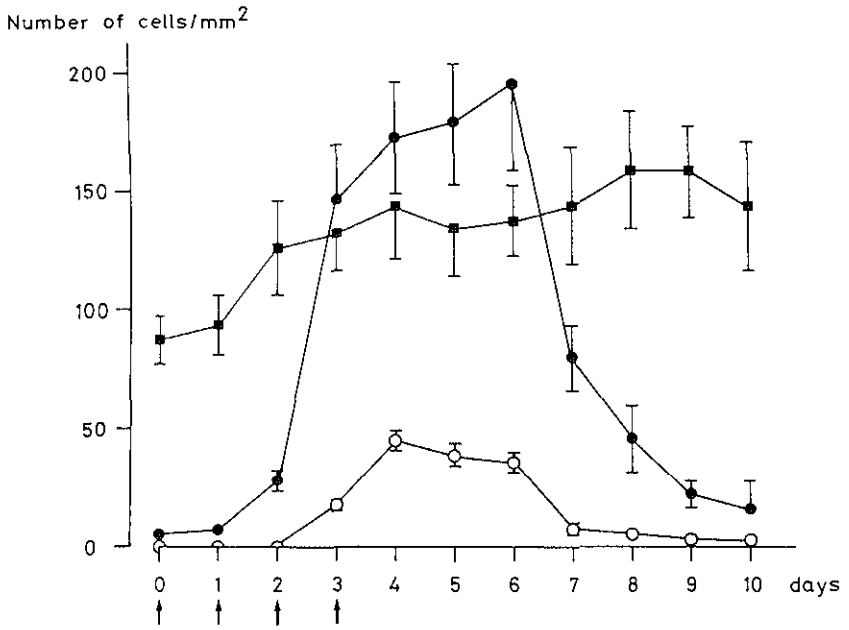

Figure 13.1. Number of cells per area measured in light microscopical bone marrow sections during and after PHZ treatment (arrows). Only cells displaying a nucleus in the plane of section were counted. Bars indicate 1 SEM of the arithmetic mean of at least 6 mice per experimental point.
o, intravascular macrophages; ■, extravascular macrophages; ●, intravascular erythroblasts.

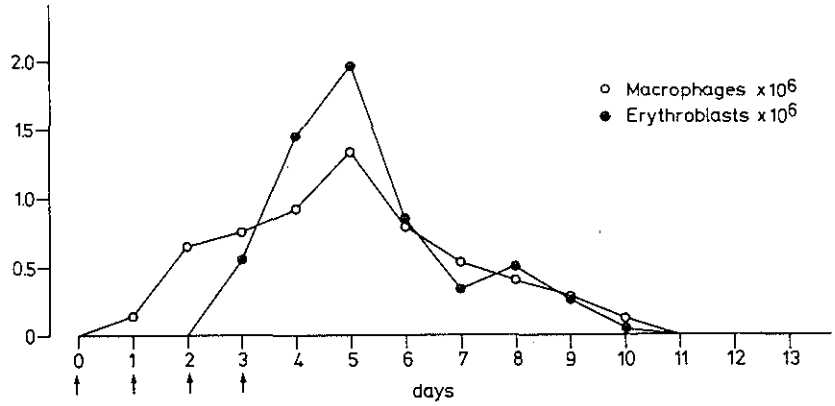

Figure 13.2. Correlation between the number of erythroblasts and monocytes/macrophages in the central veins of the adult liver during and after PHZ treatment (arrows). Each point represents the average cell number per liver in at least 7 animals as determined in a morphometric study of light microscopical sections.

Both in the bone marrow sinusoids (Fig. 13.1) and in the hepatic sinusoids and central veins (Fig. 13.2) erythroblasts were located around erythrophagocytic macrophages. The PHZ-induced increase of macrophages correlated with the appearance of erythroblasts in these sites (Figs. 13.3 and 13.4) and was preceeded by an increase of monocytic cells in various stages of macrophage development as judged by electronmicroscopy. Especially in central veins of the liver and bone marrow sinusoids it was evident that non-resident macrophages served as central macrophages (CM) in erythroid clusters, since in these locations no, respectively few, macrophages are encountered in control mice. Therefore, our observations suggest that part of the erythroid microenvironment is capable of migration.

CM in the liver, central veins and bone marrow sinusoids covered parts of the surrounding erythroblasts with long thread-like cytoplasmic projections, which often showed branching. In the hepatic sinusoids, CM extended filopodia of various length and plate-like protrusions in between the surrounding erythroblasts. These latter cytoplasmic sheaths (Fig. 13.5) had a very regular thickness of about 840 Å, while a regular distance of 250 Å existed between the outer membranes of CM and erythroblasts, and no specialized membrane contacts were observed. The significance of these close contact areas is not yet resolved.

Macrophages in erythroid clusters are a distinct subpopulation of the mononuclear phagocyte system

Using cultured primary adherent cells from normal bone marrow as antigenic source, we have prepared a hybridoma cell line (rat spleen cells x P3-X63-Ag8.653

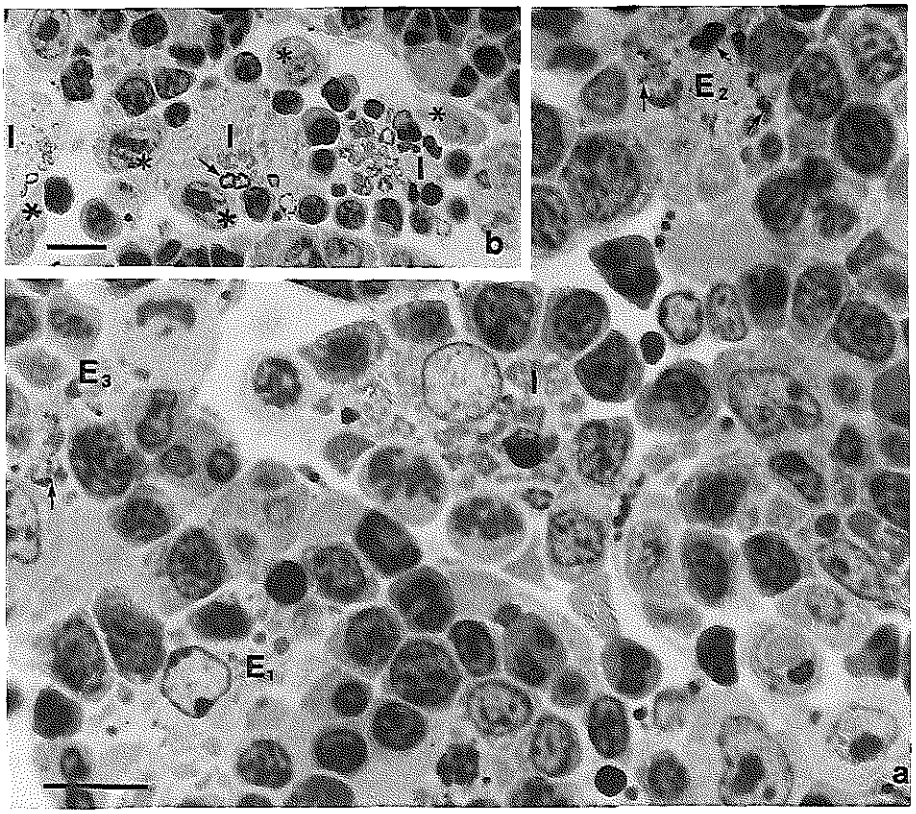

Figure 13.3. (a) Light microscopy of femoral marrow on day 6 after the first of 4 daily injections (on day 0, 1, 2, 3) of PHZ. A large intrasinusoidal central macrophages (1) surrounded by erythroblasts can be observed. Two mitotic cells are present in the erythroid islet. E, extravascular macrophages; I, intravascular macrophages. (b) Intrasinusoidal erythroid islets with ghosts of ingested erythrocytes (arrow), showing Heinz bodies. Bar, 10 μm.

murine myeloma cells; Kearney et al., 1979) secreting a monoclonal antibody (mAb) of the IgG2c subclass that mainly recognizes a subpopulation of macrophages in the splenic red pulp and bone marrow. The mAb, termed ER-HR3 (Piersma et al., 1985), was purified by protein A affinity chromatography and FPLC on a Mono Q anion exchange column and is at least directed against a plasma membrane component of mouse mononuclear phagocytes (de Jong et al., 1990a). To study the tissue distribution of the antigenic determinant reacting with ER-HR3, frozen tissue sections of whole mouse embryos, and of neonatal and adult mouse organs, were consecutively incubated with the purified mAb, sheep-α-rat-Ig conjugated to either horseradish peroxidase or galactosidase, and with diaminobenzidine (DAB method) or 5-bromo-, 4-chloro-, 3-indolyl-β-galactopyranoside (BCIG method) substrate solution, respectively. In the bone marrow relatively few ER-HR3+ve cells were encountered. Such cells showed reticular branching of their cytoplasm, and were found throughout the bone

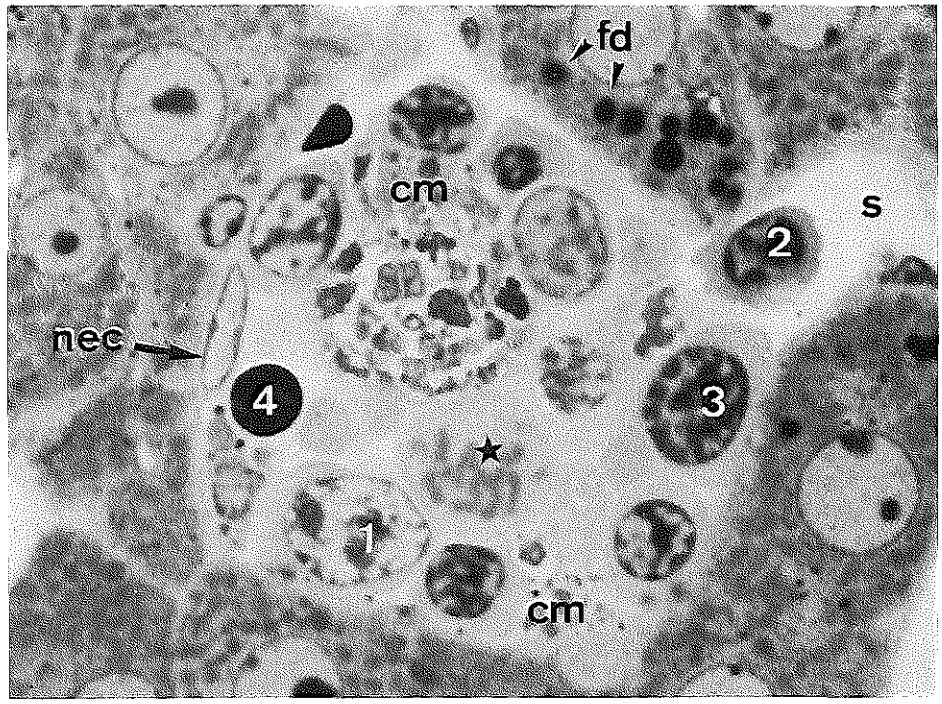

Figure 13.4. Light microscopy of an erythroid islet in a liver sinusoid on day 4 after the first of 4 daily injections of PHZ. A great variation of erythroid developmental stages is present.
asterisk, mitotic cells; nec., endothelial cell nucleus; s, sinusoid; cm, central macrophage; fd, fat droplets in parenchyme cells; 1, proerythroblast; 2, basophilic erythroblast; 3, polychromatic erythroblasts; 4, expelled normoblast nucleus. Magnification x 4356

marrow plug. The extensive layer of F4/80+ve cells (macrophages) adjacent to the subendosteal bone surface (Hume et al., 1984c) was not recognized by ER-HR3. In the spleen (Fig. 13.6) ER-HR3+ve reticular cells were found in erythroid clusters located in the red pulp, and in decreasing frequency towards the marginal zones around the lymphoid domains, which did not contain these cells. Characteristically, small groups of ER-HR3+ve cells were always found directly beneath the splenic capsule. The number of ER-HR3+ve cells in the spleen was significantly less than the number of cells reacting positively with Mac-1 (M1/70; Springer et al., 1970), Mac-2 (M3/38; Ho and Springer, 1982), Mac-3 (M3/84; Ho and Springer, 1983) or F4/80 (Austyn and Gordon, 1983). In addition to cells in the bone marrow and splenic red pulp, the mAb stained some elongated cells in the stroma of the renal medulla and in the lamina propria of the gut. The highest concentration of ER-HR3+ve cells was found in the lamina propria of villi and crypts in the lower ileum, and their number rapidly decreased towards the proximal ileum and the colon, which only showed some scattered mAb+ve cells in the lamina propria under the crypts. Furthermore, staining

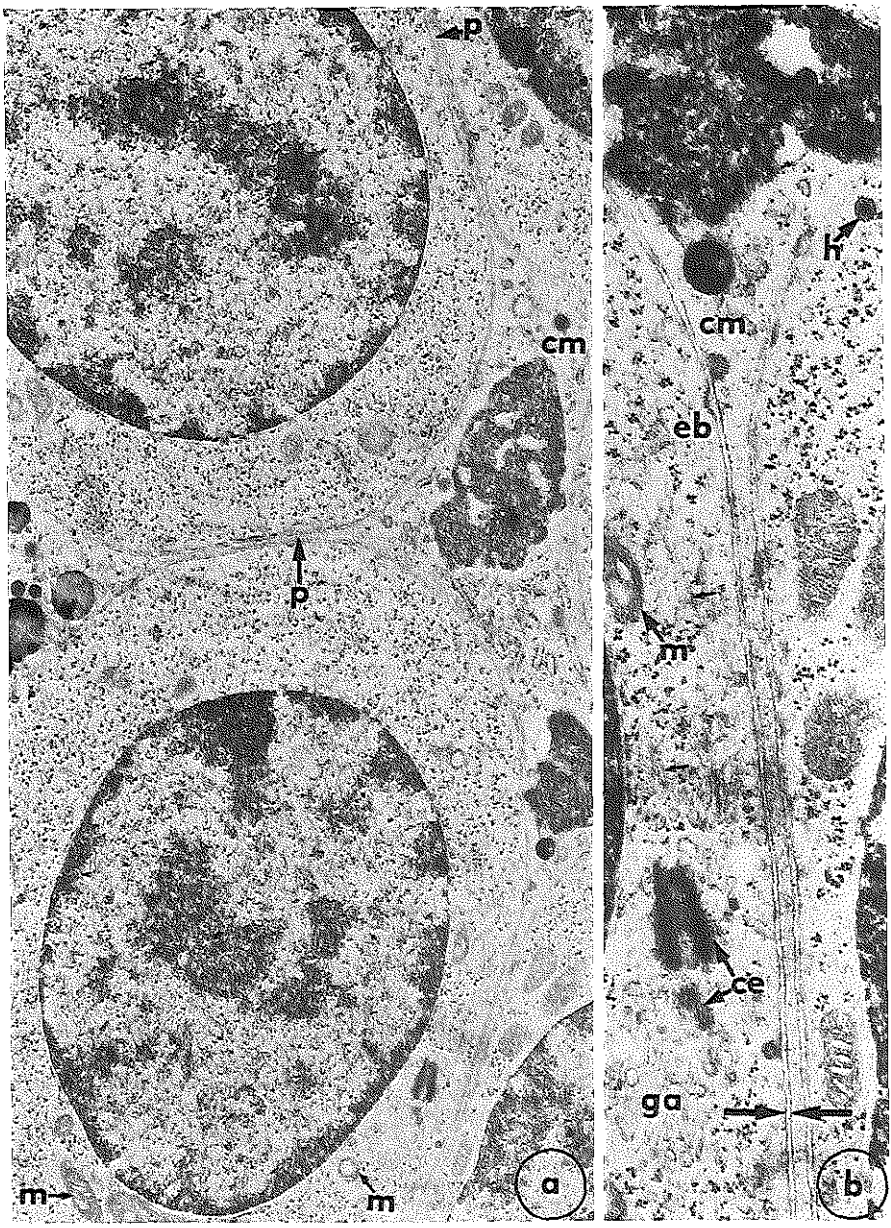

Figure 13.5. (a) Electronmicroscopy of adult mouse liver on day 7 after the first of 4 daily injections of PHZ. Illustrated is the regular thickness of the intrasinusoidal central macrophage (cm) projections (p) in between the surrounding erythroblasts. (b) Detail from Figure 13.5a. Note the 250 Å cleft (arrows) between the cm and the erythroblasts. ce, centriole. Magnification x 33,800

of some elongated cells in the dermis of the skin was noticed, and was most prominent in a mid portion of the mice between the fore and hind legs. In the capsular sinuses and paracortex of lymph nodes, especially the mesenteric nodes, a few large cells expressed the ER-HR3 antigen. Also thymus and Peyer's plaques contained some

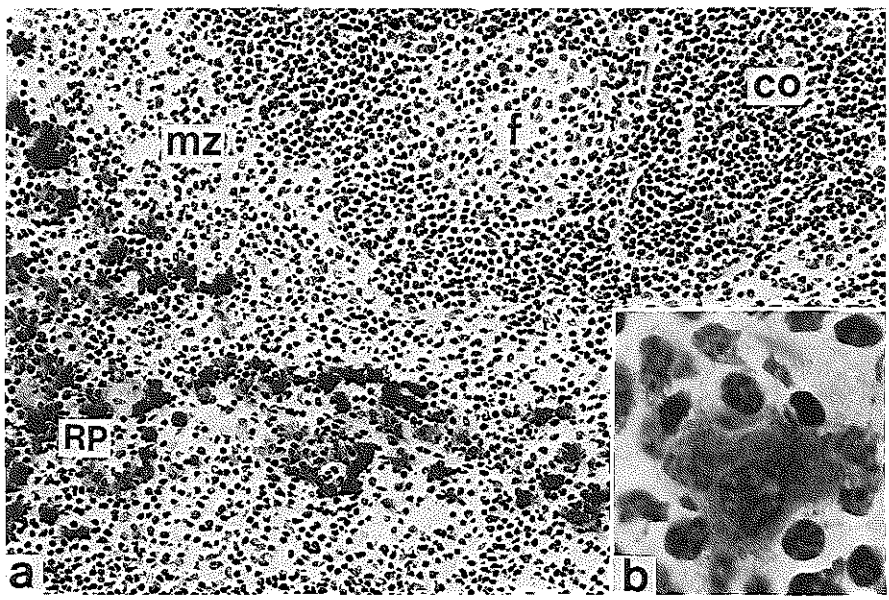

Figure 13.6. (a) ER-HR3+ve cells in the red pulp of the adult mouse spleen. DAB method. Nuclei are counterstained with hematoxylin. f, follicle center; co, corona; mz, marginal zone; RP, red pulp. Magnification x570. (b) Detail of Figure 13.6a. Magnification x 2346.

ER-HR3+ve cells. During embryogenesis, the yolk sac did not react with the mAb. As of day 14 post-gestation (Table 13.1) the fetal liver showed an increasing number of cells staining with the mAb, coinciding with the onset of erythropoiesis in the liver. ER-HR3+ve cells gradually disappeared from the neonatal liver around day 5 after birth, together with the disappearance of erythroid activity in this organ. While bone marrow granulopoiesis in mice normally occurs as of day 18 post-gestation, erythropoiesis and ER-HR3+ve cells were only observed around or directly after birth (Table 13.1). These observations suggest, that ER-HR3+ve cells in non-lymphoid parts of hemopoietic organs are associated with adult type hemoglobin erythropoiesis. The antigen recognized by ER-HR3 was absent from all other well-defined cell types in the body, including osteoclasts, osteoblasts, bone-lining cells and fibroblasts.

Within 15 minutes following i.v. infusion of colloidal carbon particles into adult mice, marginal zone macrophages had phagocytized many particles on histological examination. ER-HR3+ve cells contained less carbon particles when situated in the vicinity of the marginal zones, and showed no signs of carbon ingestion in the center of the red pulp. Four days after the first of 3 daily injections with 1 mg of PHZ per mouse, all ER-HR3+ve cells in the splenic red pulp had greatly increased their cell volume due to the phagocytosis of damaged erythrocytes (Fig. 13.7). Remarkably, scattered ER-HR3+ve cells appeared in the adult mouse liver during PHZ-induced

Table 13.1
Development of hemopoietic activity and expression of ER-HR3

	granulop.act.*		erythrop.act.*		ER-HR3 expression	
	days post-plug	days post-partum	days post-plug	days post-partum	days post-plug	days post-partum
yolk sac (Hb-E)	-	n.p.	7-12.5	n.p.	-	n.p.
liver (Hb-A)	13-21	-	>11	<7	>14	<5
spleen (Hb-A)	>17	>>	>15	>>	>16	>>
bone marrow (Hb-A)	>17.5	>>	-	>1	-	>1

*	after Metcalf and Moore (1971).
>	as of day indicated.
<	until day indicated.
>>	throughout adult life.
n.p.	not present.
Hb-E/A	embryonic or adult type hemoglobin.

hemolytic anemia (Fig. 13.8a). A part of ER-HR3+ve cells in the liver was located in the center of the scattered erythroid clusters that are transiently found in PHZ-treated adult mice (Fig. 13.8b).

In vitro, primary macrophage colonies in CSF-M-stimulated cultures of spleen and bone marrow contained scattered cells expressing the ER-HR3 antigen. Varying percentages of cells in cultures of a series of monocyte/macrophage cell lines stained positively with ER-HR3, i.e. 13 per cent in P388D1 (Koren et al., 1975), 10 per cent in J774 (Ralph et al., 1975), 19 per cent in Pu5-1.8 (Ralph et al., 1974), 14 per cent in WEHI-3B (Warner et al., 1969) and 88 per cent in AP284 (cloned in our laboratory from mouse spleen). In semi-solid cultures of unstimulated bone marrow cells, adherent fibroblast colonies develop within 1-2 weeks (Friedenstein et al., 1970; Wilson et al., 1974). Clusters of macrophages preferentially adhere to these fibroblasts due to locally produced activities, including colony-stimulating factor (Brockbank and van Peer, 1983; Brockbank et al., 1985; Piersma et al., 1984). Granulopoietic activity was absent from these cultures. We followed the appearance of Mac-1+ve fibroblast colonies as an indication of the presence of macrophages in such colonies, and compared this with the presence of ER-HR3+ve cells (Table 13.2). All cells reacting with mAb's to Mac-1 and ER-HR3 were typical monocyte/macrophage as judged by morphological

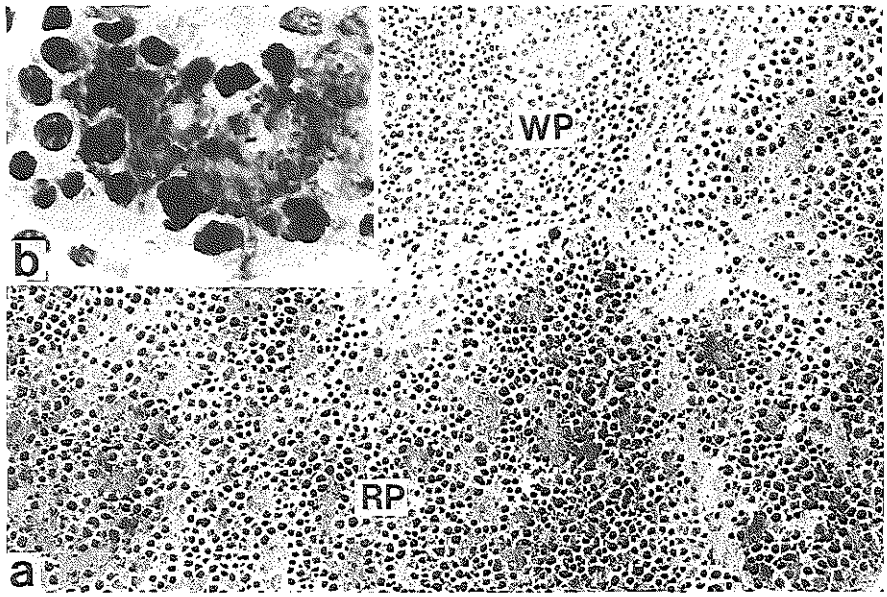

Figure 13.7. (a) ER-HR3+ve cells in the red pulp of an adult mouse on day 6 after the first of 3 daily injections of PHZ. Cells reacting with the mAb are swollen due to phagocytosis of effete erythrocytes, indicating that ER-HR3+ve cells are phagocytic. Method as in legend of figure 13.6. Magnification x 250. (b) Detail of figure 13.7a. Magnification x 650

criteria, i.e. small spherical or elongated to reticular cells with small nuclei as compared to fibroblastic cells. Both the number of colonies containing cells expressing the Mac-1 or ER-HR3 antigen, and the number of cells per colony recognized by the mAb's, increased between day 7 and 14 of culture. However, at all time points the number of ER-HR3+ve colonies and ER-HR3+ve cells per colony was less as compared to Mac-1. After incubation of 3-day-old cultures with silica, the typical macrophages disappeared from the cultures, but reappeared in the following days (Table 13.2). Similar to Mac-1+ve cells, ER-HR3+ve cells were significantly depleted from the cultures, indicating that cells expressing ER-HR3 are phagocytic cells. Antisera directed against Mac-2 and Mac-3 were found to be less useful than the α-Mac-1 used in these studies, since a part of the fibroblastic cells was stained by these mAb's.

Effects of ER-HR3 injection on hemopoietic activity in vivo

We investigated a possible role of the ER-HR3 antigen in hemopoiesis by repeatedly injecting neonatal mice with the mAb (Tables 13.3, 13.4). One and 3 days after

cessation of the i.p. injections on day 16 and 14, respectively, we determined some blood parameters and the number of hemopoietic stem cells (CFU-S), early

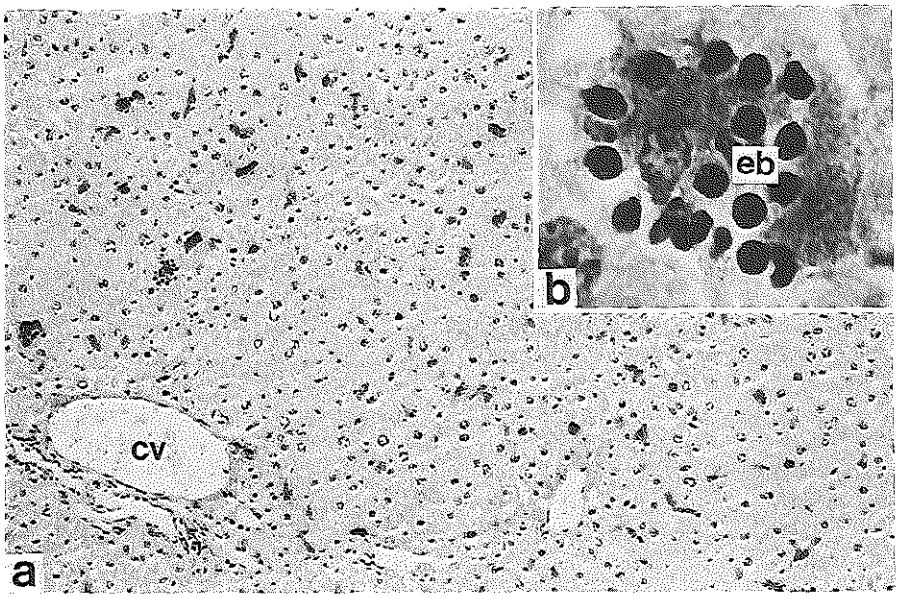

Figure 13.8. (a) ER-HR3+ve cells are present in the liver of adult mice on day 6 after the first of 3 daily injections of PHZ. cv, central vein. Magnification x 250. (b) Detail of Figure 13.8a. A ER-HR3+ve cell is surrounded by erythroid cells (eb). Magnification x 1340

progenitors (CFU-Mix, BFU-E, CFU-GM) and nucleated cells in their spleen and femurs. The number of fibroblastic progenitors (CFU-F) in the spleen was also determined. After the last mAb injection (Table 13.3) a significant transient decrease was observed in the number of erythrocytes per ml of blood and an increase in MCV. The number of femoral nucleated cells and hemopoietic progenitors, but not of CFU-S was decreased as well (Table 13.4). In contrast, the splenic content of CFU-S and CFU-GM was significantly increased after cessation of the ER-HR3 injections, while splenic CFU-F numbers were much lower than in the control spleens. These observations suggest that injection of ER-HR3 depresses bone marrow hemopoietic activity in neonatal mice. The compensatory increase of splenic hemopoiesis was apparently not sufficient to avoid the development of a moderate macrocytic anemia.

The effect of ER-HR3 injection on the burst of erythropoietic activity in Epo-injected hypertransfused adult mice is presented in Table 13.5. In hypertransfused mice, ^{59}Fe incorporation was severely depressed and erythropoietic activity was virtually absent. Following injection of Epo, erythropoiesis was stimulated in spleen and bone marrow despite the high hematocrit (65-70 after day 2). In the blood, ^{59}Fe-incorporating reticulocytes appeared. Both in the presence of polyclonal rat IgG as a control, or ER-HR3, the Epo-induced ^{59}Fe-incorporation was significantly decreased in blood and spleen. In the bone marrow, ER-HR3 injection elicited a specific decrease of the Epo-induced ^{59}Fe-incorporation, which was not observed following injection of polyclonal

Table 13.2

Relative numbers of fibroblast colonies containing adherent cells recognized by ER-HR3 or Mac-1 in short-term semi-solid bone marrow cultures

	ER-HR3				Mac-1+			
	A	B	C	D	A	B	C	D
no silica:								
day 7	12	42	27	19	0	47	41	12
day 10	12	6	55	27	6	6	32	56
day 13	8	25	52	15	0	8	3	89
with silica:								
day 7	46	15	31	8	0	80	12	8
day 10	55	27	18	0	26	40	11	23
day 13	41	28	20	11	36	24	26	12

Cat. A: no mAb+ve cells in fibroblast colony;
Cat. B: less than 10 mAb+ve cells;
Cat. C: centrally located cluster of mAb+ve cells:
Cat. D: colony overlayed with mAb+ve cells.

2.5×10^5 nucleated BMC per ml were cultured in 24-well Costar culture plates containing α-medium supplementd with 20% fetal calf serum and 0.8% methylcellulose (MC) as viscous medium. After 3 days of culture at 37°C, the culture medium was removed from the cells that adhered to the plastic by washing twice with PBS. Cultures were than incubated for 2 hrs with or without 0.1 mg of silica in PBS + 20% FCS to induce lysis of phagocytic cells. Subsequently, the wells were washed twice with PBS and complemented with fresh medium and 0.8% MC. After a total culture period of 7, 10 or 13 days the medium was removed and the cells stained for ER-HR3 or Mac-1 after fixation with 0.05% glutaraldehyde in PBS.

rat IgG. These data suggest that the presence of the ER-HR3 epitope is favourable for optimal erythropoietic activity in the bone marrow.

DISCUSSION

In adult mice suffering from a hemolytic anemia, ectopic erythropoietic islets were observed in the bone marrow sinusoids, the liver sinusoids and central veins. Within one day after the first of 4 daily injections of PHZ, monocytic cells appeared at these locations and differentiated into macrophages within another 2 days. Erythroblasts were observed to surround these macrophages and form characteristic erythroid clusters. Although CM showed internalization of effete erythrocytes, no evidence of phagocytosis of nucleated cells was obtained. These observations suggest that CM are a prerequisite for erythropoiesis in vivo. Apparently, the intravascular CM were not derived from the resident macrophage populations, but originated from immigrating

monocytes.

As defined by the expression of the ER-HR3 antigen, CM belong to a subpopulation of macrophages that has a very restricted distribution pattern in the body. The proposal that ER-HR3 is a macrophage specific antigen, at least in spleen and bone marrow, evolves from a variety of observations. Without exception, ER-HR3+ve cells accumulated large numbers of effete erythrocytes during PHZ-induced hemolytic anemia, whereas part of these cells phagocytized colloidal carbon particles. ER-HR3+ve cells could be largely depleted from adherent bone marrow cell cultures by silica treatment in a way similar to the depletion of Mac-1+ve cells (Table 13.2). In addition, ER-HR3 is expressed by part of the cells from a series of established monocyte/macrophage cell lines. Using immuno-electronmicroscopy we have recently

Table 13.3
Effect of repeated ER-HR3 administration on some blood parameters in neonatal mice

	hematocrit (L/L)	erythrocytes x 10^{12}/L	MCV (μm^3)	leucocytes x 10^9/L
control	28.8 (1.6)*	5.6 (0.2)	51.4	9.1 (1.2)
day 1	27.6 (1.0)	4.8 (0.1)	57.5	11.5 (0.9)
day 3	31.4 (1.1)	5.5 (0.1)	57.1	10.5 (0.7)

* Arithmetic mean (1 SEM) of 8 individually assayed mice.
All animals were assayed on day 17 after birth. Fifty μg of IgG (ER-HR3) per mouse was injected in the suborbital plexus on day 0, 2 and 4 after birth, and 100 μg of mAb was injected i.p. on day 7, 9, 11, 14 and 16. The last injection was omitted in the case of the 3 day group. Control mice were injected ith the vehicle, i.e. either 50 or 100 μl of PBS, on the same days, including day 16.

demonstrated that ER-HR3 in the spleen and bone marrow is exclusively expressed by macrophages including the CM of erythroid clusters (de Jong et al., 1990a). Since the number of ER-HR3+ve cells both in bone marrow cultures and in freeze sections of embryonic, neonatal and adult mouse tissues was much smaller than the number of cells reacting with mAb's to Mac-1, Mac-2, Mac-3 and F4/80, we conclude that ER-HR3+ve macrophages are a small subpopulation of body macrophages.

It is not yet clear whether the ER-HR3 antigen has a function in hemopoiesis. However, the present study provides various clues to a role of the antigen in hemopoietic organs. The expression of ER-HR3 (Table 13.1) strictly coincided with the presence of erythropoietic activity (Petrakis et al., 1969; Metcalf and Moore, 1971). In

view of this, and the absence of ER-HR3+ve cells from the yolk sac, we tentatively

Table 13.4
Effect of repeated ER-HR3 administration on the population size of various hemopoietic progenitor cells in neonatal mice

	nucleated cells ($x10^7$)	CFU-S d.7	CFU-S d.12	CFU-GM d.7	CFU-GM d.12/13	BFU-E d.12/13	CFU-Mix d.12/13	CFU-F d.10
Femur:								
control	100	100	100	100	100	100	100	n.d.
day 1	86*	89	103	40	53	69	43	n.d.
	(6)	(20)	(15)	(25)	(15)	(22)	(16)	
day 3	95	102	125	147	101	135	148	n.d.
	(6)	(21)	(26)	(69)	(27)	(37)	(69)	
Spleen:								
control	100	100	100	100	n.d.	n.d.	n.d.	100
day 1	118	144	132	116	n.d.	n.d.	n.d.	41
	(13)	(23)	(13)	(16)				(11)
day 3	108	167	190	164	n.d.	n.d.	n.d.	36
	(11)	(26)	(19)	(31)				(11)

* Arithmetic mean (1 SEM) of 8 individually assayed mice, expressed as a percentage of control.

Absolute numbers in control femurs were: CFU-S (colonies counted on day 7): 3100; CFU-S (day 12): 1850; CFU-GM (day 7): 4468; CFU-GM (day 12/13): 14,264; BFU-E: 4294; CFU-Mix:3502.
Absolute numbers in the control spleens were: CFU-S (day 7): 4725; CFU-S (day 12): 1438; CFU-GM (day 7): 15,277; CFU-F: 1346.

concluded that ER-HR3 is associated with adult type hemoglobin production. The in vivo effects of ER-HR3 in neonatal mice were not in full support of this conclusion, since ER-HR3 did not only decrease erythroid progenitor cell (BFU-E) numbers in the bone marrow, but that of granulocyte/macrophage (CFU-GM) and the early mixed colony-forming cells (CFU-Mix) as well. One day after the last ER-HR3 injection the mice had a moderate macrocytic anemia. Due to the large amounts of purified mAb required for injection, we were unable to study the effect of higher doses of ER-HR3 or a daily injection schedule on in vivo blood cell formation. The observed effects are considered to be specific to ER-HR3, and not due to an immune reaction to the mAb, as neonatal mice are tolerant to mAb injection. Since macrocytic anemias are a consequence of metabolic deficiencies (e.g., vitamin B12, folic acid) or defects in either

the erythropoietic precursors, including stem cells or regulatory cells in erythropoietic

Table 13.5
Effect of ER-HR3 injection on the erythropoietin-induced ^{59}Fe-incorporation in hypertransfused mice

Injected with:	blood (1 ml)	spleen (g^{-1})	spleen (total)	bone marrow (total)
PBS	0.9(0.1)*	32.5(6.7)	3.5(0.7)	6.7(2.6)
PBS + Epo	18.2(1.6)	75.1(16.2)	11.1(2.8)	23.0(2.7)
IgG + Epo	12.7(0.8)	57.6(6.3)	10.3(1.3)	21.3(3.3)
mAb + Epo	12.3(1.6)	59.7(9.1)	9.3(1.6)	15.3(0.9)

* Arithmetic mean (1 SD) of 4 individually assayed mice per experimental group.
Epo: erythropoietin; 6 U i.v. per mouse.
IgG: polyclonal rat IgG; 0.8 mg i.v. per mouse.
mAb: anti-ER-HR3; 0.8 mg i.v. per mouse.
Adult mice were made polycythemic by an infusion of 0.56 ml of packed isologous erythrocytes i.v. on day 1 and 2. On day 4 and 5 mice were injected i.v. with either PBS, Epo, rat IgG or ER-HR3. One μCi of $^{59}FeCl_3$ was injected on day 6 and measurement of radioactivity was done on day 7.

organ stroma, these data provide evidence that ER-HR3 does not affect the systemic Epo level.

Injection of ER-HR3 also affected the Epo-induced erythropoietic activity in the bone marrow of adult hypertransfused mice. It should be noted here, that we included relatively crude rat polyclonal IgG as a control, since we had no irrelevant mAb of the IgG2c class available at the time of the study. This polyclonal IgG is known to elicit a significant larger immune response as compared to the monospecific ER-HR3. It is therefore possible that the polyclonal IgG control group may have obscured a specific effect of ER-HR3 on the splenic ^{59}Fe-incorporation following Epo injection. It is evident that further studies have to be carried out to elucidate the presumptive role of ER-HR3 in hemopoiesis.

Acknowledgements. The authors are indebted to Dr. P.J.M. Leenen for characterization of macrophage cell lines with respect to ER-HR3, and to Mrs. J.S.A. Voerman and Mr. N.H.C. Brons for excellent technical assistance and to Mrs. C. Meijerink-Clerkx for typing the manuscript.

14. GENERAL DISCUSSION

More detailed discussions on the results obtained in these studies are represented in the discussion sections of Chapters 6-13. This general discussion will mainly be restricted to some general objectives and observed trends.

14.1. Fixation with hexazotized pararosanilin

The fixation method used in the practical work of this thesis was recently developed in our laboratory. Fixation of frozen tissue sections was performed with the diazonium salt of pararosanilin. The compound often is referred to as 'hexazotized' pararosanilin since it contains a total of six nitrogen atoms present in three diazonium groups. These diazonium groups are able to react with the phenol, indole and imidazole group of tyrosine, tryptophan and histidine, respectively (Burnstone, 1962; Pierce, 1968). Binding of one molecule hexazotized pararosanilin with amino acids present in distinct proteins may result in protein cross-linking and thus fixation. However, fixatives have to fulfil multiple criteria: **(1)** they must have good preservative properties on the tissue morphology; **(2)** they must immobilize the antigen; **(3)** they must preserve the antigen reactivity and **(4)** they must allow adequate permeability of the tissue to the immunological reagents. The first two criteria often demand a fixative with rapid and complete fixation properties, whereas the latter two criteria demand the lowest degree of fixation that is practically possible. All fixation procedures in immunohistochemistry so far attempt to reduce fixation to a point where antigenicity is preserved while maintaining structural integrity.

Fixation with hexazotized pararosanilin complies with most of the above mentioned criteria. The fixative has very good preservative properties on tissue morphology without loss of antigenicity for many antigens (see Chapters 6 and 7). After immunostaining, positivity is confined to specific cell types and no diffusion artefacts are observed. However, the permeability for the immunochemical reagents is somewhat reduced in regions with high cell density. Consequently, the staining intensity of the enzymatic reaction in these regions is less intense when compared to that obtained with acetone fixation. Rinsing the sections with phosphate-buffered saline just before pararosanilin fixation strongly improved the immunostaining of these regions. It is assumed that rinsing with phosphate-buffered saline results in a slight tissue shrinkage. Micro spaces formed by this process facilitate the penetration of the immunoreagents

to the antigenic sites after fixation with hexazotized pararosanilin. Another approach to overcome the problem of the reduced immunostaining in regions with high cell density, not considered in the practical work of this thesis, is the use of other diazonium salts as fixatives. Two possible candidates which are also used in enzyme histochemistry are the diazonium salts of O-dianisine (Fast Blue B) and p-p-diaminodiphenylamine (Fast Black B). Both diazonium salts contain two diazonium groups (Fig. 14.1) and thus, like pararosanilin, should be able to cross-link proteins. However, cross-linking will be less intense because both compounds lack one diazonium group compared to pararosanilin. As a consequence, reduced cross-linking may improve the penetration of immunoreagents in cell dense tissues.

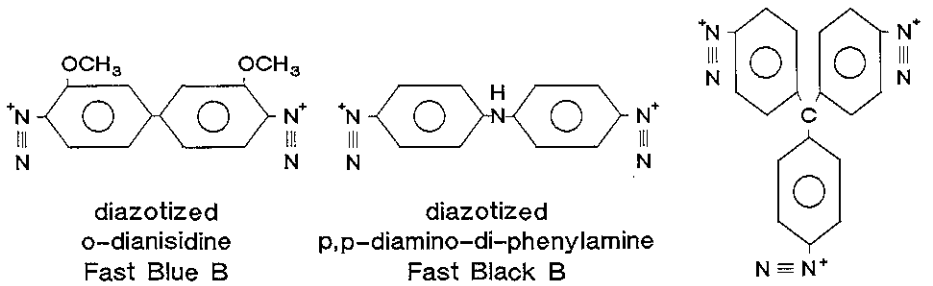

Figure 14.1. Chemical structure of various diazonium salts.

Despite rinsing of the sections with phosphate-buffered saline, pararosanilin fixation reduces the antigen reactivity of for example ER-TR6 and FcRII (Chapter 6) compared to acetone fixation. The precise mechanism by which this happens is not known. The assumption that pararosanilin-reactive amino acids are abundant in the recognized epitopes might explain the observed phenomenon since extensive cross-linking of the antigen may alter its reactivity. In contrast to ER-TR6 and FcRII, the immunoreactivity of other antigens is improved after fixation with hexazotized pararosanilin. For example, Thy-1-positivity is increased greatly in many organs (Chapters 6, 8, 9, and 10). Especially the staining of connective tissue components by 59-AD2.2 is improved. Whether this was due to better preservation of extracellular matrix components or to the introduction of false positivity due to cross reactivity with a pararosanilin-altered antigen remains a matter of speculation. However, the observation that in human tissues the antigen is present on fibroblasts and extracellular matrix components

(Rettig et al., 1988) favours the former proposal. Moreover, hexazotized pararosanilin especially improves the morphology of stromal regions. Within these regions extracellular matrix components are abundant.

It should be noted that hexazotized pararosanilin is only suitable for the fixation of frozen sections. The poor penetrative properties of the compound do not allow in situ fixation by perfusion.

In conclusion, the developed fixation method with hexazotized pararosanilin is effective in that **(1)** the antigenicity of most antigens is preserved; **(2)** the tissue preservation is good; **(3)** the fixation time is short, **(4)** the method permits the use of fluorescence techniques; and **(5)** the stock solutions can be stored at 4°C for a longer period.

14.2. Localization and characterization of macrophage subsets in murine organs

As outlined in the Chapter 2, mechanisms accounting for macrophage heterogeneity are rather complex. Local stimuli, e.g. specific cell-cell interactions and produced factors are likely to contribute to or to influence heterogeneity. As a result, so-called 'microenvironments' or 'niches' are formed. Within these niches the various macrophage populations develop with their own characteristics and functional properties. Assuming that macrophages present in a niche are influenced by the same stimuli, their phenotypes should be identical or at least greatly overlap. This hypothesis predicts that at specific micro-anatomical sites specific macrophage subsets are present.

The objective of this thesis was to characterize the macrophages present in murine organs in detail with special attention to the occurrence of macrophage subpopulations.

The characterization of the splenic, gastro-intestinal and renal macrophage populations is described in the Chapters 8, 9, and 10, respectively. In general, two populations of macrophage-like cells were recognized. The first population consisted of small dendritic cells that constitutively expressed the Ia antigen and lacked acid phosphatase and nonspecific esterase activity. Part of these cells additionally expressed other macrophage antigens such as F4/80, Mac-1, BM8 and FcRII (Chapters 9 and 10). The second population consisted of larger cells that expressed most macrophage antigens investigated. Many cells of this population exhibited nonspecific esterase and acid phosphatase activity. Cells of the first population share many characteristics with dendrocytes. Their possible relationship with macrophages will be discussed in section 14.3. Cells of the second population likely represent macrophages. With exception of some micro-anatomical sites in the spleen, a further

subdivision of this population into distinct phenotypically well-defined macrophage subsets could not made. The discrepancy between the spleen and the other organs with regard to this subject will be discussed below.

An attempt to describe the precise location of distinct macrophage subsets in the organs was made by subdividing them in specific regions. Compartmentalization of the spleen was based on its anatomical diversity (van Ewijk and Nieuwenhuis, 1985), whereas compartmentalization of the gastro-intestinal tract and kidney is based mainly on the presence of histologically different tissues. In the spleen compartments such as the marginal zone and the germinal center probably represent specific functional

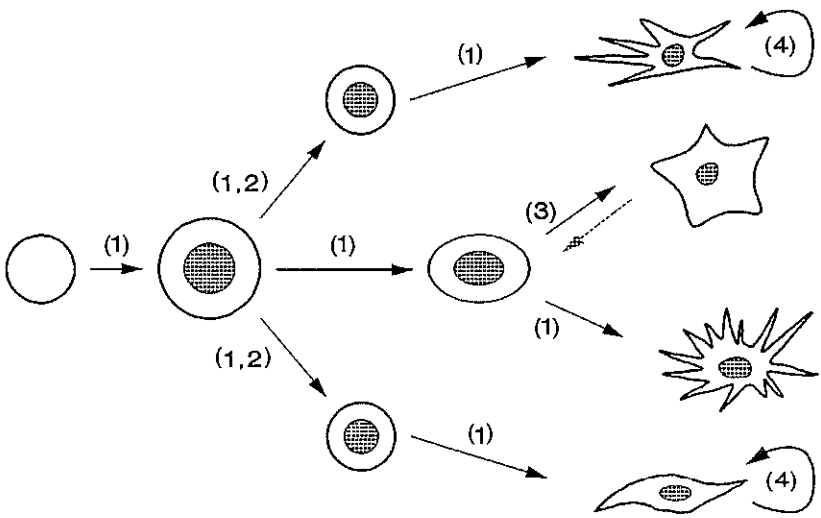

Figure 5. Processes involved in macrophage heterogeneity.
(1) maturation, (2) differentiation, (3) activation, (4) local proliferation

microenvironments and this explains why defined macrophage subpopulations can be recognized here. However, little is known about the functional organization in other organs at a local level. This hampers the adequate compartmentalization of functional niches.

Inadequate compartmentalization is certainly not the only reason why distinct phenotypically well-defined macrophage subpopulations could not be demonstrated. For example, at some micro-anatomical sites of the spleen such as the T cell area in the white pulp, the macrophage populations are rather heterogeneous. Likely, in the generation of macrophage heterogeneity processes such as maturation, activation and differentiation are involved. A schematic view of these processes is presented in Figure 14.2 and they will be discussed shortly.

Maturation of macrophages is accompanied by functional and phenotypical changes as has been demonstrated by many investigators. Maturing macrophages evolve in their function, enzyme activity, secretion of substances, expression of surface receptors and antigens. For example, the expression of the antigens F4/80 (Hirsch et al., 1981), BM8 (Malorny et al.,1986) and ER-HR3 (Chapter 11) and the M-CSF receptor (Nicola, 1987) increases during maturation. Additionally, the number of lysosomes (Cohn and Benson, 1965), the secretion of Apoprotein E (Werb and Chin, 1983) and β-galactosidase activity (Bursuker et al., 1982) increase. However, the natural cytotoxic activity against tumor cells and micro-organisms (Lohman-Matthes et al., 1979; Decker et al., 1986; Baccarini et al., 1988) and the production of plasminogen activator and IFN (Neumann and Sorg, 1980; Werb and Chin, 1983), are processes related to immature and intermediate stages of maturation, respectively.

Macrophage activation is a multistep process aimed at initiating or enhancing specific macrophage functions necessary to annul the pathogenic stimulus. Distinct levels of activation can be recognized and each level is accompanied by specific functional and phenotypical characteristics. Therefore, activation greatly enlarges macrophage heterogeneity. Examples of phenotypical and functional changes occurring upon activation have been given before on pages 30 to 32, and will not be discussed further.

Macrophage differentiation also contributes to heterogeneity. This process is associated closely with the former two processes. However, differentiation is defined as an irreversible process that only occurs in dividing cells and is accompanied by clear morphological, phenotypical and functional differences.

Two other phenomena are likely to be involved in the generation of macrophage heterogeneity although their role is less convincingly demonstrated, i.e., the early delineaging of different macrophage precursor populations from stem cells and local proliferation of distinct macrophage subpopulations.

The existence of more than one pre-defined macrophage progenitor cell is proposed by Bursuker and Goldman (1982, 1983). In their view, resident peritoneal macrophages, expressing a high 5'nucleotidase activity are derived from another precursor than exudate macrophages, expressing a low 5'nucleotidase activity. Under steady state and inflammatory conditions both lineages may be differentially expanded, depending on which type is requested. A more recent study indicates that nucleotidase activity is probably not a reliable clonal marker (de Water et al., 1985) and therefore their results must be interpreted with care. However, other data are available that support their concept of multiple differentiation lineages. For example, spleen and bone marrow-derived macrophage precursors as well as their progeny differ markedly in their cytotoxic activity and 'target' selectivity (Baccarini et al., 1986). Furthermore,

splenic macrophage colonies cultured in soft-agar differed in their Mac-2 expression and their ability to present antigen to antigen-specific T cells. This latter characteristic correlates well with the number of MHC class II-positive macrophages in a particular colony (Walker, 1987). Finally, kinetic studies related to the reappearance of macrophages in the spleen after selective macrophage elimination show marked differences between distinct subpopulations (van Rooijen et al., 1989).

Local proliferation of macrophages also contributes to macrophage heterogeneity. Although the self-maintenance of resident macrophage populations is a matter of debate, there are experimental data that support this view. For example, in the lung over 20% of the macrophages proliferate locally (Evans et al., 1987).

In conclusion, the heterogeneity among macrophages in vivo results mainly from selective modulation by various environmental influences acting on macrophages at different stages of their development. However, evidence is accumulating that clonal diversification and local proliferation are additional mechanisms that influence macrophage heterogeneity. Simultaneous performance of these processes and macrophage migration between different compartments increases the complexity.

14.3. Relationship between macrophages and dendrocytes

At present, the interrelationship between macrophages and dendrocytes is rather unclear. Evidence from ontogenetic studies (Janossy et al.,1986; van Rees et al., 1988) suggests that macrophages and dendrocytes emerge as separate lineages early in development. Other studies suggest that dendrocytes have a monocytic origin and thus belong to the MPS (see below). Indications for such origin were obtained from studies in which the primary humoral immune response was investigated (Kamperdijk et al., 1978). It was observed that after immunogenic stimulation the veiled cells of the afferent lymph and the interdigitating cells in the draining lymph node contained Birbeck granules. This observation led to the assumption that these cells were derived from Langerhans cells of the skin. The latter cells resemble macrophages in many respects. They originate from the bone marrow (Katz et al., 1979), exhibit ATP-ase and nonspecific esterase activity (Wolff and Stingl, 1983) and possess the receptors for iC3b and FcRII (Austyn, 1987). In addition, they express various macrophage markers, like Mac-1 and Mac-2 (Haines et al., 1983), F4/80 (Hume et al., 1983a), BM8 (Malorny et al., 1985), MOMA-2 (Kraal et al., 1987) and ER-HR3 (Chapter 11). Furthermore, it has been demonstrated that Langerhans cells in vitro are capable to differentiate phenotypically to veiled and dendritic cells that perform an immunostimulatory function (Schuler and Steinman, 1985). Isolated veiled cells, when intravenously injected, home

in the lymph node paracortex and become interdigitating cells (Fossum, 1988). Taken together these data indicate that Langerhans cells most likely delineage from monocytes and give rise to the interdigitating cells as proposed by Hoefsmit et al. (1982).

A comparable development of interdigitating cells from monocytes has also been proposed for the spleen (Veerman et al., 1974) and the intestine (Sminia et al., 1983). Various other arguments can be put forward to sustain the monocytic origin of dendrocytes. As described in various reports, including Chapters 8, 9 and 10 both cell types share a number of surface markers indicating their close relationship (Wood et al., 1985). For example, the NLDC-145 antigen is expressed by macrophages after the continuous stimulation with L-cell conditioned medium (Breel et al., 1987), as well as by a subpopulation of alveolar macrophages (Breel et al., 1988). Furthermore, it has been shown that interdigitating cells can become highly phagocytic under certain experimental conditions (Duijvestein et al., 1982; Fossum et al., 1984). Recent studies indicate that phenotypical and functional dendrocytes can develop from cultured monocytes under serum-free conditions (Peters et al., 1986) or after metrizamide treatment (Kabel et al., 1989). These findings, together with the observation that distinct murine organs contained a continuum of cells that express macrophage, macrophage/dendrocyte and dendrocyte antigens favour the hypothesis that dendrocytes have a monocytic origin.

15. SUMMARY - SAMENVATTING

Macrophages are constituents of the mononuclear phagocyte system. Cells belonging to this system are the progeny of the myelo-monocytic progenitor cell, which develops from hemopoietic stem cells in the bone marrow. Macrophages are widely distributed throughout the body and they perform a variety of functions. Generally, these can be classified into three categories, i.e., phagocytosis, immunoregulation and the secretion of biologically active products. It is expected that distinct macrophage populations differ in their phenotypical characteristics and function as a result of exposure to different local environmental signals. Likely, this functional heterogeneity is reflected by their phenotype. The aim of the experimental work described in this thesis was to localize and characterize phenotypically distinct macrophage subpopulations in murine organs. For this purpose an immunohistological approach was chosen. A number anti-monocyte/macrophage antibodies is utilized for the immunostaining of frozen tissue sections from various organs.

For the fixation of frozen tissue sections a fast single-step fixation method was developed using hexazotized pararosanilin as a fixative. This fixative appeared to be superior to conventional acetone fixation. Especially, the tissue morphology of the stromal regions of lympho-hemopoietic organs was improved. Simultaneaously, this resulted in an increased number of positively staining cells for many monoclonal antibodies in these cell-poor regions (Chapter 6). Also the tissue morphology of other organs was preserved well by hexazotized pararosanilin (Chapter 7).

Fixation with hexazotized pararosanilin was used in studies that were performed to characterize the macrophage populations of the spleen (Chapter 8), the gastro-intestinal tract (Chapter 9) and the kidney (Chapter 10). The determination of the precise localization of macrophages in these organs was optimized by subdividing them in specific regions on the basis of micro-anatomical or functional criteria. Although the macrophages in the various regions were characterized extensively, discrete macrophage subsets could only be demonstrated in the spleen. It is concluded from these studies that macrophage heterogeneity is a rather complex phenomenon that is caused by selective modulation of macrophages at different stages of their development by various environmental influences. However, evidence is accumulating in the literature that clonal diversification may also contribute to macrophage heterogeneity.

Chapter 11 describes the reactivity of a new macrophage-specific antibody, ER-HR3, in fetal and adult mice. Cells recognized by the antibody are abundant at

hemopoietic sites such as adult spleen and bone marrow. In the fetus, ER-HR3-positive cells are also present in the liver but within one week after birth these cells disappear from the liver along with erythropoietic activity and appear in the bone marrow. In the spleen the ER-HR3-positive cells are restricted to the red pulp. ER-HR3-positive cells are also observed in the mesenteric lymph node, the thymic cortex, the Peyer's patch and the connnective tissues of the intestine, the renal medulla and the dermis.

As described in Chapter 12, the ER-HR3 monoclonal antibody recognizes two naive proteins of 55 and 69 kD. At the cellular level the antigen(s) is (are) present on the outer plasma membrane as well as on cytoplasmic membraneous structures. The antigens also occur in cytoplasmic vesicles. During the maturation process of macrophages the ER-HR3 antigen is increasingly expressed. The remarkable coincidence of ER-HR3 antigen expression and the presence of adult-type hemoglobin synthesis suggests that the ER-HR3 antigen is involved in the erythropoiesis (Chapter 13). Possible functions of the ER-HR3 antigen expressed by macrophages in non-hemopoietic sites remain speculative.

SAMENVATTING

Macrofagen behoren tot het zgn. mononucleaire fagocytensysteem. Dit systeem bestaat uit cellen die afstammen van de myelomonocytaire voorlopercellen die een rijping hebben ondergaan in de monocytaire richting. Myelomonocytaire voorlopercellen ontstaan uit pluripotente hemopoietische stamcellen, die aanwezig zijn in het beenmerg. Macrofagen komen door het gehele lichaam verspreid voor waar ze een groot aantal verschillende functies uitoefenen. Deze functies kunnen globaal in drie hoofdgroepen worden onderverdeeld, te weten, fagocytose, regulatie van de immuunrespons en het secerneren van biologisch actieve stoffen.

Bij het formuleren van de vraagstelling van dit proefschrift is er van uitgegaan dat de heterogeniteit in fenotype die wordt waargenomen bij macrofagen gerelateerd is aan verschillen in functionaliteit. Verder is aangenomen dat macrofagen gelegen op nagenoeg dezelfde plaats onder invloed zullen staan van dezelfde locale omgevingsfactoren. Verwacht mag worden dat deze macrofagen een (nagenoeg) gelijk fenotype zullen vertonen. Het doel van het in dit proefschrift beschreven onderzoek was om tot een nadere karakterisering te komen van deze macrofaag-subpopulaties. Voor deze karakterisering is een aantal monoclonale antistoffen gebruikt gericht tegen antigenen op monocyten/macrofagen. De verdeling van antigeen-positieve cellen in verschillende organen van de muis is onderzocht. Hiertoe werden de organen uitgeprepareerd, bevroren en in dunne weefselcoupes gesneden.

Voor de fixatie van deze weefselcoupes werd gebruik gemaakt van een snelle fixatiemethode, die ontwikkeld is in ons laboratorium. De coupes werden gefixeerd met gediazoteerde pararosaniline. Indien deze fixatiemethode wordt vergeleken met de gebruikelijke acetonfixatie dan blijkt dat de weefselmorfologie sterk verbeterd is (Hoofdstuk 6 en 7). Dit is met name het geval in de stromale gebieden van lymphohemopoietische organen. Tegelijk met deze verbeterde morfologie werd met de meeste van de geteste monoclonale antilichamen een verhoogd aantal positieve cellen in de stromale gebieden gevonden (Hoofdstuk 6).

In het beschreven onderzoek zijn de macrofaagpopulaties van de milt (Hoofdstuk 8), het maag-darmkanaal (Hoofdstuk 9) en de nier (Hoofdstuk 10) gekarakteriseerd. Uit deze studies blijkt dat alleen in de milt duidelijk fenotypisch verschillende subpopulaties te definiëren zijn. Het lijkt waarschijnlijk dat macrofaag-heterogeniteit een zeer complexe verschijnsel is, bij de totstandkoming waarvan diverse processen betrokken zijn. Cellen in verschillende stadia van de monocytaire ontwikkeling worden geacht op een voor hen specifieke wijze op locale stimuli te reageren. Verder zijn er

steeds meer aanwijzingen dat al vroeg in de ontwikkeling van monocytaire cellen veranderingen optreden die worden overgedragen op hun nakomelingen. Deze zgn. klonale diversiteit zou kunnen bijdragen aan de macrofaag-heterogeniteit.

Het laatste deel van dit proefschrift (Hoofdstuk 11, 12 en 13) beschrijft een nieuw macrofaag-specifiek antigeen dat herkend wordt door monoclonaal antilichaam ER-HR3. De verdeling van antigeen-positieve cellen in de foetale en de volwassen muis wordt beschreven in Hoofdstuk 11. ER-HR3-positieve cellen zijn overvloedig aanwezig in de hemopoietische organen. In de foetus zijn dit de lever en de milt. Gelijktijdig met de afname van de erythropoietische activiteit na de geboorte verdwijnen de ER-HR3-positieve cellen uit de lever en verschijnen deze in het beenmerg. In de milt beperkt de aanwezigheid van positieve cellen zich tot de rode pulpa. ER-HR3-positieve cellen komem ook voor in de mesenteriale lymfklier, de schors van de thymus, de Peyerse platen, het bindweefsel van het maagdarmkanaal, het merg van de nier en de lederhuid.

Het monoclonale antilichaam ER-HR3 herkent twee eiwitten ter grootte van respectievelijk 55 en 69 kD (Hoofdstuk 12). Op cellulair niveau zijn deze eiwitten aantoonbaar op de celmembraan en op membraneuze structuren in het cytoplasma. De antigenen komem ook voor in vesicels in het cytoplasma. Verder wordt tijdens het rijpingsproces van monocyt naar macrofaag een toename in ER-HR3 antigeen expressie waargenomen. Er zijn enkele aanwijzingen dat in de hemopoietische organen het ER-HR3 antigeen betrokken is bij de productie van erythrocyten welke het zgn. adult-type hemoglobine bevatten (Hoofdstuk 13). De functie van het ER-HR3 antigeen zowel de hemopoietische organen als in andere organen dient verder te worden onderzocht.

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ABBREVIATIONS

ATP	adenosine-tri-phosphate
BCG	Bacillus Calmette Guérin
BPA	burst-promoting activity
C3	complement factor 3
CR	complement receptor
CSF	colony-stimulating factor
DC	dendritic cell
DNA	deoxyribonucleic acid
Fc	constant part of Ig
FcR	Fc receptor
FIM	factor increasing monocytopoiesis
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte/monocyte colony-stimulating factor
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
LFA	lymphocyte function-associated antigen
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAF	macrophage activating factor
M-CSF	monocyte colony-stimulating factor
MFR	mannose/fucose receptor
MNP	mononuclear phagocytes
MHC	major histocompatibility complex
NAD	nicotinamide-adenine-dinucleotide
NSE	nonspecific esterase
PGE	prostaglandin E
TNF	tumor necrosis factor
VLA	very late antigens

CURRICULUM VITAE

Johannes Pieter de Jong werd geboren op 20 april 1955 te 's-Gravenhage. Na het volgen van het lager en middelbaar onderwijs werd in 1974 aangevangen met een hogere beroepsopleiding als zoölogisch analist. Deze werd in 1976 met goed gevolg afgesloten. In 1977 werd middels een colloquium doctum toelating verkregen tot het volgen van onderwijs aan de Rijksuniversiteit van Utrecht. Dit resulteerde in het behalen van het doctoraal examen Biologie in januari 1983. In deze maand werd tevens een aanvang gemaakt met de werkzaamheden als wetenschappelijk assistent op de afdeling Celbiologie II van de EUR. In maart 1988 werden deze werkzaamheden beëindigd en werd een dienstverband gestart bij Duphar b.v., alwaar de schrijver van dit proefschrift nog steeds werkzaam is.

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