STROMAL CELLS IN THE MOUSE THYMUS.

AN IMMUNOCYTOCHEMICAL CHARACTERIZATION OF THE THYMIC MICROENVIRONMENT

STROMALE CELLEN IN DE THYMUS VAN DE MUIS.
EEN IMMUNOCYTOCHEMISCHE KARAKTERISERING VAN DE MICRO-OMGEVING IN DE THYMUS

PROEFSCHRIFT

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De lichtmicroscopische opname op de omslag (T. van Os, afdeling Celbiologie en Genetica, Erasmus Universiteit, Rotterdam) toont een vriescoupe van de thymus van de muis, die behandeld werd met monoclonale antistoffen gericht tegen medullaire epitheelcellen.

Voor mijn ouders

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Chapters 4-9 represent the appendix papers of this thesis

ABBREVIATIONS

Ab	antibody
APC	antigen presenting cell
BM	bone marrow
CFU-S	colony forming units in spleen
CMR	cortico-medullary region
CRT	cortisone resistant thymocytes
CTL	cytotoxic T lymphocyte
CTL-p	cytotoxic T lymphocyte precursor
dGuo	deoxyguanosine
FITC	fluorescein-isothiocyanate
FLS	forward light scatter
HEV	high endothelial venule
HSC	hemopoietic stem cell
IDC	interdigitating cell
IL-2	interleukin-2
MHC	major histocompatibility complex
PNA	peanut agglutinin
PTL-p	precursor of proliferating T lymphocyte
PLS	perpendicular light scatter
SBL	soya bean lectin
TdT	terminal deoxynucleotidyl transferase
Th	T helper
Ts	T suppressor
TL	thymus leukemia
TNC	thymic nurse cell

PREFACE - VOORWOORD

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1. *GENERAL INTRODUCTION*

Vertebrates possess a surveillance system, called the immune system, that protects them from invading foreign substances and organisms. Immune protection in vertebrates is mediated by a dual system that maintains two basic defenses against foreign invaders. The humoral immune response defends primarily against the extracellular phases of bacterial and viral infections. The cellular immune response is particularly effective against fungi, parasites, intracellular viral infections, cancer cells and foreign tissue. One class of lymphocytes, the B lymphocytes, initiates the humoral immune response. Individual B cells, when activated by recognition of a foreign invader, differentiate to plasma cells which secrete antibodies, proteins that bind specifically to the foreign substance. The other class of lymphocytes, the T lymphocytes, mediates the cellular immune response. When the organism is invaded by a foreign substance (antigen), the T cells that recognize it are activated and initiate a reaction that includes binding to and eliminating the substance. Thus, humoral immunity can be transferred with serum, cellular immunity only with cells. Besides the specificity of its responses the immune system has another important feature that is known as immunological memory. After primary antigenic stimulation effector lymphocytes are induced that mediate the immune functions. Among the progeny of the activated lymphocytes are also cells, that retain the capacity of being (re)stimulated by the original antigen. After a renewed contact with antigen, the specific immune response (secondary response) is faster and of greater magnitude than the primary response. Hence, the antigen will be removed more rapidly and efficiently. Protection against infectious diseases obtained by vaccination is mainly dependent upon the memory lymphocytes. Both B and T lymphocytes, together with all other classes of cells that constitute the lymphohemopoietic system, derive from pluripotent hemopoietic stem cells (HSC) in the bone marrow (BM) (Fig. 1) (Till and McCulloch, 1961; Ford et al., 1966; Curry and Trentin, 1967; Moore and Owen, 1967; Moore and Metcalf, 1970). B lymphocytes proliferate and differentiate mainly within the BM, whereas T lymphocytes do so in the thymus. They exert their effector functions mainly within the peripheral lymphoid organs such as spleen, lymph nodes, Peyer's patches and tonsils.

This thesis deals with the differentiation and maturation of T (thymus-derived) lymphocytes. The development of mature immunocompetent T lymphocytes requires 3 steps. Firstly, HSC differentiate to prothymocytes, precursors committed to the T cell lineage. These cells leave the BM and settle in the thymus. Secondly, upon entry into the thymus, these prothymocytes proliferate and give rise to a population of immature thymocytes (thymic lymphocytes). Thirdly, thymocytes differentiate into mature T cells that emigrate from the thymus to peripheral lymphoid organs. After activation by antigen these mature T cells function as effector cells in the cellular immune response, and as regulatory cells in both the humoral and the cellular immune response (Cantor and Weissman, 1976; Stutman, 1978). This thesis deals mainly with intrathymic events in T cell differentiation. A summary of current knowledge of T cell differentiation is given in Chapter 2.

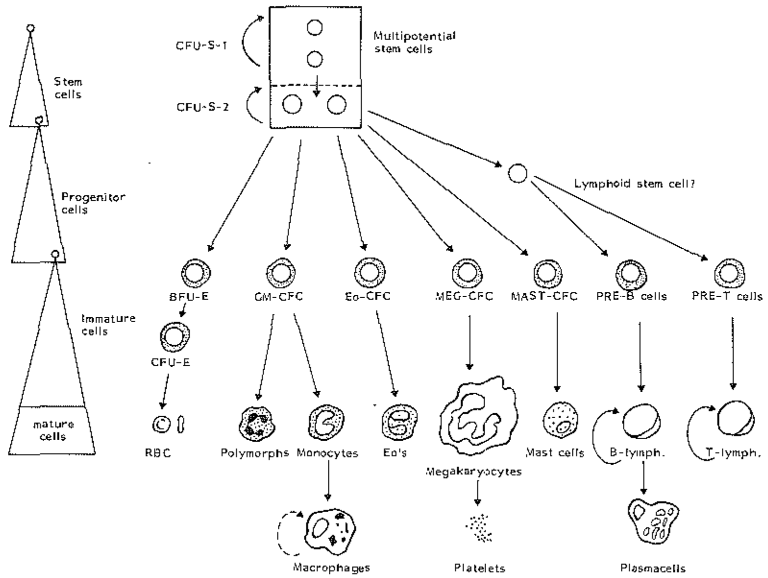

Figure 1. Differentiation of murine hemopoietic cells (Metcalf, 1984, modified). For explanation of progenitors c.f. Metcalf, 1984.

2. *T CELL DIFFERENTIATION*

2.1 *Prethymic T cell differentiation*

HSC and prothymocytes are not easily identified in the BM because of their low frequencies and lack of distinctive morphological properties. Therefore, these early precursor cells have been defined in functional assay systems. In the sixties, BM-derived colony forming units in spleen (CFU-S) were defined as putative HSC (Till and McCulloch, 1961; see also review by Metcalf and Moore, 1971). These cells were shown to seed the spleen of lethally irradiated mice. Prothymocytes, on the other hand, were defined as those cells which, when transplanted into a lethally irradiated animal, give rise to a thymus cell population of donor origin (Kadish and Basch, 1976). In their assay system mouse prothymocytes had the size of medium to large lymphocytes. These cells did not react with antibodies to the pan-T cell antigen Thy1. Cano Castellanos et al. (1983) observed similar characteristics for rat prothymocytes. These authors furthermore showed that prothymocytes lacked other markers characteristic for thymocytes, such as (1) the nuclear enzyme terminal deoxynucleotidyl transferase (TdT), (2) cell surface receptors for peanut agglutinin (PNA) and (3) reactivity with a thymocyte specific antiserum. They also showed that prothymocytes, when recovered from the thymus, lose their capacity for specific homing to the thymus; they accumulate mainly in the liver. Results obtained with a short term *in vivo* assay, in which flowcytometry was used, confirmed the *absence* of T cell antigens from prothymocytes and the induction of such antigens within 3 hrs after entry into the thymus (Lepault et al., 1983).

Prothymocytes and CFU-S have a number of physical characteristics in common (for instance buoyant density, sedimentation velocity and electrophoretic mobility) (Boersma et al., 1981b). Therefore these parameters cannot be used to separate them. Also, analysis of sensitivity to corticosteroids revealed that prothymocytes and CFU-S cannot be separated by a difference in sensitivity to these compounds (Basch and Kadish, 1977; Greiner et al., 1982). However, expression of cell surface antigens in combination with relative cell size provides a means of separating prothymocytes from HSC. Mulder et al. (1984) isolated HSC and prothymocytes by fluorescence-activated cell sorting of BM cells labeled with an antibody directed to H-2K antigens. Almost all CFU-S were found to be present among a subpopulation of 4% BM cells with high forward light scatter (FLS), low perpendicular light scatter (PLS) and bright H-2K immunofluorescence characteristics. In contrast, the fraction of cells in the same FLS/PLS window with dull H-2K fluorescence contained few CFU-S and gave rise to a transient thymus regeneration. Thus, prothymocytes have fewer H-2K antigens on their cell surface than do CFU-S.

From extrapolation of thymocyte regeneration in irradiated BM transplanted mice, Boersma et al. (1981a) calculated that the mouse BM contains 3 prothymocytes per 10^4 cells (0.03%). This figure corresponds well to the 0.075% prothymocytes in the short-term *in vivo* assay of Lepault and Weissman (1981).

In addition to the regenerating thymus in radiation BM chimeras, the embryonic development of the thymus has also provided substantial knowledge of prothymocytes and their entry into the thymus. This approach allows investigation of the stage at which the very first prothymocytes enter the thymic anlage. During the course of embryonic development, the hemopoietic system is built up from HSC which primarily reside in yolk sac and fetal liver (Moore and Owen, 1967; Owen and Ritter, 1969). Boersma (1983) analyzed the production

of prothymocytes in the fetal liver and showed that the frequency of prothymocytes in fetal liver is 11% compared to normal bone marrow.

Several studies have shed light on the question of the actual timing and mechanism of entry of prothymocytes into the thymic anlage. The first prothymocytes enter the embryonic thymus of the mouse from the bloodstream on day 11 of gestation (Owen and Ritter, 1969; Fontaine-Perus et al., 1981). The immigrant cells were characterized as large basophilic cells, which go through a series of blast cell generations. Extensive studies on the entry of prothymocytes into the thymic anlage have been performed in avian embryos. LeDouarin et al. (1976) studied the colonization of the avian thymus using embryonic chicken-quail chimeras. In these chimeras the donor and host cells can be distinguished by structural differences in their nuclei. Their experiments showed that the first influx of quail prothymocytes into the chicken thymus occurs on day 7 and lasts for 36 hours. They furthermore showed that cells with the ability to home to the thymus, are circulating in the blood at least 2 days before they actually enter the thymic anlage, indicating that the onset of colonization is determined by an intrinsic thymic mechanism and not by a hypothetical change in precursor cell properties. In more recent studies from the same group (Jotereau et al., 1980; Jotereau and LeDouarin, 1982), it was shown that the avian thymus is colonized during successive 'receptive' periods. The authors proposed that the thymic anlage, in its receptive period produces a chemotactic factor, the production of which is interrupted when a certain number of precursors have entered the organ.

2.2 Intrathymic T cell differentiaton

2.2.1 Architecture of the thymus

The thymus consists of cells derived from several sources. Of the stromal components, the epithelium of the mouse thymus is thought to have a dual derivation, receiving contributions from the 3rd pharyngeal pouch and neuro-ectodermal components from the neural crest (Cordier and Haumont, 1980) (See Fig. 2). Thus, the epithelium of the mouse thymus consists of components of both ecto- and endodermal origin. However, LeDouarin et al. (1984) consider the avian thymus to be fully endodermally derived. The mesenchymal elements are, at least in part, derived from the pharyngeal arch mesenchyme. Early in thymus development these components assume a relatively simple architecture with an inner epithelial mass surrounded by a capsule of mesenchyme (Jenkinson et al., 1981). During progression of ontogeny this anlage is seeded by lymphoid precursors and antigen presenting cells (APC) from the fetal liver (Bartlett and Pyke, 1982). Gradually both lymphoid and stromal components expand, and during the course of ontogeny, the thymic anlage assumes the architecture of the adult thymus.

The architecture of the adult thymus has been thoroughly investigated (Hoshino, 1963; Hwang et al., 1974; van Ewijk, 1984; van de Wijngaert et al., 1984). The mouse thymus consists of two separate lobes which histologically demonstrate two major compartments, a peripheral, cortical area and a central, medullary area, as judged by two criteria: the presence of distinct lymphoid as well as stromal, mainly epithelial, cells. This is illustrated in Fig. 3. We will first consider the stromal (nonlymphoid) cell types in the thymus.

Cortical epithelial cells have long thin cell processes interconnected by desmosomes and constitute a fine meshwork. Medullary epithelial cells are more spindle-shaped and closely packed. Other stromal cells in the thymus are

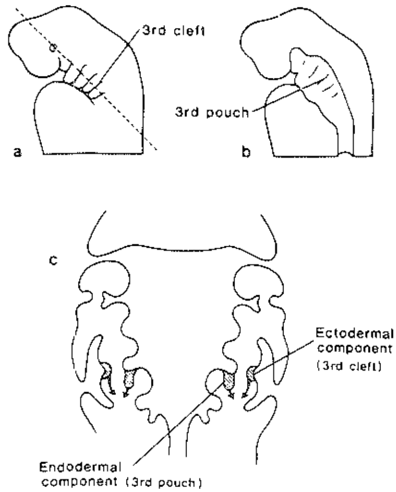

Figure 2. Diagrams showing the origin of the epithelial components of the mouse thymus from pharyngeal endoderm (b and c) and ectoderm (a and c). The gestational age illustrated is about 9-10 days. The broken line in (a) indicates the plane of section illustrated in c (Owen and Jenkinson, 1984).

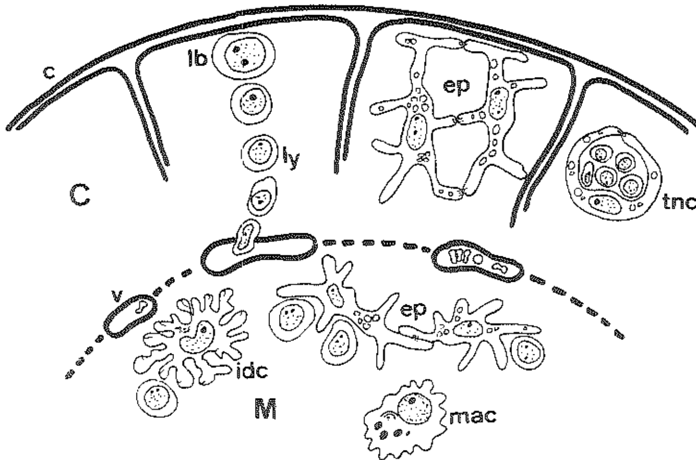

Figure 3. Architecture of the thymus. c = capsule; C = cortex; ep = epithelial cells; idc = interdigitating cell; M = medulla; mac = macrophage; lb = lymphoblast; ly = lymphocyte; tnc = thymic nurse cells; v = vessel.

two types of BM-derived antigen presenting cells (APC), i.e. (a) macrophages, located in both cortex and medulla (Beller and Unanue, 1980; Duijvestijn et al., 1982) and (b) interdigitating cells (IDC), exclusively present in the medulla (Kaiserling et al., 1974; Duijvestijn et al., 1982).

The anatomical localization of the two major classes of thymocytes corresponds to the anatomical organization of the thymic stroma. In the cortex, small immature thymocytes localize, which comprise 85% of the total thymic lymphoid population. In the medulla medium-sized cells are found, which behave like functionally mature peripheral T cells. These cells comprise 15% of the total thymocytes (Weissman, 1973).

The thymus contains a third type of region, areas devoid of stromal cells, demonstrated in frozen tissue sections (Rouse et al., 1979; Van Ewijk, 1984). These areas occur randomly in the cortex and medulla and have no yet identified function. A fourth type of region that can be mentioned is the socalled cortico-medullary region (CMR), the ill-defined boundary between cortex and medulla.

The vascular supply of the thymus occurs via blood vessels carried by connective tissue septae, which penetrate as far as the CMR. Here, arterioles branch into capillaries which ascend into the cortex and the subcapsular region, and curve back towards the interior of the lobe. These vessels merge into large postcapillary venules in the CMR and in the medulla. In addition, the medulla is supplied by some capillaries which originate directly from the arterioles in the CMR. In the cortex, the endothelial cells of capillaries show tight junctions, which are impermeable to macromolecules. In contrast, the walls of postcapillary venules allow passage of macromolecules (Raviola and Karnovsky, 1972). The continuous investment of cortical capillaries with epithelial cells and the strategic location of macrophages along them further prevents access of antigen to the cortex. Therefore, a blood-thymus barrier to circulating macromolecules does exist, but is limited to the cortex, resulting in medullary lymphocytes, unlike cortical lymphocytes, being freely exposed to blood-borne substances. In the CMR and medulla lymphocytes have been observed infiltrating the endothelium of the venules (Raviola and Karnovsky, 1972; Van Ewijk, 1984) indicating that cell passage from blood to the thymus or thymus to the blood occurs in these regions.

Recent evidence indicates that prothymocytes enter the thymus from the bloodstream in both the subcapsular outer cortex and the medulla. Studies of Jotereau and LeDouarin (1982) using embryonic chicken-quail chimeras provided the first histological evidence for precursors migrating simultaneously into cortex and medulla. Ezine et al. (1984) obtained further evidence to support this notion in radiation BM chimeras. These authors reconstituted irradiated mice with small numbers of BM cells from Thy1 congenic donors and they subsequently observed distinct clones of cells within the thymus. Four different types of reconstitution patterns were observed in frozen sections of the thymus stained with an antiserum which specifically detects donor-type thymocytes: (1) discrete confluent subcapsular foci of donor cells, which sometimes extend to the cortico-medullary region and medulla, (2) complete reconstitution of an entire thymic lobe, (3) focal accumulations of scattered cells in cortex and medulla, (4) accumulation of donor-type thymocytes only in the medulla. The latter pattern of reconstitution is suggestive of a medulla-specific precursor. Together, these studies indicate that (only a few) prothymocytes enter the thymic lobes and that these cells can give rise to different subpopu-

lations in the thymus, located in different areas. Such a phenomenon was already suggested in 1974, when Shortman and Jackson provided evidence for two independent T cell populations in the cortex and the medulla. We will now focus on these thymic subpopulations in a more specific way.

2.2.2 *Cell surface antigens of T cells*

Many conventional and monoclonal antibodies are now available which identify distinct cell surface molecules that are differentially expressed on subpopulations of mouse thymocytes and peripheral T cells. Therefore, they provide a means of studying the differentiation of T cells. They can be applied with appropriate second stage antibodies to study subpopulations in lymphoid cell suspensions by immunofluorescence and flowcytometry. Alternatively, they can be used to localize subpopulations in frozen tissue sections by immunohistology. The most common cell surface antigens of mouse T cells are listed in Table 1. This table also shows the percentages of positive cells in various lymphoid organs. The most prominent T cell markers are briefly discussed below.

T cells express the Thy1 antigen, which, in the mouse, is a pan-T cell marker, i.e., antibodies to this marker react with virtually all T cells. For a long time it was regarded as an unambiguous marker of commitment to the T cell lineage in mice. However, Thy1 is also expressed on murine CFU-S and myeloid progenitor cells (Schrader et al., 1982), on nervous tissue (Reif and Allen, 1964), and on the connective tissue of the skin (Morris and Ritter, 1980).

The T200, or Leucocyte Common Antigen (LCA) is a marker which is expressed on most hemopoietic cells (Trowbridge, 1978; Ledbetter and Herzenberg, 1979). Its function has not yet been resolved.

T cells express antigens encoded for by genes of the major histocompatibility complex (MHC) which govern transplantation reactions and which determine the peak of the immune response. The MHC of the mouse, called the H-2 complex, is localized on chromosome 17. For a detailed description of the H-2 complex the reader is referred to Klein et al. (1983). T cells express antigens encoded for by the K and D regions (class I) and the I region (class II). Class I antigens are the classical transplantation antigens, expressed on most nucleated cells, that are responsible for graft-rejection of unmatched grafts. Class II molecules are predominantly expressed on cells of the immune system and provide an efficient intercellular recognition system. Adjacent to the cluster of loci on chromosome 17, the genes of which encode for class I and II MHC antigens, 2 loci are located of which the genes code for the thymus leukemia (TL) and Qa antigens. The TL antigens were defined by their expression on leukemic cells and thymocytes. These markers are, however, absent from peripheral T cells (except TL5) and from any other cell types. In contrast, the Qa antigens are expressed mainly on peripheral T cells, although some of them also on B cells. Qa1 and Qa2, in addition, are expressed within the thymus. The function of these antigens has not yet been elucidated.

Like the TL and Qa antigens, B14 is differentially expressed on T cells. B14 is expressed on thymocytes, but not on peripheral T cells. In contrast, ThB is a marker expressed not only on thymocytes, but also on B cells.

The most extensively studied differentiation antigens in the mouse are the Lyt series of antigens. They were the first T cell antigens to be linked to functional subpopulations of T cells. Cantor, Boyse and others initially

Table 1
Markers of mouse T cells. Percentage positive nucleated cells in lymphoid organs of adult mice

	BM	thymus	spleen	lymph nodes	References
H-2K	60-100	41-68	100	100	Scollay et al., 1980b Lepault et al., 1983
H-2D	nt	80-96	100	100	Scollay et al., 1980b
I-A	13	34-43	58	60	Scollay et al., 1980b Lepault et al., 1983
TL1,2,3	0	63-85	0	0	Old and Stockert, 1977 Shen et al., 1982
TL3	0	69-74	10-20T[1]	10-20T	Scollay et al., 1980b Lepault et al., 1983
TL4	only expressed on leukemic cells				
TL5	nt	65	nt	40	Flaherty et al., 1977
Qa-1	-	+	20	28-35	Stanton and Boyse, 1976
Qa-2	nt	16-50	75	65-75	Flaherty, 1976 Flaherty et al., 1978
Qa-3	nt	0	nt	35	Flaherty et al., 1978
Qa-4	nt	0	60	70T	Hämmerling et al., 1979
Qa-5	nt	0	40	30T	Hämmerling et al., 1979
Thy1	4.5	96-100	26-30	52-57	Ledbetter et al., 1980 Van Ewijk et al., 1981 Lepault et al., 1983
T200	98	97	56	88	van Ewijk et al., 1981 Lepault et al., 1983
B14	0	70-80	0	nt	Sidman et al., 1983
ThB	21	40-60	38-54	nt	Stout et al., 1975 Lepault et al., 1983
Lyt1	nt	97	22-34	59	Ledbetter et al., 1980 Van Ewijk et al., 1981
Lyt2,3	nt	81-82	8-13	16-21	Ledbetter et al., 1980 van Ewijk et al., 1981
L3T4	36	87	23	nt	Dialynas et al., 1983
MEL14	nt	3	nt	100	Gallatin et al., 1983 Reichert et al., 1984
LFA-1	79	97	97	nt	Kaufmann et al., 1982
PNA	45	85-88	38	nt	Reisner et al., 1976 Chervenak and Cohen, 1982
SBL	nt	1	nt	nt	Raedler et al., 1983
TdT	nt	85	0	0	Kung et al., 1975

1. Percentage positive T cells
nt = not tested

defined the Lyt1, 2 and 3 antigens on the surface of functionally active T cells by evaluating the effects of both allo-antisera and monoclonal anti-Lyt Ab plus complement on the cells mediating a number of immune responses (Cantor and Boyse, 1977). In this way T cells involved in both T-dependent Ab (T helper (Th) cells) and delayed-type hypersensitivity (DTH) responses were judged to be Lyt1+2-3- and cytotoxic T cells (CTL) and suppressor T cells (Ts) to be Lyt1-2+3+. Cantor and Boyse suggested that both classes were derived from a common Lyt1+2+3+ precursor which lost either Lyt1 or Lyt2,3 during differentiation. However, recent flowcytometric studies have suggested that the Lyt1 antigen is expressed on nearly all T cells but at quantitatively different levels on different T cell subpopulations (Mathieson et al., 1979; Ledbetter et al., 1980; Van Ewijk et al., 1981). Thus, the T cell subpopulations characterized by complement-mediated cytotoxicity as Lyt1- are 'Lyt1 dull' by flowcytometry while the Lyt1+ subsets are 'Lyt1 bright'. Lyt2,3, abbreviated to Lyt2 (since Lyt2 and Lyt3 are expressed in parallel and possibly are covalently linked (Ledbetter and Herzenberg, 1979)), therefore, is a more absolute marker for the identification of T cell subsets.

The correlation noted in early studies between the Lyt1-2+ phenotype and CTL and Ts activities, and between the Lyt1+2- phenotype and Th function was challenged by Swain (1981), who noted that most of the CTL analysed in these studies were directed at class I MHC alloantigens whereas Th or DTH T cells were generally specific for class II alloantigens. Swain suggested that the Lyt phenotype might instead correlate most directly with the class of MHC determinants recognized by a T cell rather than with the T cell's effector function. Thus, Lyt2 expression would correlate with T cell specificity for class I MHC products, while the Lyt1+2- phenotype would correlate with specificity for class II antigens.

Recently, Dialynas et al. (1983) described a new monoclonal Ab which detects a cell surface molecule designated L3T4. L3T4 is expressed on a population of T cells which appears to correspond precisely to the Lyt2- subpopulation. This suggests that L3T4 may be a more reliable marker for helper T cells than Lyt1. Studies employing anti-L3T4 Ab and either cloned alloreactive T cells or antigen-specific T cell hybridomas have indeed shown that the expression of the L3T4 antigen correlates with specificity of the cell for class II MHC products and that addition of the Ab blocks its helper function. This suggests that L3T4 is a marker on T helper cells which is linked to a receptor on the helper cell's surface for class II MHC products on other cells. Pierres et al. (1984) have described a monoclonal Ab with a similar reactivity.

Gallatin et al. (1983) have recently developed a monoclonal Ab, which detects the MEL14 antigen, which is associated with a 'homing' receptor for peripheral lymph nodes, expressed on peripheral T cells. Pretreatment of T cells with the Ab prevents adherence of the T cells to postcapillary high endothelial venules (HEV), that mediate entry into the lymph node parenchyma. In the thymus, MEL14 is only expressed on 3% of thymocytes including both cortical and medullary cells (Reichert et al., 1984; van Ewijk, 1984), presumably relatively mature intrathymic cells.

Another cell surface antigen with a functional role is the leucocyte function-associated antigen 1 (LFA-1) (Kaufmann et al., 1982). This marker is expressed on most B and T cells. Antibodies directed to LFA-1 inhibit the responses of bulk T cell cultures to allogeneic, xenogeneic and modified self-MHC antigens, as well as the responses of helper and cytotoxic T cell

clones to either class I or class II MHC antigens.

In addition to antibodies, lectin binding properties have been used to define cell populations. Peanut agglutinin (PNA) binding has become a convenient way to separate cortical thymocytes, that bind high levels of PNA (PNA+), from medullary thymocytes, that bind low levels of PNA (PNA-). Peripheral T cells are PNA- except for activated T cells (Chervenak and Cohen, 1982). Soya bean lectin (SBL) binding defined a very small subpopulation of intrathymic lymphoblasts which are located directly under the thymic capsule.

Furthermore, the nuclear enzyme terminal deoxynucleotidyl transferase (TdT) has proven to be a useful marker to distinguish subsets of T cells. Only cortical thymocytes express TdT, medullary cells and peripheral T cells do not.

The thymocyte subpopulations defined with several of these markers and their relationships will be discussed in Chapter 2.2.4.

2.2.3 The T cell receptor

T cells recognize foreign antigens in combination with MHC antigens (so-called *'MHC restriction'* (see Chapter 2.2.5). To account for this MHC-restricted antigen recognition, two types of structures for the antigen receptor of T cells have been proposed. Single receptor theories state that the T cell receptor recognizes neither antigen nor MHC-encoded molecules alone, but does recognize a complex formed between them (Zinkernagel and Doherty, 1975). Dual receptor theories propose that the T cell has two receptors: one for antigen and one for self-MHC molecules (Zinkernagel et al., 1978a). The consensus of current immunological studies favour a single receptor (reviewed by Parham, 1984).

It has been postulated that the receptors on T cells must have recognition sites that are essentially the same as those of antibodies. Consequently, structures related to immunoglobulin (Ig) variable (V) domains should be found on T cells (Jensenius and Williams, 1982). Monoclonal antibodies have been raised that specifically recognize particular T cell lines, hybridomas or T cell tumours (Allison et al., 1982; Meuer et al., 1983; Haskins et al., 1983). Some of these antibodies have been shown to inhibit the immunological response of these cells in a clone-specific manner and therefore presumably react with the T cell receptor. Several groups have used these clonotypic antibodies to immunoprecipitate disulphide-linked heterodimers, composed of two distinct glycoproteins of molecular weights $\pm$ 40 kD: an alpha chain and a slightly smaller beta chain (Allison et al., 1982; Meuer et al., 1983; Haskins et al., 1983). Both chains appear to have variable and constant regions (McIntyre and Allison, 1983) (Fig. 4). Other groups have used the techniques of molecular biology to isolate from a series of antigen-specific, MHC-restricted mouse T helper (Th) hybridomas, the genes that encode the beta chain of their receptors. Hedrick et al. (1984a, b) isolated membrane bound polysomal RNA of a Th hybridoma, synthesized ^{32}P-labelled DNA copies (cDNA) of this RNA and removed DNA sequences also expressed in B cells by hybridization with B cell RNA ('subtraction'). The resulting cDNA probe was likely to contain T cell specific DNA sequences and to encode the T cell receptor. This probe was used to screen a previously constructed library of cloned T cell specific cDNA's. It would be expected that similar to immunoglobulin genes, the genes encoding for the T cell receptor would rearrange as a mechanism to generate diversity in the T cell repertoire. To test this possibility the DNA of 7 selected clones were hybridized with the DNA of a T cell hybridoma and liver cells. One of these clones showed a significant difference between the hybridization patterns

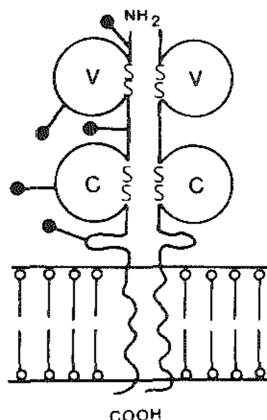

Figure 4. Model of the T cell receptor (Williams, 1984). c = constant region; v = variable region.

obtained with T cell DNA and liver DNA. In subsequent hybridization experiments with DNA blots from T cells of different antigen-specificity, this clone gave different patterns for each of the antigen-specific T cells. This observation indicates a process of somatic gene rearrangement during T cell development and provides strong evidence that this cDNA includes the sequence of the T cell receptor. Comparison of the nucleotide sequence of the cloned T cell receptor specific cDNA with the DNA coding for immunoglobulins revealed a striking homology. Not only was the T cell receptor built from two disulphide-bridged domains of exactly the same size and general structure as immunoglobulin domains, but there was strong amino acid sequence homology at several sites. The N-terminal domain was variable, the C-terminal domain constant, and there was evidence for independent J segments between the two domains. This homology indicates that evolutionary, the genes encoding the antigen receptors of B and T cells have arised from a common ancestral gene.

2.2.4 Thymocyte subpopulations and their relationships

Three requirements must presumably be met by thymocytes before they are permitted to leave the thymus, i.e. (1) they must be selected on the basis of appropriate MHC-restricted antigen receptors, (2) they must be *phenotypically* mature, (3) they must be *functionally* mature. MHC-restricted antigen recognition will be reviewed in Chapter 2.2.5. The phenotypical maturation of T cells within the thymus will now be briefly discussed, followed by the functional characteristics of thymocytes.

The phenotypes of the two major thymocyte populations, i.e. the cortical and medullary cells, have been thoroughly studied and compared with the phenotype of mature peripheral T cells. In general, small cortical thymocytes express high levels of Thy1 and low levels of T200. Cortical thymocytes are PNA+, TL+ and most of them are H-2K/D- (Ledbetter et al., 1980; Van Ewijk et al., 1981; Scollay et al., 1980b, Scollay, 1982). On the other hand, medium-sized medullary cells express low levels of Thy1 and high levels of T200. They express H-2K/D antigens but do not express PNA receptors or TL antigens. Thus,

medullary cells closely resemble peripheral T cells and have a 'mature' phenotype; cortical thymocytes are 'thymus-unique' in phenotype ('immature').

The markers Lyt1, Lyt2 and L3T4 not only define functional subpopulations but they also further subdivide thymocytes in the cortex and medulla (see Mathieson et al., 1979; Ledbetter et al., 1980; Van Ewijk et al., 1981; Dialynas et al., 1983; Scollay, 1983; Scollay and Shortman, 1983). In the peripheral lymphoid organs T cells are either Lyt2+ or L3T4+, they never express both markers at the same time. Thymic medullary thymocytes appear to obey the same rules, whereas cortical thymocytes are distinct in expressing all three of these antigens. Five major subpopulations can be defined using these functional markers, two are present in the medulla and are as such equivalent to the two major peripheral T cell populations, and three subpopulations are present in the cortex. These five subpopulations are: (1) the major population of small cortical cells (70% of total thymocytes) expressing Lyt1, Lyt2 and L3T4; (2) large cortical blasts (15% of thymocytes) also concomittantly expressing Lyt1, Lyt2 and L3T4; (3) lymphoblasts (3%), which are Lyt1 dull or Lyt1-, Lyt2-, L3T4- and are located predominantly in the subcapsular cortex; (4) medullary cells with the phenotype of 'helper' cells (8%) and which are Lyt1+, Lyt2-, L3T4+; (5) cells with the phenotype of cytotoxic/suppressor cells (5%) which are also located in the medulla expressing Lyt1 dull, Lyt2+, L3T4- (Table 2, Fig. 5). In addition several minor subpopulations have now been identified: i.e. (6) Lyt1-, Lyt2-, L3T4- lymphoblasts, located in the medulla (Goldschneider et al., 1982; Huiskamp and van Ewijk, 1985); cortical Lyt1-, Lyt2-, L3T4- lymphoblasts (3) have been further subdivided into (a) 'null' cells, i.e. blasts that do not express any pan T cell markers (T200, Thy1); (b) T200+ Thy1- blasts; (c) T200-, Thy1+ blasts; (d) T200+, Thy1+

Table 2

Thymocyte subpopulations

Percentage of thymocytes	Phenotype	Size	Localization
70	T200+, Thy1+, Lyt1+, Lyt2+, L3T4+	small	thymic cortex
15	T200+, Thy1+, Lyt1+, Lyt2+, L3T4+	large	thymic cortex
8	T200+, Thy1+, Lyt1+, Lyt2-, L3T4+	medium	thymic medulla, peripheral lymphoid organs
5	T200+, Thy1+, Lyt1 dull, Lyt2+, L3T4-	medium	thymic medulla, peripheral lymphoid organs
1	T200-, Thy1-, Lyt1-, Lyt2-, L3T4-	large	thymic medulla
?	T200-, Thy1-, Lyt1- or dull, Lyt2-, L3T4-	large	thymic subcapsular and outer cortex
?	T200+, Thy1-, Lyt1- or dull, Lyt2-, L3T4-	large	thymic subcapsular and outer cortex
?	T200-, Thy1+, Lyt1- or dull, Lyt-2, L3T4-	large	thymic subcapsular and outer cortex
?	T200+, Thy1+, Lyt1- or dull, Lyt2-, L3T4-	large	thymic subcapsular and outer cortex

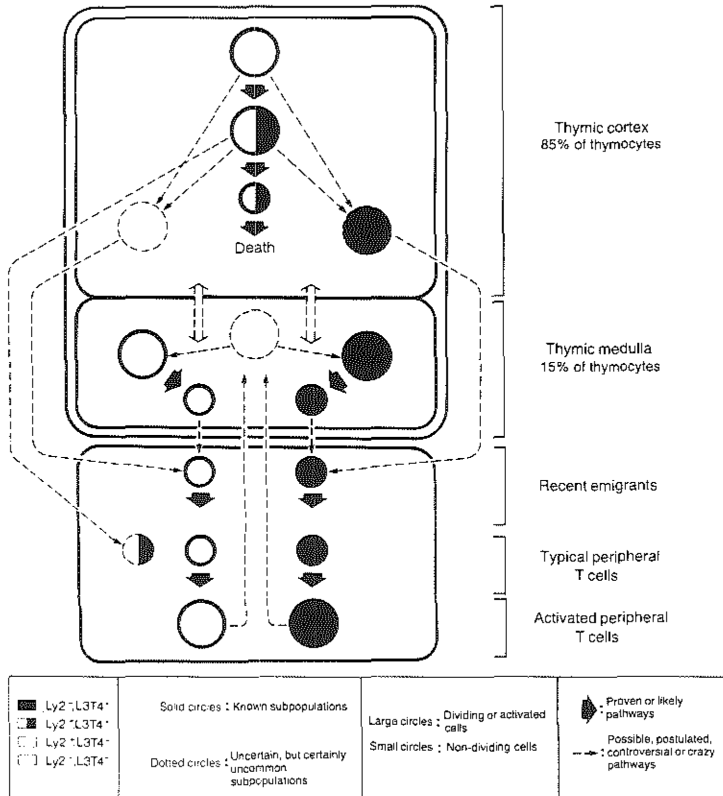

Figure 5. T cell subpopulations and their relationships (Scollay and Shortman, 1984).

blasts (van Ewijk et al., 1981; Huiskamp and van Ewijk, 1985). Scollay (1983) suggests that the cortex may contain a few cells with a medullary phenotype (L3T4+, Lyt2- or L3T4-, Lyt2+).

It seems likely that the subpopulations 7, 3, 2, 1 represent, in that order, the typical maturation lineage in the cortex, with progression from no T cell specific markers to all markers and from large cells to small ones. This is based on the following observations. As already discussed, prothymocytes enter the thymic cortex at the subcapsular region and do not express any T cell specific markers. Furthermore, Weissman (1973) traced the descendants of subcapsular blasts that were labelled radioactively by topical application of radioactive DNA precursors. He showed that thymocytes in the inner cortex derive from these subcapsular blasts. The sequence of appearance of T cell markers on null cells was demonstrated with two models: (1) During embryonic development of the mouse thymus, T cell antigens appear on the surface of thymocytes in the following order: Thy1, Lyt1, Lyt2 (van Ewijk et al., 1982);

(2) In sublethally irradiated mice the regenerating intrathymic null cells first express Thy1 and/or T200, and then L3T4 and Lyt antigens (Huiskamp and van Ewijk, 1985).

What is less clear is the relationship of the cortical cells to the two medullary populations 4 and 5. To analyze this relationship several aspects of the intrathymic generation of T cells must be taken into account. Therefore, the following aspects will be considered in detail now: the functional properties of cortical versus medullary cells, the rate of T cell production and the characteristics of cells that leave the thymus. Thereafter, the various models proposed to explain T cell differentiation and the relationship of cortex and medulla will be discussed.

2.2.4.1 Functional characteristics of thymocyte subpopulations

Chen et al. (1982) analyzed the functional capability of cortical-type (PNA+) and medullary-type (PNA-) thymocytes (i.e. proliferation capacity and cytotoxicity) in limiting dilution cultures stimulated by concanavalin A (con A) in the presence of T cell growth factors. Limiting dilution microculture techniques, with a cloning efficiency of near 100% for mature peripheral (PNA-) T cells, were used to measure the frequency of all thymocytes capable of proliferation (PTL-p) and all thymocytes capable of generating CTL (CTL-p). In unfractionated thymus the PTL-p frequency was 1 in 9.9, the CTL-p frequency 1 in 28. Ninety-nine % of this activity was concentrated in the 14-15% medullary-type PNA- subpopulation. Thus, these data indicate that most cortical cells are immature and non-functional, whereas the medulla contains virtually all precursors of functionally mature cells.

However, in this experimental system 20-25% of medullary-type cells appeared incapable of proliferation (Chen et al., 1983a). This suggests that the absolute size of the functional pool is 7-12% of all thymocytes. To further investigate the functional properties of medullary thymocytes, PTL-p and CTL-p frequencies were also measured in thymocyte populations of cortisone-injected mice, because cortisone resistant thymocytes (CRT) appeared to be a random selection of medullary cells by phenotypic criteria (Benner et al., 1974; Scollay and Shortman, 1983). Cortical thymocytes and 70% of medullary thymocytes are depleted by cortisone injection, the remaining cells (4%) being designated CRT. Only 20-25% of the total thymic PTL-p and CTL-p could be recovered from the cortisone resistant fraction of the thymus. This indicates that functional precursors are within both the cortisone sensitive and the cortisone resistant subgroups of medullary-type thymocytes.

Chen et al. (1983b) also correlated the functional capability with the Lyt phenotype of thymocytes. The majority of PTL-p was among PNA- (medullary) cells, both Lyt2+ and Lyt2-, whereas CTL-p frequency was almost entirely confined to PNA-Lyt2+ cells. Using a similar limiting dilution microculture system, Ceredig et al. (1983a) showed that virtually all precursors of interleukin-2 (IL-2) producing cells (Th cells) were in the L3T4+, Lyt2- population. Their frequency was 1/192. Together these data indicate that the large majority of cortical cells are non-functional, whereas the large majority of medullary cells are functional in terms of susceptibility to antigenic/mitogenic activation and capacity to proliferate and to generate helper and cytotoxic effector T cells. Thus, the medulla contains virtually all precursors of functional Th cells and CTL. Of the medullary thymocytes, the Lyt2-, L3T4+ subpopulation represent precursors of Th cells, whereas the Lyt2+, L3T4- sub-

population contains CTL-p. However, recent data published by Reichert et al. (1984) and van Ewijk (1984) indicate that a minority of cortical cells do express mature characteristics. In these studies MEL14 expression served as an indication of maturity. Both studies demonstrated that scattered large MEL14+ cells localize only in the cortex, not in the medulla, and that they constitute less than 3% of total thymocytes. Reichert et al. also showed that these MEL14+ cells have a mature phenotype despite their cortical localization. The relationship of these cortical MEL14+ cells with medullary thymocytes and peripheral T cells is not yet clear.

2.2.4.2 Quantification of T cell production

Several investigators have analyzed the thymic balance of immigration, proliferation, death and emigration of cells. Bryant (1972) and others injected adult mice with two radioactive DNA precursors, i.e. ^{125}I-deoxyuridine (^{125}I-UdR) and ^{3}H-thymidine (^{3}H-TdR) (see also Joel et al., 1977; McPhee et al., 1979). Since ^{125}I-UdR is very poorly reutilized, whereas ^{3}H-TdR is reutilized efficiently, the relative intrathymic decline of the two isotopes reflects the extent of local reutilization of thymidine. This is an index of the degree of local cell death: if all cells produced emigrate from the thymus both isotopes should be lost in parallel, whereas intrathymic death would result in maintained ^{3}H-TdR levels. With this technique it was found that 60-95% of the total population of thymocytes dies in situ. Proliferation was found mainly in the cortex where 96.8% of thymic mitoses are located (Bryant, 1972). ^{125}I-UdR is not lost from the thymus until 2 days after injection, indicating that this is the minimal maturation time of cortical cells (Joel et al., 1977).

In another approach, Scollay et al. (1980a) used intrathymic injection of fluorescein-isothiocyanate (FITC) to label thymocytes in situ and to trace the descendants that emigrate from the thymus towards peripheral lymphoid organs. Their data show that the rate of migration varies with age. Newborn mice produce 1.4×10^5 thymic migrants per day, whereas young adult animals (3.5-5.5 wks) produce 19×10^5 migrants per day. In adult animals (3-6 mths) the rate of emigration has decreased to $1\text{-}2 \times 10^5$ per day, like in newborn mice. When these numbers are expressed relative to thymus size, the thymus of newborn, young adult and adult mice produces 4.7, 9.5 and 2×10^3/day/10^6 thymocytes (less than 1%), respectively. So the maximum rate of production, expressed both as absolute numbers and per 10^6 thymocytes, was observed at an age when thymus growth is maximal. As mentioned above, the massive intrathymic death, a process which is difficult to document morphologically, is an almost exclusive feature of cortical thymocytes (Bryant, 1972; McPhee et al., 1979). This phenomenon is often explained as being the result of a selection process, which only allows cells with appropriate MHC selection specificities to enter the peripheral pool of lymphocytes (see Chapter 2.2.5).

2.2.4.3 Intrathymic origin and phenotype of thymic emigrants

Consistent with the notion that peripheral T cells derive from medullary cells, not cortical ones, is the finding that the PNA, Thy-1, TL and H-2 phenotype of migrants is indistinguishable from the phenotype of peripheral T cells and medullary cells but quite different from the phenotype of cortical cells (Scollay et al., 1980b, Scollay, 1982). Furthermore, migrants are divided into Lyt2+ and Lyt2- subpopulations (Scollay and Weissman, 1980), the same as medullary thymocytes.

In a more recent study, Scollay et al. (1984a) demonstrated that there are

characteristics by which migrants differ from peripheral T cells. These are the physical parameters, size and density, and sensitivity to killing with the monoclonal antibody B2A2 in the presence of complement. Medullary type cells, whether defined as PNA- or CRT, are slightly larger and less dense than peripheral T cells and they are killed by B2A2 in the presence of complement, whereas most peripheral T cells are not. Examination of thymic migrants showed them, in each parameter, to be more like medullary thymocytes than T cells. The observation that recent migrants are sensitive to B2A2 whereas cells which have resided in the periphery for a longer period are not, provides strong circumstantial evidence that migrants come from the medulla and that B2A2 expression changes over several weeks after the cells have left the thymus. Nevertheless, data from thymus grafts have suggested that medullary cells remain in the thymus for very long periods (i.e., 6 months or more) (Elliot, 1973; Stutman, 1978). This has been used in arguing the possibility that all medullary cells are long-lived intrathymic cells and therefore not a source of thymic emigrants. In those experiments, however, the T6 chromosomal marker was used to distinguish graft from host cells, so only cells that were dividing were detected, leaving the possibility of a considerable bias in the sample. Scollay (1984) has repeated these experiments, but has used donor/host combinations differing at the Thy1 locus, so that 99% of graft cells could be analyzed. He showed that medullary cells with a lifespan of 4 weeks or more constitute considerably less than 1% of the total thymocyte population. A small but significant population remains for at least 3 months, constituting 0.1% of the total thymocyte population. Thus, the long-lived thymocyte, as measured in this system, is not representative of the medullary thymocyte pool as a whole. Taken together these data do allow for the possibility that the medulla is a source of emigrant T cells. However, there is recently published evidence, which suggests that thymocytes may leave the thymus from the cortex. Van Ewijk (1984) and Reichert et al. (1984) speculated that the few bright MEL14+ thymocytes in the thymic cortex could represent the selected cells which are allowed to leave the thymus and give rise to bright MEL14+ peripheral T cells. Although no formal proof exists, this hypothesis is supported by the recent observation that migrants are bright MEL14+ (Reichert et al., 1984).

For a long time it was thought that the traffic of T cells between the thymus and periphery is unidirectional (Stutman, 1978), i.e., once emigrated, mature T cells cannot re-enter the thymus. So far, leukemic cells were the only documented exception (Stutman, 1978). A recent publication (Naparstek et al., 1982) has shown that activated T cells may re-enter the thymus and remain for a long period in this organ. It remains to be investigated whether all populations of sensitized T cells are represented in the thymus or whether some of these cells are specifically destined to return to the thymus because they bear particular receptors for thymus homing.

2.2.4.4 *Models of the relationship between cortex and medulla*

Until recently there were two competing views to explain the relationship between cortex and medulla (reviewed by Mathieson, 1982 and Scollay, 1983). In the first model, both functional medullary subpopulations are believed to derive from a common cortical precursor expressing Lyt1, Lyt2 and L3T4. Basically this is the model put forward by Cantor and Boyse (1977). The Cantor and Boyse hypothesis predicts that during differentiation, Lyt1+2+ cells lose Lyt2 and become Lyt1+2-. Alternatively, they lose Lyt1 and become Lyt1-2+. This hypothesis is based on autoradiography studies of Weissman (1973). Weissman demonstrated that (some) medullary thymocytes derive from cortical cells. Thus, immature cells would express both functional antigens, whereas mature

cells would express either of the two markers. This, however, is not in accordance with recent data published by three groups. Mathieson et al. (1981) and van Ewijk et al. (1982) showed that in ontogeny Lyt1+ cells appear before Lyt2+ cells in the embryonic thymus. Mathieson et al. (1981) suggested an independent origin of Lyt1+ and Lyt2+ T cell lineages. Ceredig et al. (1983b) have recently shown that Lyt1+2- cells can become Lyt1+2+ *in vitro*. Therefore the Lyt1-2+ subpopulation could, at least in part, be derived from the Lyt1+2- subset. A second model, proposed by Shortman and Jackson (1974) argues that prothymocytes are already split into two lineages, i.e., cells that populate the cortex or the medulla. They based this model on their observation that at least some medullary cells are not derived from cortical cells, but instead are generated independently. They suggested that precursor T cells that enter the medulla, mature and give rise to functional T cells which can emigrate towards peripheral organs. In contrast, precursor T cells that enter the cortex differentiate, but give rise to cells of which most are not functional and therefore cannot leave the thymus ('dead-end' cells). In these models both functional subpopulations develop simultaneously in the same compartment(s).

A third model has recently been proposed by Mathieson (1982) and Scollay (1983). They have suggested that the two functional subpopulations may develop independently in the cortex and the medulla. One subpopulation, assumed by Scollay to be the Th population, would develop in the cortex, and a selected minority of these cells would pass through the medulla to enter the periphery, whereas the other one, the CTL population, developed only in the medulla. This model allows for a minority migration from cortex to medulla (model 1), but also for an independent medullary population (model 2).

Very recently, a fourth alternative was proposed by Reichert et al. (1984). They propose that the cortex is the major contributor of thymus emigrants. In this model the rare cortical MEL14+ cells are thought to represent the emigrants. The observations that these cells are larger than the majority of MEL14- cortical thymocytes, mature in phenotype and enriched in CTL-p are in concordance with this model. They also suggested that the cortex and medulla may both contribute to the peripheral T cell pool, but produce quite different classes of migrants. Reichert et al. (1984) and van Ewijk (1984) propose that the cortex produces virgin T cells in a 'sterile' environment, whereas the antigen-accessible medulla may be responsible for antigen-driven T cell generation.

2.2.5 *Lympho-stromal interaction*

As discussed in Section 2.2.1, the thymus is a lympho-epithelial organ, in contrast to bone marrow and peripheral lymphoid organs. The epithelial cells contribute a large part of the thymic stroma. In this section the role of the thymic stroma in the process of intrathymic T cell differentiation will be discussed.

Various *in vivo* and *in vitro* experiments have unraveled the function of the thymic stroma in the maturation of T cells. The thymic stromal cells exert their effects on certain steps in T cell differentiation by secretion of various hormones, such as thymosin, facteur thymique sérique (reviewed by Kruisbeek, 1979; Trainin, 1983). These hormones act upon three different types of target cells: prothymocytes, thymocytes and 'post thymic precursor cells'. Currently, the existence of the latter category of cells is controversial, because it is not known if thymic emigrants need to undergo further maturation

steps in the periphery (Stutman, 1978; Scollay et al., 1984b). Among the effects of thymic hormones are induction of T cell markers, proliferation and effector T cell function. Beardsley et al. (1983) recently reported that a thymic epithelial cell line produces a soluble factor capable of inducing immature (PNA+) thymocytes into IL-2 producers. Furthermore, it was postulated that the thymic stroma produces soluble agents, which attract precursor T cells. The postulation of a chemotactic factor was based on the following observations: (1) precursor T cells enter the thymic anlage in successive 'receptive' periods (Jotereau et al., 1980); (2) *in vitro* migration experiments demonstrated that the homing of murine fetal liver cells (containing prothymocytes) into thymic rudiments is specific (Pyke and Bach, 1979; Fontaine-Perus et al., 1981). Recently, such a soluble attractant was indeed isolated from thymic tissue: Potworowski et al. (in press) identified a glycoprotein, solubilized from thymic epithelial cells, as the thymic element responsible for the chemotactic effect *in vitro*. It is a molecule consisting of two subunits with molecular weights of 55 and 140 kD. Preliminary information on its intracellular localization indicates that it is associated with intracytoplasmic vacuoles of thymic epithelial cells.

On the other hand, some developmental steps require direct, receptor mediated, *cell-cell contact* of the stroma with differentiating T cells. This was documented with the following evidence. Immunological restoration of neonatally thymectomized mice was shown to be possible only with free thymus grafts and not with thymi implanted in diffusion chambers (reviewed by Stutman, 1978).

The interaction of thymocytes with thymic stromal cells has been the subject of a number of studies using various approaches. It has been shown that the intimate contact between stromal cells and thymocytes *in vivo* is represented by lympho-stromal complexes which can be isolated *in vitro*. Examples of these complexes are 'thymic nurse cells' (TNC), which are epithelial cells enclosing up to 30 thymocytes (Wekerle and Ketelsen, 1980) and the so-called 'thymocyte rosettes', complexes of thymocytes surrounding a central macrophage or 'dendritic' cell (Kyewski et al., 1982). Hiai et al. (1984) have investigated the *formation* of lympho-stromal complexes *in vitro*. The study involved complex formation between an epithelial cell line and thymocytes. They demonstrated that in this model formation of complexes between epithelial cells and thymocytes is predominantly associated with thymic blast cells, not small cortical thymocytes. After differentiation into small thymocytes, the blast cells appeared to lose the ability to form complexes with epithelial cells. Jordan et al. (1979) have developed a monolayer culture technique to obtain thymic stroma free of lymphoid cells and they have attempted to effect a recombination of stroma and immigrant progenitor cells by transplanting the stroma back into an *in vivo* environment. On grafting beneath the kidney capsule of syngeneic mice, lympho-stromal structures developed within the transplanted thymic stromal cell aggregates. However, they could not establish whether the lymphoid cells present in the lympho-stromal structures were of host origin. Jenkinson et al. (1982) demonstrated unequivocally the immigrant nature of prothymocytes in fetal liver, that recolonize organ cultures of fetal thymic lobes depleted of lymphoid cells *in vitro*. Together these studies illustrate the close contact between the thymic stroma and differentiating T cells, but they do not reveal the nature of their interaction. Morphological as well as functional studies suggest that the MHC antigens expressed on stromal cells in the thymus are the elements which are essential mediators of signals to differentiating T cells. These studies will be discussed below.

Immunohistological studies of both murine and human thymus demonstrated that both class I and class II MHC antigens are expressed in a confluent pattern on the medullary stroma, whereas only class II antigens were detectable in a reticular pattern on the cortical stroma (Rouse et al., 1979; Janossy et al., 1980). The expression of class I antigens on the cortical stroma, however, is controversial. Two studies of the human thymic stroma (Rouse et al., 1982; Müller et al., 1983) demonstrate the presence of class I antigens on the cortical stroma. Immunoelectronmicroscopic studies of the mouse thymus demonstrated the presence of class II antigens on thymic epithelial-reticular cells in the thymic cortex (Van Ewijk et al., 1980). Class II MHC antigens in the medulla were demonstrated on more than one cell type. Our group (Van Ewijk et al., 1980) and others (Rouse et al., 1982) located these antigens on epithelial cells. On the other hand, other groups have shown that Ia bearing cells in the medulla are bone marrow-derived antigen presenting cells (APC) (Barclay and Mayrhofer, 1981; Bartlett and Pyke, 1982). These APC are considered to be interdigitating cells (IDC) (Duijvestijn and Barclay, submitted for publication).

Indirect evidence that MHC antigens play a role in T cell differentiation was provided by Jenkinson et al. (1981) and Van Ewijk et al. (1982), who showed that in the embryonic thymus, the initial expression of T cell differentiation antigens, such as Lyt1 and Lyt2, on the cell surface of thymocytes, is preceded by the acquisition of MHC antigens by stromal cells. Moreover, Farr and Sidman (1983) found that expression of Ia antigens was reduced in thymic tissue of aged mice and that much of this loss was associated with cortical epithelial cells. This age-related decrease of Ia antigen expression by thymic epithelial cells is accompanied by loss of thymic weight and cellularity and reduced capacity of thymic tissue to promote T cell differentiation (Hirokawa and Makinodan, 1975). Furthermore, reappearance of Lyt1 and Lyt2 antigens on thymocytes in the repopulating thymus of sublethally irradiated mice correlated with reappearance of normal membrane-associated MHC staining patterns (Huiskamp and Van Ewijk, 1985; Huiskamp et al., 1985). Another morphological indication for the role of MHC antigens in T cell differentiation is the phenomenon that the thymic rudiment of nude mice differs markedly from its normal counterpart in the sense that class I, but not class II MHC antigens are expressed on its epithelial cells (Jenkinson et al., 1981).

There are many functional studies reported in the literature which emphasize an instructive role of stromal cells in the development of T lymphocytes. These studies show that most T lymphocytes recognize conventional (non-MHC) foreign antigens (X) only in the context of the polymorphic gene products of the MHC (self) (Blanden et al., 1975; Zinkernagel and Doherty, 1975; Fink and Bevan, 1978; Katz et al., 1978; Zinkernagel et al., 1978a,b; Sprent and von Boehmer, 1979; Sprent, 1980; Kindred, 1980, 1981; Kruisbeek et al., 1981, 1983a,b; Singer et al., 1981, 1982). The particular MHC antigens that T cells recognize in association with conventional antigens are not necessarily the MHC determinants encoded within their own genotype but rather appear to be those expressed by the *host environment* in which the T cells matured. Thus, somewhere during differentiation the T cell 'learns' to see host MHC determinants as 'self' determinants. Experiments with two types of *semiallogeneic* chimeric mice suggest that the *thymus* is the host element that determines the specificity of the self-recognition repertoire of the T cell (Zinkernagel et al., 1978a; Fink and Bevan, 1978):

(1) Chimeras in which (AxB)F1 T cell depleted BM cells are used to reconstitute an irradiated recipient of the same haplotype as one of the hybrid's

parents AxB → A, and which are subsequently immunized with vaccinia virus (Zinkernagel et al., 1978a) or minor histocompatibility antigens (Fink and Bevan, 1978), generate antigen-specific CTL that kill only those target cells, which present antigen in association with A-type, not B-type MHC antigens (Fig. 6a). The CTL thus recognize only the recipient parental haplotype. In contrast, the chimeras made by reconstituting A with adult *spleen* cells of AxB origin generate virus-specific cytotoxicity to infected A and B targets. This indicates that once differentiated, mature T cells do not *change* their restriction specificity.

(2) Chimeras analogous to the AxB → A can also be generated by grafting a parental A thymus into an (AxB)F1 that has been thymectomized, irradiated and protected with AxB T cell depleted BM (Fig. 6b). These animals differed from the previous chimeras in that not all host tissues but only their thymus bore the parental haplotype A.

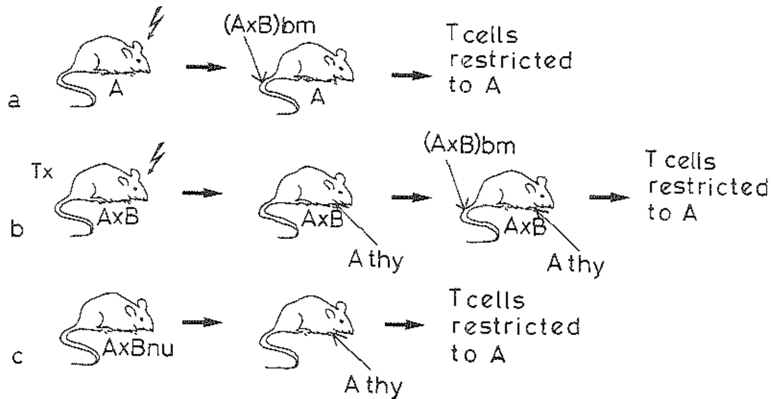

Figure 6. Radiation BM chimeras and nude chimeras used to assess the MHC restriction specificity of T cells. Tx = thymectomize.

In contrast to semiallogeneic chimeras of the AxB → A type, fully *allogeneic* A → B chimeras, made by reconstitution of irradiated B recipients with A-type BM stem cells, failed to develop CTL responses against virus-infected targets (Zinkernagel et al., 1978a). To address the question why the allogeneic chimeras were not immunologically reconstituted, Zinkernagel et al. (1978b) made various types of chimeras and tested their spleen cells by transfer into secondary freshly irradiated and virus-infected (AxB)F1 recipients. They concluded that, although the thymus determines which range of H-2 antigens might be recognized as self, it is mainly the H-2 of the BM-derived cells (i.e. macrophages or interdigitating cells) that determines the actual, phenotypically expressed and measurable specificity for self-H-2 of the T cell. Thus, if T cells of any H-2 type learn to recognize A as self, they cannot express their immunocompetence unless the same A is expressed, at least partially (in (AxB) F1), on the cells they interact with, i.e. the lymphocytes and their host's BM-derived APC. This can also explain why long-term A → B chimeras,

completely reconstituted by donor cells, are severely T cell immuno-incompetent (Zinkernagel et al., 1978a). In A → B irradiated allogeneic chimeras, the precursor T cells learn to recognize B as self-H-2, but since the T cells and the rest of the BM-derived cells (including APC) are made up of cells expressing A exclusively, no MHC restricted antigen recognition or cell interactions can occur.

Another experimental model to examine the influence of the thymus on the development of the self-recognition repertoire of T cells is provided by thymic chimeras made by engrafting congenitally athymic *nude* mice with thymic lobes of syngeneic, semi-allogeneic or allogeneic donors. AxB nude mice that received semi-allogeneic (A) fetal thymus grafts (AxBnu ← A) (Zinkernagel et al., 1979) and A nude mice that received AxB fetal or newborn thymus grafts (Anu ← AxB) (Zinkernagel et al., 1980) respond to antigen in the context of the MHC haplotype shared by host and donor (A) only (Fig. 6c). Sofar the findings in nude chimeras are consistent with those obtained in radiation BM chimeras. However, the results obtained in fully allogeneic nude chimeras contradicted those obtained in radiation bone marrow chimeras (Kindred, 1980, 1981; Zinkernagel et al., 1980): they disputed an important instructive role of the implanted thymus in these animals.

The data reported above have been collected using different experimental systems, mouse strains and antigens, antigen-primed or unprimed donors, as well as various time points after reconstitution and doses of irradiation. These differences in experimental approaches may give different answers: Several studies demonstrate that the *time* allowing the chimeras to reconstitute their lymphoid system and the *dose* of irradiation used to deprive recipients of lymphoid cells, influence the self-recognition repertoire of the chimeric T cells to a great extent. T lymphocytes from A → AxB *longterm* chimeric mice can only generate and display cytotoxic activity to infected A cells but not to infected B cells. However, when these chimeric lymphocytes are sensitized in irradiated and freshly infected AxB recipients they respond to both infected A and B targets (Zinkernagel et al., 1978b). This phenomenon can be explained as follows: in these irradiated and freshly infected recipient mice, BM-derived cell types of host origin (AxB) are still present, in contrast to longterm radiation BM chimeras, in which the greater part of the original BM-derived cells has been replaced by cells descending from the transplanted hemopoietic stem cells. Zinkernagel (1978) then used a higher dose of irradiation to test whether this dose depletes the thymic host AxB BM-derived cells more effectively. AxB mice were irradiated with 1025 R (i.e. 75-100 R more than previously used) and reconstituted with A BM stem cells. When these chimeras were infected with vaccinia virus, the T cells lysed infected A but not B targets, as expected. However, upon adoptive transfer into freshly irradiated and infected AxB recipients, these lymphocytes did not respond to infected B targets. When the usual dose of 925-950 R was used, adoptively transferred chimeric lymphocytes lysed both infected A and B targets. Consequently, the few BM-derived cells of AxB host origin, presumably APC, that usually survive irradiation appear to be sufficient to promote the differentiation of donor A cells expressing restriction specificity for B. If these cells are eliminated, no mature T cells restricted to the second H-2 type (B) are generated. The studies of Katz et al. (1978) and Sprent and Von Boehmer (1979) clearly show that the dose of irradiation, *lethal* or *sublethal* respectively, also determines if Th cells recognize host- or donor-type MHC antigens. Together, these data illustrate the relevance of the dose of irradiation used to construct chimeras for the self-recognition specificity of both Th cells and

CTL. To analyze the presence of radiation-resistant APC or T cells in the thymus, Longo and Schwarz (1980) and Longo and Davis (1983) have directly assessed the donor or host origin of thymic APC in radiation bone marrow chimeras and the restriction specificity of their T cells. They demonstrated that with increasing doses of irradiation, host-type APC are more quickly depleted and replaced by donor-type APC. They furthermore showed that, even when APC have become donor-type, a population of host T cells with restriction specificity for host-type H-2 may survive in radiation chimeras, that is resistant to the conventional doses of 900-975 R. The latter cells are effectively depleted by 1200 R.

It has been shown that subpopulations of murine T cells may use different MHC antigens to recognize foreign antigen. The activation of Th cells for both antibody (Katz and Benacerraf, 1975; Sprent, 1980) and CTL (Zinkernagel et al., 1978) responses is restricted by products of the I region of the MHC, whereas the effector function of CTL is restricted by products of the K/D regions of the MHC (Zinkernagel and Doherty, 1975; Blanden et al., 1975). The following series of studies has been performed to further examine the possibility that the thymus might play a critical role in determining the self-specificity expressed by some T cells but not necessarily all. Kruisbeek et al. (1981) and Kruisbeek et al. (1983a) have compared the self-recognition repertoire of CTL-p in the thymus and peripheral lymphoid organs of thymus-engrafted nude mice and radiation bone marrow chimeras. In both experimental models it was shown that K/D-restricted CTL in peripheral lymphoid organs consist of two populations, one that differentiated intrathymically and whose repertoire was dictated by its intrathymic differentiation environment and one that differentiated outside the engrafted thymus and whose repertoire was dictated by its extrathymic differentiation environment. Singer et al. (1981, 1982) examined the self-recognition repertoire of Th cells of radiation BM chimeras and nude chimeras to test if this were also true for Th cells. They demonstrated that the self-recognition repertoire expressed by Th cells is determined by the H-2 phenotype of the intrathymic environment in which the T cells had differentiated. Thus, H-2K/D-restricted CTL, but not Ia-restricted Th cells, can differentiate into functional competence either intrathymically or extrathymically. Kast et al. (1984) extended these studies using mouse strain combinations that allowed them not only to examine the restriction specificity of T cells, but also their immune response gene phenotype. They showed that the thymus dictates MHC specificity and immune response gene phenotype of T cells restricted to class II MHC determinants, but not of T cells restricted to class I MHC determinants. Collectively, these chimera studies demonstrate that the MHC antigens expressed on the thymic stroma determine, at least in part, the restriction specificity of mature cells.

Two elegant studies have directly proven that during T cell differentiation, a receptor-mediated interaction between T cells and Ia molecules on the stroma takes place. This was achieved by *in vivo* blocking of Ia antigens on stromal cells through administration of antibodies directed to these Ia antigens. The first study examined the role of Ia antigens in the induction of Th cells in peripheral lymphoid organs. For this purpose, peripheral T cells from unprimed ($H-2^k \times H-2^b$)F1 donors were injected into irradiated F1 hosts, together with SRBC and antibodies directed against I-A/E determinants of the k haplotype (Sprent, 1980). After 5 days these SRBC-specific Th cells were transferred into irradiated F1 mice with either SRBC-primed $H-2^k$ or $H-2^b$ B cells. These T cells were markedly reduced in their ability to cooperate with $H-2^k$ B cells, however, they gave good responses with $H-2^b$ B cells. These fin-

dings were taken to imply that anti-Ia^k antibody bound to Ia determinants on host (F1) APC and thus interfered with the activation of the Ia^k-restricted subpopulation of F1 T cells, but did not affect the activation of F1 T cells restricted to I-A/E^b determinants on APC. In the second study, the influence of anti-MHC antibodies was tested during the neonatal T cell development in the thymus. More specifically, Kruisbeek et al. (1983b) examined the effect of chronic injection of anti-I-A^k Ab in neonatal mice and tested the ability of these injected mice to generate splenic CTL responses. These responses were almost completely abrogated after 2-3 weeks *in vivo* treatment with Ab. Addition of IL-2, the putative non-specific soluble Th factor, restored the defect. This indicates that the defect is due to the absence of immunocompetent Ia-specific Th cells, and not to absence of K/D-restricted CTL themselves. Thus, these two types of experiments imply that in peripheral lymphoid organs as well as in the thymus, the generation of functional Th cells require nonlymphoid (stromal) cells which express class II MHC determinants on the cell surface. *In vivo* injection of anti-class II antibodies most probably leads to masking of MHC molecules on the stromal cells. Hence, developing Th cells do not recognize these determinants and are therefore not clonally amplified in the thymic microenvironment. Kruisbeek et al. (in preparation) recently confirmed the validity of this hypothesis. Neonatal mice, injected with anti-I-A antibodies, lacked the thymic and splenic subpopulations of L3T4+ Lyt2- (helper) T cells.

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3. *AIM OF THE STUDY*

As demonstrated in Chapter 2 of this thesis, the thymic stroma mediates essential intercellular signals to differentiating thymocytes. The cell types constituting this stroma and their respective contributions to development of various subpopulations of T cells, however, have only been poorly characterized. Whereas murine T cell subpopulations can be studied with a large panel of monoclonal antibodies directed to surface markers of T cells, no such characterization of subpopulations of murine stromal cells was possible. Therefore, we decided to raise a panel of monoclonal antibodies directed against specific antigens of various types of stromal cells of the mouse thymus. The reactivity of the monoclonal antibodies obtained, using immunohistology and flowcytometry, is reported in Chapter 4. In Chapters 5-7 these monoclonal antibodies are used for further characterization of the murine thymic stroma.

In Chapter 5 the monoclonal antibodies, directed to various types of thymic stromal cells, together with monoclonal antibodies directed against T cell antigens, were applied to study the phenotype of both stromal and lymphoid components of *in vitro* isolated thymic nurse cells (TNC) in order to analyze the relationship of TNC to stromal cell types defined *in situ*.

In Chapter 6 the monoclonal anti-stroma antibodies were used to study the development of the stroma of the embryonic thymus of the normal and nude mouse. In this paper we analyzed when the various types of stromal cells can first be identified during embryonic development of the normal thymus. Furthermore, we studied whether any of the stromal cells is lacking in the thymic rudiment of the nude mouse embryo.

Chapter 7 deals with the effects of various doses of dexamethasone on both lymphoid and stromal cells of the mouse thymus. To this purpose monoclonal antibodies directed to thymic stromal cells (Chapter 4) and T cell antigens were used in combination with flowcytometry and immunohistology. This chapter analyzes whether dexamethasone-induced changes in the thymocyte subpopulations can be related to changes in the stromal composition of the thymus.

Our efforts to produce monoclonal antibodies to thymic stromal cells resulted in two other monoclonal antibodies recognizing stromal cells in minority within the thymic microenvironment. These antibodies, however, react predominantly with stromal cells in *peripheral* lymphoid organs. Chapter 8 reports a detailed description of the reactivity of one of those monoclonal antibodies (ER-TR7), which is directed against reticular fibroblasts in peripheral lymphoid organs. Finally, Chapter 9 deals with the reactivity of the other monoclonal antibody, ER-TR9, which primarily reacts with a specialized macrophage population, the so-called marginal zone macrophage, in the mouse spleen. The anatomic localization of ER-TR9+ cells is correlated with that of another subpopulation of macrophages, defined by the antigen Mac-1, and with that of IgD+ and IgM+ B cells.

CHAPTER 4

MONOCLONAL ANTIBODIES TO STROMAL CELL TYPES OF THE MOUSE THYMUS

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SUMMARY

Seven hybridoma cell lines secreting monoclonal antibodies (mAb) to non-lymphoid cells of the mouse thymus have been prepared. These mAb clearly demonstrate the heterogeneity of the thymic stroma. Based on their anatomical distribution patterns observed with the immunoperoxidase technique on frozen tissue sections, they were subdivided into four groups. The first group of mAb, ER-TR1, 2 and 3, detects antigens encoded for by the I region of the major histocompatibility complex. These antigens are expressed on both stromal and lymphoid cells in lymphoid organs. mAb of the second category, ER-TR4, react with epithelial cells in the thymic cortex. mAb of the third group detect stromal cells of the thymic medulla. One antibody of this group, ER-TR5, exclusively reacts with medullary epithelial cells. ER-TR6, the other antibody of this group, reacts with medullary interdigitating cells and macrophages. The fourth type of antibodies, ER-TR7, detects the reticular fibroblasts of the thymus. The possible role of the thymic cell types detected by the present antibodies in T cell differentiation is discussed.

1. *INTRODUCTION*

It is well established that the thymus plays an essential role in the differentiation, maturation and selection of T lymphocytes (1). During their thymic sojourn lymphocytes differentiate in interconnected compartments lined by a framework of stromal cells. At present there is ample evidence, indicating that these stromal cells support the process of differentiation and maturation of T cells. The maturational effect of the thymus is mediated by secretion of various thymic hormones by the thymic stroma (2,3) as well as by direct cell-cell contact with the stroma (4). *In vivo* studies have shown that antigens encoded for by the major histocompatibility complex (MHC), expressed on the thymic nonlymphoid cells, are essential for the development of self tolerance and MHC restriction, though the precise mechanism and the nonlymphoid cell types involved are controversial (5-9).

We have undertaken immunohistological studies to analyse the thymic microenvironments responsible for T cell differentiation (10,11). These studies defined epithelial-reticular cells as the major elements, which bear MHC antigens and indicated that I-A and H-2K antigens define different compartments in the thymus. Another indication for the relevance of the thymic stroma in T cell education is the demonstration of lympho-stromal complexes in thymic cell suspensions *in vitro*. Thymic nurse cells (TNC) (12) and thymocyte rosettes (13) may represent the tight *in vivo* association between

stromal cells and lymphocytes *in situ*. However, many questions remain unanswered concerning the complex relationship between thymic lymphoid cells and the surrounding stromal cells. The identity and the heterogeneity of stromal cells within the thymus are not yet well defined. The purpose of the present paper is to analyse the cell types which constitute the thymic microenvironments responsible for T cell differentiation.

Monoclonal antibodies provide an excellent approach to study the heterogeneity of cells based on the presence of cell surface determinants. In the present paper we report a panel of monoclonal antibodies which detect various classes of thymic stromal cells. We present the localization of these cell types using immunocytochemistry.

2. *MATERIALS AND METHODS*

2.1. *Immunisation*

2.1.1 *Mice and rats*

Male and female C3H/HeJ, AKR, BALB/b, BALB/c, DBA/1, DBA/2, SWR/J, A.TL, A.TH, A.SW, B10.Br, B10.ScSn, B10.RIII, B10.M, B10.G, B10.D2/n, B10.AQR, B10.T(6R) mice, age 6-12 weeks, were used for the present study. For immunization female Louvain rats, age 10-25 weeks, were used. The animals were kept in our animal colony under routine laboratory conditions with free access to food and water.

2.1.2 *Isolation of thymic stroma and immunisation of rats*

Thymic stromal cells were isolated as follows: Thymuses of C3H mice were placed on a nylon sieve and minced with a pair of scissors, while phosphate buffered saline (PBS) was added. The stroma remaining on the sieve was further dissociated at 37°C with 0.6 mg/ml collagenase (Millipore, Bedford, MA, type IV) in 0.1 M Tris, pH 7.1, containing 1% fetal calf serum (FCS), 5mM CaCl2 and 15µg/ml DNAse (Boehringer, Mannheim, FRG, grade II). Female Louvain rats of 4 months old were injected intraperitoneally (ip) with stromal cells isolated from five thymuses. After 4 weeks they were boosted ip. Animals were then selected which had high titers of serum antibodies directed against stromal cells. After 8 weeks these animals were boosted intravenously (iv). Spleens were excised aseptically three days later.

2.2. *Cell fusion*

Immune spleen cells were fused to P3-X63-Ag8.653 myeloma cells (14) as described before (15). Peritoneal macrophages were used as feeder cells (16).

2.3. *Antisera and conjugates*

Affinity column purified rabbit-anti-rat immunoglobulin (RαRa-Ig, Dako, Copenhagen, Denmark) and rabbit-anti-rat Ig coupled to horse radish peroxidase (RαRa-Ig-HRP) were used. Sheep-anti-rat Ig coupled to β-galactosidase (ShαRa-Ig-βGal) was obtained from the Radiochemical Center (Amersham, GB). Fluorescein-conjugated rabbit-anti-rat serum (RαRa-Ig-FITC) was obtained from Nordic Immunological Laboratories (Tilburg, NL). All conjugates were routinely deaggregated by centrifugation in a Beckman (Palo Alto, CA) Airfuge at 10^5 xg. Antibodies and conjugates were optimally diluted in PBS containing 0.5% bovine serum albumin and 1% normal mouse serum, in order to prevent aspecific binding.

2.4. *Assay procedures for selection of hybridomas*

2.4.1 *General remarks*

For isolation of hybrid clones, which produced antibodies against stromal cells we chose the following screening procedure: First, hybridoma superna-

tants were tested for Ig secretion and secretion of antibodies against thymocytes, the major contaminant in stromal cell suspensions. For both screening assays we used a micro enzyme-linked immunosorbent assay (ELISA) system, as described by Van Soest et al. (17). Second, the reactivity of the Ig+ve supernatants which did not react with thymocytes was screened with frozen sections of thymic tissue, as described elsewhere (18). Hybrid clones producing antibodies which reacted with stromal cell types were subcloned using limiting dilution, expanded and stored under liquid nitrogen.

2.4.2 ELISA procedure to detect production of rat Ig in hybridoma supernatants

The ELISA method (17) was modified for detection of rat Ig. Briefly, Terasaki plates were coated with optimally diluted RαRa-Ig in PBS overnight. They were rinsed with PBS containing 0.2g/l gelatin (PBS-gel). Next, hybridoma supernatants were applied to the Terasaki plate using a replicator (Biotec, Basel, Switzerland). After incubation for 60 min plates were rinsed in PBS-gel containing 0.05% Tween-20 and incubated with optimally diluted ShαRa-Ig-βGal. Next, the plates were incubated with 4-methyl-umbelliferyl-galactoside. The reaction was terminated by adding 0.5M sodiumbicarbonate buffer (pH 10.4) to each individual well. Binding in the individual wells was then quantified, using a scanning inverted microfluorometer connected to a microcomputer (19).

2.4.3 Miscellaneous methods

To detect mAb binding to cell surface antigens the ELISA method (17) was used. Frozen sections of lymphoid organs were prepared and stained using an indirect immunoperoxidase method, and photographs were recorded as reported elsewhere (18). Immunofluorescence of lymphoid cell suspensions and flowcytofluorometric analysis were performed as described by Van Ewijk et al. (18,20).

2.5. Determination of Ig class

Ig secreted by hybridoma cells were determined by Ouchterlony double diffusion in agar on culture supernatants concentrated approximately 5-10 fold. Reagents specific for rat IgM, IgG1, IgG2a, IgG2b and IgG2c were obtained from Miles Laboratories (Slough, GB).

TABLE 1

RAT MONOCLONAL ANTIBODIES DIRECTED AGAINST MOUSE THYMIC STROMAL CELLS

Antibody	Reacts with	Immunoglobulin class
ER-TR1[x]	cortical medullary stromal cells	IgG2c
ER-TR2	" " "	IgG2b
ER-TR3	" " "	IgG2b
ER-TR4	cortical stromal cells	IgM
ER-TR5	medullary stromal cells	IgM
ER-TR6	" " and lymphoid cells	IgM
ER-TR7	reticular fibroblasts	IgG2a

x ER = Erasmus University Rotterdam
TR = Thymic Reticulum

3. *RESULTS*

3.1 *Reactivity of anti-stroma mAb with frozen thymus sections*

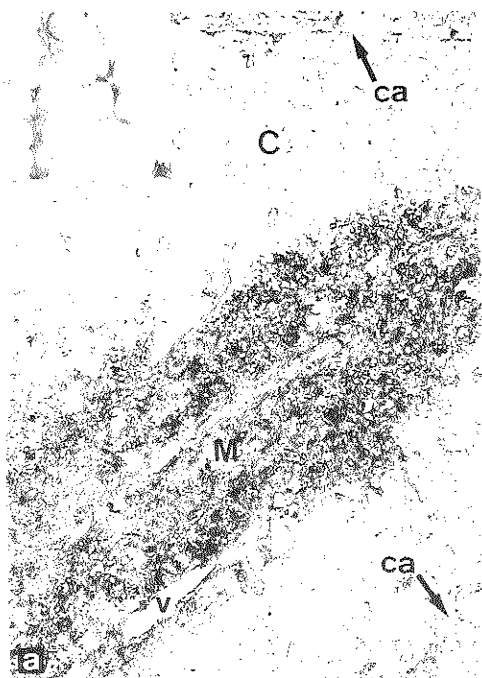
Based on the selection procedure described in section 2.4 seven hybridomas were isolated which produce antibodies reacting with nonlymphoid cell types in frozen thymus sections. Based on the reactivity patterns obtained in these frozen sections the present hybridomas can be divided into four groups (Table 1). Table 1 also includes the immunoglobulin class of each mAb. We will subsequently discuss the reactivity of each group of antibodies with thymus sections.

The first group of clones (ER-TR 1, 2 and 3) produces antibodies which give a fine reticular staining pattern in the thymic cortex and confluent staining in the medulla (Fig. 1a). This reactivity pattern is similar to the reactivity pattern of anti-H-2 antibodies (11). As these antibodies react with B cells, but not with T cells in peripheral lymphoid organs (not shown), they presumably detect Ia antigens. The second category of clones (ER-TR4) produces antibodies which detect antigens, which are expressed in a reticular pattern in the thymic cortex, whereas the medulla is virtually negative (Fig. 1b). In contrast, the third group of antibodies (ER-TR5 and ER-TR6) reacts with cells with dendritic morphology in the medulla in thymus sections. However, the staining patterns, obtained with ER-TR5 and 6, differ substantially. ER-TR5 fails to detect any cortical stromal or any lymphoid cells (Fig. 1c), whereas ER-TR6 reacts with stromal cells in the medulla and with medullary lymphoid cells (Fig. 1d). Furthermore, this antiserum reacts with the thymic capsule, with the blood vessel walls and occasional cortical nonlymphoid cells. Monoclonal antibodies produced by the fourth category of hybrid cells ER-TR7 detect 'reticular' cells in the medulla of the thymus (Fig. 2a). However, these antibodies also react with the thymic capsule and the walls of blood vessels. A negative control section, incubated with 2nd stage antibodies and diaminobenzidine tetrahydrochloride (DAB) only, is demonstrated in Fig. 2b. Flowcytometry on thymocyte suspensions confirmed the immunohistological observations (data not shown).

3.2 *Reactivity of the anti-stroma antibodies with various mouse strains*

To study the reactivity of the anti-stroma antibodies with various mouse strains we chose two approaches, i.e. flowcytometry and immunohistology. Since ER-TR1, 2 and 3 react with B lymphocytes in spleen and lymph node, and presumably detect polymorphic MHC determinants (21) we performed comparative flowcytometry on spleen cell suspensions of various inbred mouse strains using ER-TR1, 2 and 3. Since ER-TR4, 5, 6, and 7 do not react with lymphoid cells (except for ER-TR6), we tested the reactivity of these latter antibodies with various mouse strains using immunohistology.

Figure 1. Immunoperoxidase staining pattern of serial frozen thymus sections. (a), (b), (c), (d) represent frozen sections incubated with monoclonal antibodies ER-TR3, ER-TR4, ER-TR5 and ER-TR6, respectively. Insets represent higher magnifications of the cortex of (a), (b), and medulla of (c), (d), respectively. c = cortex, m = medulla, ca = capsule, v = blood vessel in medulla, t = trabeculae. Magnification: (a), (b), (c), (d): 140x; Inset: 875x.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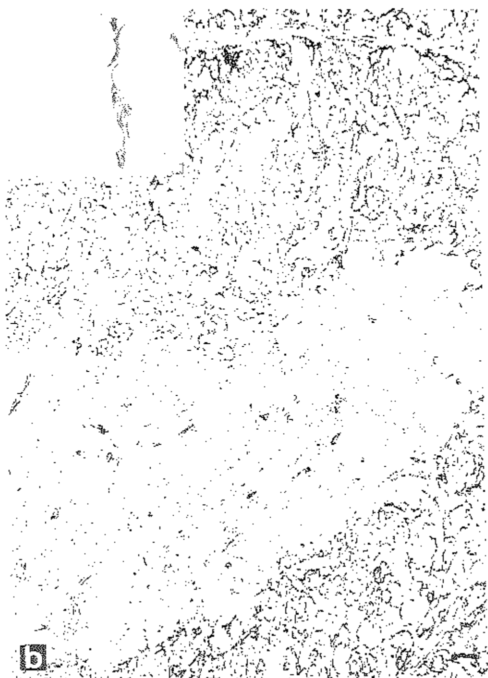
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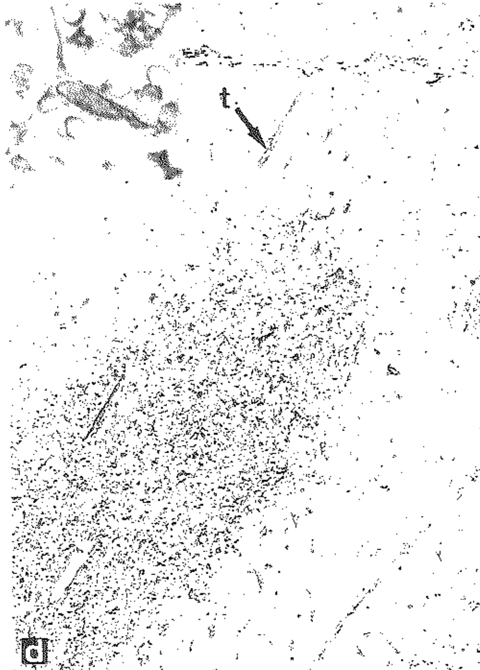
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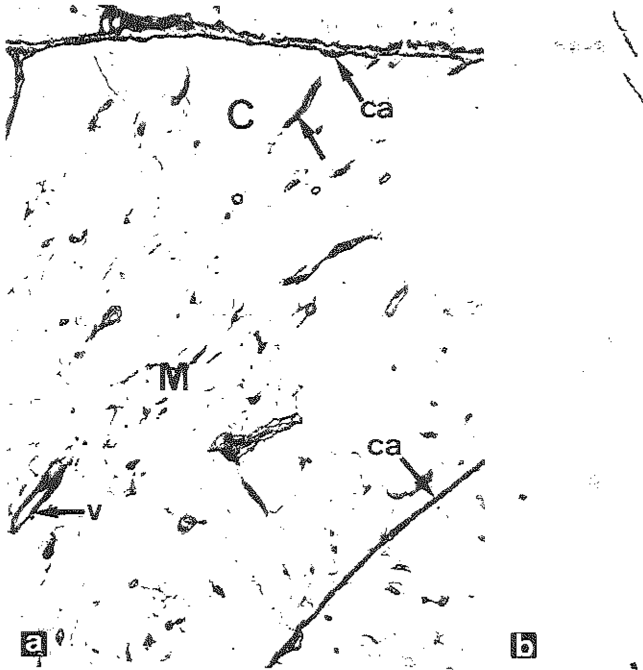

Figure 2. Immunoperoxidase staining pattern of serial frozen thymus sections. (a), (b), represent frozen sections incubated with monoclonal antibodies ER-TR7 and a negative control section, incubated with 2nd step and DAB only, respectively. c = cortex, m = medulla, ca = capsule, v = blood vessel in medulla, t = trabeculae. Magnification: 140x.

Table 2 summarizes the flowcytometry results obtained with ER-TR1, 2 and 3. The data obtained with various mouse strains carrying H-2 haplotypes of independent origin indicate that positive/negative reactivity correlates with H-2 haplotype. The reactivity of ER-TR1 and 3 with A.TL, but not with A.TH demonstrate the detection of antigens encoded by the Ia region of the H-2 complex. Strong reactivity of ER-TR2 with B10.AQR and lack of reactivity with B10.T6R indicates that this antibody also detects products of the Ia region. Taken together, the results show that ER-TR1, 2 and 3 detect antigens which are shared by several, but not all mouse strains and that these antibodies detect different Ia specificities. ER-TR2 possibly reacts with Ia specificity M17, 32 or 33 and ER-TR3 with Ia specificity 50 (22). ER-TR1 apparently detects a new specificity.

We incubated frozen sections of thymuses of mouse strains reported in Table 2 with ER-TR4, 5, 6 and 7. These antibodies react with frozen thymus sections of all mouse strains tested. Thus, the antigens detected by these antibodies are not polymorphic.

TABLE 2

DISTRIBUTION OF ER-TR1, 2 AND 3 AMONG MOUSE STRAINS WITH INDEPENDENT AND RECOMBINANT HAPLOTYPES

Strain	Haplotype							Clone		
	K	A	B	J	E	C	D	ER-TR1	ER-TR2	ER-TR3
C3H/HeJ	k	k	k	k	k	k	k	48[x]	46	46
AKR	k	k	k	k	k	k	k	54	52	54
B10.BR	k	k	k	k	k	k	k	59	58	62
B10.ScSn	b	b	b	b	b	b	b	4	5	50
Balb/b	b	b	b	b	b	b	b	4	3	39
B10.D2/n	d	d	d	d	d	d	d	56	5	54
Balb/c	d	d	d	d	d	d	d	45	3	44
DBA/2	d	d	d	d	d	d	d	27	4	47
B10.G	q	q	q	q	q	q	q	53	4	46
DBA/1	q	q	q	q	q	q	q	52	6	54
SWR/J	q	q	q	q	q	q	q	49	3	49
A.SW	s	s	s	s	s	s	s	4	20	6
B10.M	f	f	f	f	f	f	f	4	5	3
B10.RIII	r	r	r	r	r	r	r	39	39	40
B10.AQR	q	k	k	k	k	d	d	52	52	51
B10.T(6R)	q	q	q	q	q	q	d	50	3	52
A.TL	s	k	k	k	k	k	d	29	52	51
A.TH	s	s	s	s	s	s	d	5	49	7

x Data are expressed as percentage labeled cells, determined by flowcytofluorometric analysis of spleen cell suspensions as described in Materials and Methods.

4. *DISCUSSION*

In the present study we report the production of seven hybrid cell lines secreting rat mAb directed against stromal cells of the mouse thymus. On the basis of their anatomical distribution pattern in the immunoperoxidase technique on frozen tissue sections these antibodies were subdivided into four groups. We will subsequently discuss which nonlymphoid cell types are detected by the presently reported mAb.

From their anatomical distribution patterns, we conclude that the mAb ER-TR1, 2 and 3 of the first category detect antigens encoded for by the H-2 complex expressed on both cortical and medullary stromal cells. In line with this assumption is the following evidence. Similar reactivity patterns in thymus sections have been reported previously (11,23,24), using genetically defined mAb and conventional antisera against H-2 and HLA antigens, respecti-

vely. Furthermore, ER-TR1, 2 and 3 react with B cells. This is a feature of antigens encoded for by the Ia region of the H-2 complex (21). Data obtained with spleen cell suspensions of recombinant mouse strains confirm the detection of products of the Ia region. ER-TR1 detects a new specificity, whereas ER-TR2 and 3 presumably detect known specificities (22).

mAb ER-TR4 of the second category exclusively react with *epithelial-reticular* cells in the cortex of the mouse thymus. The following evidence supports this view. Double-labelling studies demonstrated the presence of ER-TR4 antibodies on I-A+ve cortical stromal cells (Van Vliet, unpublished observation). These I-A+ve cortical cells were shown to be epithelial-reticular cells (11). Two more observations favour the epithelial nature of the ER-TR4 positive cortical cells. First, ER-TR4 antibodies appear to react with isolated thymic nurse cells (Van Vliet, unpublished observation), which have been shown to be epithelial-reticular cells (25). Second, ER-TR4 antibodies crossreact with epithelia of other organs tested, such as intestinal epithelium (unpublished observation).

The medullary localization of the cells detected by the third group of antibodies, suggests that these antibodies are directed against IDC and/or medullary epithelial cells. We assume that ER-TR5 selectively reacts with epithelial cells of the medulla, because (1) ER-TR5 also reacts with epithelial cells in various organs tested, such as epidermis (unpublished observation), (2) ER-TR5 does not react with isolated IDC or macrophages (Dr. E. Hoefsmit, personal communication). Preliminary studies have shown that, in contrast, ER-TR6 reacts with isolated IDC and macrophages. The reactivity of ER-TR6 with nonlymphoid cells in the lymph node medulla indicates that this antibody detects a marker on a subpopulation of macrophages. However, ER-TR6 crossreacts with nonepithelial tissues, as well as with epithelia.

The present study further indicates that the fourth category of antibodies ER-TR7 detects antigens on reticular fibroblasts in the thymus. ER-TR7 also reacts with reticular fibroblasts in peripheral lymphoid organs (unpublished observations). Evidence to support this notion is that ER-TR7 reacts with connective tissue components in various nonlymphoid organs (unpublished observations). Strikingly, the thymus contains little ER-TR7 positive mesenchymal tissue in comparison with peripheral lymphoid organs. This mesenchymal tissue is mostly confined to the medulla. Obviously the epithelial cells constitute the major stromal compartment of the thymus.

As outlined above the presently described mAb provide a correlation between the localization of various stromal cell types and subsets of differentiating T cells. ER-TR7 outlines the reticular fibroblasts of the thymus. ER-TR1, 2 and 3 define the same environments as previously reported by us for I-A antigens (11). Although they outline both cortex and medulla, they do not selectively react with stromal cells of either cortex or medulla. ER-TR4, 5 and 6, however, demonstrate a clear demarcation of cortex and medulla. These antisera distinguish between specific stromal subpopulations of both compartments. Perhaps it is significant that antigen expression on stromal cells, as demonstrated in this study, parallels antigen expression on T cells (18,24,26). ER-TR4 expression on cortical epithelial cells correlates with the major cortical T cell population. On the other hand, immunocompetent medullary thymocytes (± 15% of the total lymphocyte population) reside in an environment, which is ER-TR5 and 6 positive.

Is there any direct evidence for the involvement of the thymic stroma in T cell differentiation and selection? At present there is evidence which indicates that the thymic stroma bears cell surface antigens which are recognized by differentiating thymic lymphocytes (5,27). It was demonstrated that for development of Ia-specific T helper cells to occur, precursor T cells need to

interact with Ia products in the thymus. Apparently, this development can be blocked by monoclonal antibodies. Kruisbeek et al. (27) showed that intraperitoneal injection of anti I-A antibodies into neonatal mice prevented the development of T cells which were able to recognize self or allogeneic I-A determinants. However, the stromal elements responsible for the self-MHC selection process have not yet been identified. We hope that the presently described mAb will provide tools to further unravel the role of stromal components in microenvironments which dictate T cell differentiation.

ACKNOWLEDGMENTS

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CHAPTER 5

IMMUNOHISTOLOGY OF THYMIC NURSE CELLS

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SUMMARY

The demonstration of Thymic Nurse Cells (TNC), complexes between stromal cells and thymocytes, in cell suspensions of murine thymuses, prompted us to investigate 1) the relationship of TNC to other thymic stromal cell types defined *in situ*, and 2) the maturation stage of the enclosed thymocytes. To this purpose we incubated *frozen sections* of TNC suspensions with various monoclonal antisera directed to T cells and stromal cell types, using immunohistology. This approach enabled us to study antigen expression on the 'nursing' cell itself and to analyze the phenotype of the enclosed lymphocytes in cross sections of TNC. The results show that lymphocytes enveloped by TNC express high levels of Thy-1, moderate levels of T200 and variable amounts of Lyt-1. Due to enzymatic degradation Lyt-2 expression could not be studied. The enveloped cells also bear PNA receptors, but no detectable I-A/E antigens. Expression of H-2K antigens on enclosed thymocytes varied from weak to absent. The 'nursing' cells react with ER-TR4, a monoclonal antibody which detects cortical epithelial-reticular cells. In addition TNC express I-A/E and H-2K antigens. In contrast, TNC do not react with ER-TR 5 and 7, monoclonal antibodies, which detect medullary epithelial cells and reticular fibroblasts, respectively. TNC do not express the macrophage antigens Mac-1 and Mac-2. We conclude that TNC *in vitro* represent the *in vivo* association of epithelial-reticular cells with cortical thymocytes. However, the enclosed thymocytes do not constitute a phenotypically distinct subset of subcapsular or outer cortical cells.

INTRODUCTION

T lymphocytes differentiate under control of the thymus (1). While in the thymus, lymphocytes come into contact with a framework of stromal, mainly epithelial, cells. At present there is ample evidence, that the thymic nonlymphoid compartment supports the development of immunocompetent T lymphocytes. The thymic stroma mediates its effect on certain steps in T cell differentiation by secretion of various hormones (2-4). However, some developmental steps require direct receptor mediated cell-cell contact of maturing T cells with the stroma (5). Furthermore, various *in vivo* experiments have demonstrated that antigens encoded for by the major histocompatibility complec (MHC), expressed on thymic nonlymphoid cells, are crucially involved in dictating or selecting restriction specificities for self MHC of maturing T cells. However, both the mechanism and the stromal elements actually responsible for determining the specificity of the T cell repertoire are controversial (6-9).

We have previously started to investigate the thymic microenvironments responsible for T cell differentiation by immunocytochemistry (10,11). In these studies epithelial-reticular cells were shown to be the major thymic elements, which express MHC antigens. These studies further demonstrated that I-A and H-2K antigens define different thymic microenvironments. I-A antigens are present throughout both cortex and medulla, whereas K antigens are only found in the medulla. A morphological indication for the relevance of the thymic stroma in T cell differentiation is the recent demonstration of lympho-stromal complexes in thymic cell suspensions *in vitro* (12). These complexes, termed thymic nurse cells (TNC), are complexes of a single epithelial cell filled with high numbers of fully intact thymocytes. TNC formation is ascribed to subcapsular and outer cortical areas of the thymus (13), the area to which prothymocytes possibly migrate upon arrival in the thymus. This observation suggests that TNC may provide a microenvironment guiding early stages of intrathymic T cell differentiation.

Questions which arise from the above presented data are: (1) What is the relationship between TNC and stromal cell types defined *in situ?* (2) Do TNC specifically interact with a subset of lymphocytes at an early stage of intrathymic T cell differentiation? If so, do they preferentially contain subcapsular or outer cortical thymocytes?

In the present study we report upon immunohistological characterization of TNC with various monoclonal antisera directed to differentiation markers of T cells as well as antibodies directed to determinants on thymic stromal cell types, recently produced in our group (14). Our results indicate that TNC *in vitro* represent the *in vivo* association of epithelial-reticular cells with cortical thymocytes.

MATERIALS AND METHODS

Animals.
Balb/c mice of 4-6 weeks old were used. They were kept in our animal colony under routine laboratory conditions.

TNC preparation.
TNC suspensions were prepared as reported by Wekerle et al. (12). However, collagenase/dispase (Boehringer Mannheim Inc., Mannheim, FRG) was used instead of trypsin.

Antisera.
Reagents used in the present study are listed in Table 1. For immunoperoxidase studies the monoclonal antibodies were detected with rabbit-anti rat immunoglobulin conjugated to horse radish peroxidase (RaRa-Ig-HRP) (Dako, Copenhagen, Denmark). Alternatively they were detected with rabbit-anti-rat immunoglobulin conjugated to tetramethyl-rhodamine-isothiocyanate (RaRa-Ig-TRITC) (Nordic, Tilburg, The Netherlands). To prevent aspecific binding to Fc receptors the conjugates were routinely deaggregated before use by centrifugation in a Beckman airfuge at 10^5 xg. The conjugates were optimally diluted in PBS containing 0.5% BSA and 1% normal mouse serum in order to prevent aspecific binding. Peanut agglutinin conjugated to fluorescein-isothiocyanate (PNA-FITC) (Vector Laboratories, Burlingame, USA) was optimally diluted to distinguish cortical from medullary thymocytes.

Preparation of frozen sections
TNC were fixed in 0.5% paraformaldehyde in phosphate buffered saline (PBS) at

Table 1. REAGENTS USED IN THE PRESENT STUDY

Reagent code[x]	Reacts with	Reference nr.
59-AD-22	Thy-1 antigen	15
53-7-313	Lyt-1 antigen	15
53-6-72	Lyt-2 antigen	15
30-G12	T-200 antigen	15
PNA	Cortical thymocytes	16
ER-TR4	Cortical epithelial cells	14
ER-TR5	Medullary epithelial cells	14
ER-TR7	Reticular fibroblasts	14
M5/114.15.2	$I\text{-}A/E^{b,d}$	17
M1/42.3.9.8	H-2K, all haplotypes	18
M1/70.15.11.5	Mac-1 antigen	19
M3/38.1.2.8	Mac-2 antigen	20

[x] All reagents used, except for PNA, are rat monoclonal antibodies directed against mouse determinants. They were applied in conjunction with RaRa-Ig-HRP as a 2nd stage reagent, followed by diamino-benzidine tetrahydrochloride (DAB). PNA was applied as PNA, conjugated with fluorescein-isothiocyanate (PNA-FITC).

4°C for 30 min (21). The fixed cells were mixed with a warm solution of 1.5% agar (Difco) in PBS. After setting the agar containing TNC was mounted on a cryostat specimen holder using Tissue Tek II (Miles Laboratories, Naperville, IL) as mounting medium. The mounted tissue was frozen by placing the cryostat specimen holder in solid CO_2. 5 µm frozen sections were cut using a cryostat (Bright, Huntington, UK). These sections were dried with a fan, briefly fixed in acetone, dried again and stored at room temperature in a desiccator on silicagel until use.

Immunoperoxidase staining of frozen sections
Immunoperoxidase staining of frozen sections and photography were performed as described below. Sections were soaked in PBS containing 0.5% bovine serum albumin (BSA), and 0.05% Tween-20 (rinsing buffer). Sections were then overlayed with monoclonal antibodies, and incubated for 60 min at room temperature in moist chambers. After repeated washing with rinsing buffer, sections were incubated for 60 min with appropriately diluted second stage antibodies. After rinsing antibody binding was visualized using di-aminobenzidine tetrahydrochloride (DAB), according to Graham and Karnovsky (22). The contrast of the precipitate was enhanced by incubating sections in a solution of 1% $CuSO_4$ and 0.9% NaCl for 10 min. Sections were dehydrated, coverslipped and examined using Zeiss 10x, 25x and 100x Planapo objectives. Photographs were recorded

using Ilford Pan F films. The contrast of the image was enhanced with a 490 nm interference filter (Schott).

Immunofluorescence staining of frozen sections
Two-colour immunofluorescence staining of frozen sections was performed according to Van Dongen et al. (23).

RESULTS

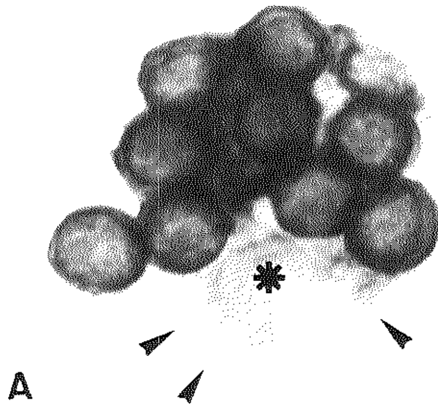
In the present paper we report the results of immunoperoxidase studies on frozen sections of suspensions of TNC of Balb/c mice. This approach did not only enable us to study antigen expression on the 'nursing' cell itself, but also enabled us to 'look inside' the TNC and to study the enclosed thymocytes in cross sections of TNC. In the first part we will present the results obtained with monoclonal antibodies against various T cell markers of the mouse, i.e. Thy-1, Lyt-1, Lyt-2 and T-200 and with peanut agglutinin (PNA). In the second part we will report the reactivity of monoclonal antibodies directed against a variety of thymic stromal cell types of the mouse with the 'nursing' cell itself. Table 1 summarizes all reagents used in the present paper.

Data for each antiserum are based on samples of 200 or more individual TNC and on three separate experiments. Only those TNC which were morphologically intact and which contained at least four thymocytes were included in the present study.

1. *Reactivity of TNC with PNA and various monoclonal antisera against T cells*

The results obtained with various T cell markers are summarized in Table 2. Frozen sections of TNC suspensions, incubated with monoclonal anti-Thy-1, show that all lymphoid cells inside TNC in 99% of TNC are strongly Thy-1 positive. (Fig. 1a) We only observed 5/590 TNC with one Thy-1 negative lymphocyte each. Frozen sections, of TNC incubated with monoclonal anti-T200 (Fig. 1b) in general reveal a similar reactivity pattern, i.e. a homogenously strong T-200 expression on all enclosed thymocytes. However, T-200 expression is weaker than expression of Thy-1. Only 5/595 TNC contained one T-200-ve lymphocyte. TNC sections incubated with monoclonal anti-Lyt-1 (Fig. 1c) demonstrate that all lymphocytes in TNC are Lyt-1 +ve. However, these sections differ markedly from sections incubated with anti-Thy-1 or anti-T-200. Large differences in antigen density among lymphocytes within one TNC were observed in 91% of TNC. However, three minor subpopulations were noted, each amounting to 3% of TNC. These TNC subpopulations contained lymphocytes with homogeneously low, intermediate or high levels of Lyt-1, respectively. Frozen sections of TNC, incubated with PNA-FITC demonstrated that all lymphocytes inside TNC are strongly PNA +ve (Fig. 2a). No PNA -ve thymocytes were observed. In addition, PNA appeared to react with TNC as well as with other nonlymphoid cells in the cell suspension, presumably macrophages. Frozen sections of TNC, incubated with monoclonal anti Lyt-2 only demonstrated that thymocytes en-

Figure 1. Immunoperoxidase staining pattern of frozen sections of TNC. Incubation with monoclonal anti-Thy-1 (a), anti-T-200 (b), anti-Lyt-1 (c). Note that the TNC themselves are negative with these antibodies (arrows). Note negative nucleus (asterisk).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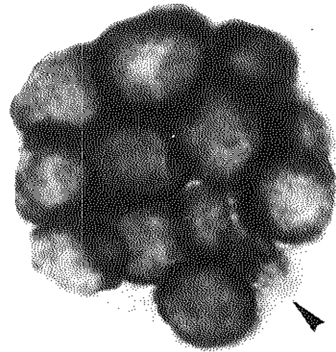
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Table 2. REACTIVITY OF BALB/c TNC WITH PNA AND MONOCLONAL ANTISERA DIRECTED TO T CELL ANTIGENS

T cell marker	n	staining intensity of thymocytes	% positive TNC
Thy-1	590	++[1]	in 99% of TNC all thymocytes were Thy-1+ve; 1 % of TNC contained one Thy-1-ve thymocyte
T-200	595	+	in 99% of TNC all thymocytes were T-200+ve; 1% of TNC contained one T-200-ve thymocyte
PNA	200	++	all thymocytes in TNC are PNA+ve
Lyt-1	500	± to ++[2]	all thymocytes in TNC are Lyt-1 +ve
Lyt-2	100	-[3]	all thymocytes in TNC are Lyt-2 -ve

1. The average number of thymocytes enclosed by TNC noted in crosssections was 13.
2. 91% of TNC: ± to ++
   3% of TNC: ++
   3% of TNC: +
   3% of TNC: ±
3. Lyt-2 staining was not detectable due to enzyme treatment of TNC.

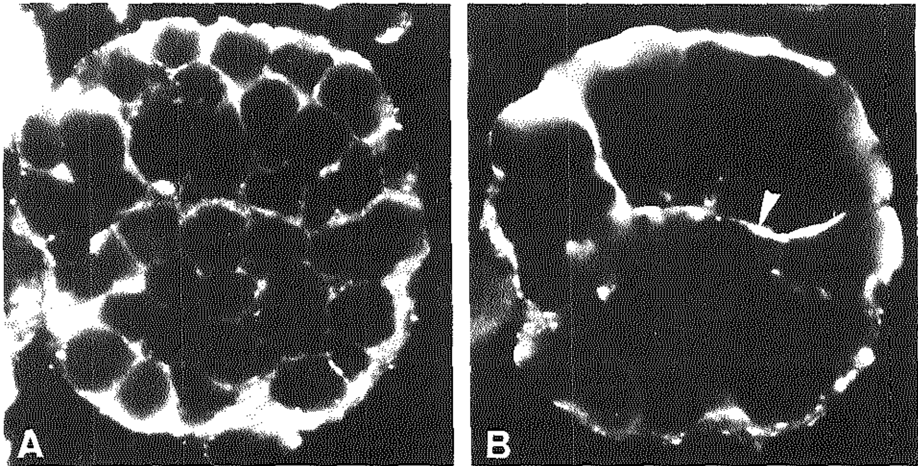

Figure 2. Two-colour immunofluorescence staining pattern of frozen sections of TNC. Incubation with PNA-FITC (a), and ER-TR4 followed by RaRa-Ig-TRITC (b). Note that not only thymocytes, but also TNC are PNA +ve. Note ER-TR4 +ve cytoplasmic extension in center of TNC ((b), arrow).

closed by TNC are virtually Lyt-2 -ve (not shown). A likely explanation for this finding is the removal of Lyt-2 from the cell surface by the enzymatic treatment during TNC isolation.

To test this hypothesis we compared Lyt-2 expression on thymocytes, which had been treated with collagenase/dispase, and untreated control lymphocytes. About 80% of control thymocytes were strongly Lyt-2 positive, whereas enzyme treated thymocytes were virtually negative (data not shown). This is in line with observations by others that Lyt-2 is very sensitive to trypsin treatment (13,23). We conclude that the enzymatic treatment used for TNC isolation destroys the Lyt-2 antigen of the enclosed lymphocytes. Thus, no data on the expression of Lyt-2 on thymocytes in frozen sections of TNC could be obtained. Comparison of enzyme treated and untreated thymocytes indicated that binding of PNA, anti-Thy-1, anti T-200 and anti-Lyt-1 were not detectably affected by the isolation procedure used.

We did not demonstrate any I-A/E positive lymphocytes enclosed in TNC. H-2K expression varied from weak to absent. In summary, thymocytes in TNC in situ are strongly PNA and Thy-1 positive, moderately T-200 positive and demonstrate variable Lyt-1 expression. They are I-A/E and H-2K negative or weakly H-2K positive. This phenotype corresponds to the phenotype of cortical thymocytes.

2. *Reactivity of TNC with monoclonal antisera against various thymic stromal cell types*

Frozen sections of TNC were incubated with monoclonal antisera directed against various thymic stromal cell types of the mouse thymus. Table 3 shows a summary of the results. Monoclonal antibodies from clone M5/114, which detect I-A/E antigens, react with all TNC studied. TNC also express H-2K antigens, as demonstrated with M 1/42. 90% of TNC react with ER-TR4, an antiserum, which detects epithelial reticular cells of the thymic cortex (Fig. 2b). TNC do not react with ER-TR5, a monoclonal antibody which detects epithelial cells of the thymic medulla, nor with ER-TR7, a monoclonal antibody which reacts with reticular fibroblasts. Furthermore, two monoclonal antisera, directed against cell surface antigens on macrophages, were tested for reac-

Table 3. REACTIVITY OF BALB/c TNC WITH MONOCLONAL ANTISERA DIRECTED TO VARIOUS STROMAL CELL TYPES OF THE MOUSE THYMUS

Clone	% +ve TNC[1]	Staining intensity
ER-TR4	90	+
ER-TR5	0	-
ER-TR7	0	-
M1/42	99	++
M5/114	99	++
M1/70	0	-
M3/38	0	-

1. Number of TNC studied: 185-205.

tivity with TNC. Table 3 demonstrates that TNC do not express either Mac-1 or Mac-2.

In summary, TNC react with antisera to I-A and K antigens and cortical epithelial cells. TNC neither react with antisera to medullary epithelial cells, nor with antisera directed to reticular fibroblasts and macrophages. Thus, TNC can be considered as the equivalent of cortical epithelial-reticular cells.

DISCUSSION

In the present paper we describe the phenotype of TNC in frozen sections of TNC suspensions, as demonstrated with various monoclonal antibodies directed against (1) T cells and (2) thymic stromal cell types. The advantage of our method is that antigen expression on thymocytes, enclosed in TNC, can be studied in cross sections of TNC. Others (13,25,26) have cultured TNC in order to release the enclosed thymocytes for phenotypic studies. However, even short periods of culture could alter surface antigen expression of the enclosed thymocytes and induce maturation.

The present results confirm and extend the data on the phenotype of the enveloped thymocytes, presented by Kyewski and Kaplan (13). Only with regard to expression of MHC antigens there is a discrepancy; in contrast to Kyewski and Kaplan (13) we did not demonstrate any I-A/E antigens and we demonstrated only weak expression or absence of H-2K antigens on the enveloped thymocytes. Culturing of TNC could have induced the expression of MHC-antigens on the thymocyte surface in their studies. Since they used flowcytometry to detect binding of antibodies, it is possible that a difference between the detection limits of the two methods used provides an alternative explanation.

The data obtained with antisera against stromal cell types are consistent with an epithelial nature and a cortical origin of the TNC, as proposed by Wekerle et al. (12) and Kyewski and Kaplan (13), respectively. The major finding in the present study is that 90% of TNC are positive when incubated with ER-TR4 antibodies, which exclusively react with cortical epithelial-reticular cells. TNC do not represent the population of TR5 +ve medullary epithelial cells, because they do not react with ER-TR5. This evidence favours the epithelial-reticular nature and the cortical origin of the majority of TNC. It is consistent with the finding that TNC neither express the macrophage antigens Mac-1 and Mac-2, nor the ER-TR7 antigen of reticular fibroblasts. However, on the basis of these results we cannot conclude if all TNC are derived from cortical epithelial cells. 10% of TNC are TR4 -ve. These TR4 -ve cells could represent a subpopulation of TNC. Alternatively, this result could merely be due to the detection limit of our method. Furthermore, we do not presently know if ER-TR4 and ER-TR5 react with all cortical or medullary epithelial cells, respectively.

The present study further shows that all TNC are I-A/E and H-2K positive. These results agree with results obtained by Wekerle et al. (12). The expression of MHC antigens on TNC, however, does not provide conclusive evidence on the origin of TNC, since MHC antigens are not exclusively expressed on cortical epithelial-reticular cells. Medullary epithelial cells and IDC also bear MHC antigens (11,27). Expression of class II MHC antigens on cortical epithelial-reticular cells is generally agreed upon (10,11,28-30). In contrast, experiments to detect the expression of class I antigens gave controversial results. 11-4.1, the antiserum used to detect H-2K antigens by Rouse et al. (10) en van Ewijk et al. (11) does not react with cortical epithelial-reticular cells. However, M1/42, the antiserum used to detect H-2K antigens in the

present study, strongly reacts with these cells (Van Vliet, unpublished observation).

From the present data, obtained with T cell markers, we conclude that TNC are associated with the major population of cortical thymocytes. This is based on the following evidence: (1) All thymocytes inside TNC strongly express PNA receptors. This is a feature of cortical thymocytes (16,31). In contrast medullary thymocytes express few PNA-receptors. (2) TNC contain thymocytes which are strongly Thy-1 and moderately T-200 positive and heterogenous in Lyt-1 expression. This phenotype is similar to the phenotype of cortical thymocytes (32). In contrast, the majority of medullary thymocytes express a high amount of Lyt-1 (33).

We did not obtain any evidence that TNC are associated with a distinct subset of immature cortical thymocytes. From previous studies we know that immature cortical cells have a special phenotype. The earliest cells in the thymic cortex are 'null cells', precursor T cells, which do not bear any T cell markers and which give rise to 'Thy-1 only's', cells only expressing Thy-1. These 'Thy-1 only's' upon their turn give rise to cells which also express Lyt antigens (32). Both populations of immature cortical cells are also pronounced in the embryonic thymus (34). In addition, it was demonstrated that Lyt positive cells develop from Lyt negative cells in cultures of embryonic thymus (34) and in cultured embryonic thymocytes (35). If TNC would be involved in early steps of T cell differentiation we would expect TNC to be preferentially associated with null cells or Thy-1 only's. Obviously, there is no case for such a preferential association.

The present study does not provide any evidence on the possible clonality of the thymocytes enclosed by TNC. However, the demonstration of TNC bearing thymocytes of both host and donor origin in radiation bone marrow chimeras refutes the notion of a monoclonal origin of the enveloped thymocytes (13). Presently, we believe that the complete envelopment of thymocytes by the epithelial cells in vitro is the result of the isolation conditions. This hypothesis is based on three findings. First, our results indicate that TNC associated thymocytes are accessible to enzyme. All markers used in the present study except Lyt-2 point to a cortical origin of TNC. Furthermore, Lyt-2 is expressed on the majority of cortical thymocytes, whereas only a minority of medullary cells are Lyt-2 +ve (32). Since TNC associated thymocytes are Lyt-2 -ve and Lyt-2 was shown to be trypsin sensitive, we presume that enzyme molecules had access to the thymocytes of the lympho-stromal complexes before complete envelopment occurred. Second, other nonlymphoid cells such as splenic follicular dendritic cells (FDC) also appeared to round up during isolation (36; Heinen and Boniver, personal communication). Third, in frozen sections of the intact thymus incubated with ER-TR4 and anti I-A antibodies we would expect to see spherical structures, if TNC exist in vivo. However, we observed a network of epithelial-reticular cells with thymocytes in their invaginations, and no spherical structures (10,11,14). The circular epithelial elements, noted in thymic sections by others (30,37,38) have not yet been proven to be true 'bags', filled with thymocytes, by serial sections.

Based on the present results we conclude that TNC represent the ER-TR4, I-A/E and H-2K positive epithelial-reticular cells throughout the thymic cortex, demonstrated in frozen sections of murine thymus (10,11,14).

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CHAPTER 6

STROMAL CELL TYPES IN THE DEVELOPING THYMUS OF THE NORMAL AND NUDE MOUSE EMBRYO

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SUMMARY

The anatomical distribution of various nonlymphoid cell types in the embryonic mouse thymus *in vivo* and *in vitro*, as well as in the thymic rudiment of the nude mouse embryo has been studied. For this purpose a panel of monoclonal antibodies, ER-TR3, 4, 5, 6 and 7, directed to various types of stromal cells of the mouse thymus was used in combination with immunoperoxidase labeling on frozen sections.

It was shown that as early as day 13 in thymic ontogeny distinction of TR4+ve cortical epithelial cells and TR5+ve medullary epithelial cells is possible. Thus, as far as stromal components are concerned, the thymus at day 13 in ontogeny is already subdivided into cortex and medulla. At day 13, Ia (TR3) was expressed in a focal pattern in the medulla subsequently appearing throughout both cortex and medulla by day 16.

The thymic rudiment of the nude mouse embryo differs markedly from the normal embryonic thymus in its lack of demonstrable Ia antigen. Furthermore, TR4 and TR5 were only expressed on occasional epithelial cells lining the cysts of the nude thymus in a mutually exclusive fashion. The majority of stromal cells of the nude thymus however, is negative for all ER-TR antisera tested.

In addition, we have shown that in organ cultures, the organization of the stroma of thymic lobes remains intact, at least for a period of 11 days. Embryonic thymi cultured in the presence of deoxyguanosine (dGuo), which causes depletion of lymphoid cells, also contain cortical and medullary areas as identified by the presence of TR3,4+ve and TR5+ve stromal cells. This indicates that the lack of organization in the nude thymus is not simply due to the absence of lymphoid cells.

1. *INTRODUCTION*

Mammalian T lymphocyte populations are derived from hemopoietic stem cell precursors (1). These cells migrate to the thymus and during ontogeny are already evident with the developing thymic rudiment by day 12 of gestation (2). Once within the thymus, proliferation occurs and the resultant progeny undergo differentiation into the various thymocyte subsets defined by anatomical location and surface marker phenotype (3,4,5). However, only a small proportion of thymocytes leave the thymus as emigrants and the lineage relationships between these and the other thymocyte populations is controver-

sial (5). Similarly, although intrathymic T cell differentiation appears to involve stromal cell/lymphoid stem cell interactions (6-9), the nature of these interactions and the influence of thymic microenvironments on T cell differentiation pathways are unclear. Characterization of the thymic stroma should, however, provide a basis for the elucidation of microenvironmental effects in T cell development.

A number of stromal cell types have been identified in the developing thymus including epithelial cells thought to be derived from both pharyngeal ectoderm and endoderm (10), cells bearing a marker characteristic of neuroendocrine cells (11,12) and macrophage and dendritic type cells of hematogenous origin (13,14). Recently, we have also prepared a panel of monoclonal antibodies distinguishing various stromal cell types in the adult thymus including cortical and medullary epithelial cells (15). In this report we use these antibodies to study the ontogeny of the thymic stroma in relation to the progression of thymocyte differentiation in normal thymus development (16) both *in vivo* and in organ culture and to examine the stromal composition of the defective nude thymic rudiment as a pointer to the role of the various stromal components in normal development. The results indicate that distinction of cortical and medullary stroma is possible as early as day 13 of development. In contrast, normal cortical/medullary representation is absent from the developing nude thymus which contains only a few cells reactive with the antibodies employed.

2. *MATERIALS AND METHODS*

2.1 *Embryonic material*

Embryonic material was obtained from inbred matings of CBA mice. Embryonic nude thymi were obtained from matings of B10.Br nu/nu and nu/+ mice. Males and females were caged overnight and checked for vaginal plugs the next morning. The day of finding a vaginal plug was designated day 0. Individual thymi were removed from 13- to 19-day embryos, as described previously (17).

2.2 *Organ cultures*

Organ cultures were carried out on the surface of Nuclepore filters (Nuclepore Corp., Pleasanton, CA) supported on gelatin foam rafts as described previously (18). To deplete organ cultures of the embryonic thymus of lymphoid cells, isolated thymic rudiments were explanted into organ culture at day 14 and cultured in 1.35 mM deoxyguanosine (dGuo) for 5 days (18).

2.3 *Antibodies*

Monoclonal antibodies used in the present study are listed in Table 1. For detection of monoclonal antibodies anti-rat-immunoglobulin conjugated to horse radish peroxidase (RaRa-Ig-HRP; DAKO, Copenhagen, Denmark) was used. To avoid aspecific binding of the conjugate it was optimally diluted in PBS containing 0.5% BSA and 1% normal mouse serum.

2.4 *Preparation and incubation of frozen sections*

Frozen sections were prepared and incubated, and photography was performed as previously reported (16).

Table 1

Monoclonal antibody[1]	Reacts with
ER-TR3	Ia antigen on cortical and medullary stromal cells
ER-TR4	cortical epithelial cells
ER-TR5	medullary epithelial cells
ER-TR6	medullary interdigitating cells, macrophages and fibroblasts
ER-TR7	reticular fibroblasts

1. A detailed description of these antibodies was given in (15).

3. *RESULTS*

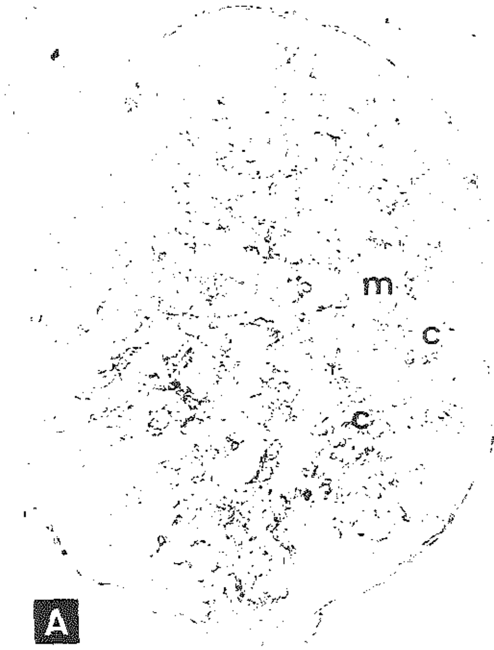
3.1 *General remarks*

Section 3.2 concentrates on the following questions: (a) at which point in the period of 13-19 days of gestation can the cell types, detected by the present antibodies, be identified in normal embryonic thymus? (b) at which stage in ontogeny can cortex and medulla first be identified in the embryonic thymus? Section 3.3 reports on the question: are any of the stromal cell types lacking in the rudimentary thymus of 14-day-old nude mouse embryos, as postulated by us in previous studies (17)? Section 3.4 reports on the following questions: is it possible to identify the various stromal cell types in embryonic thymus maintained in organ culture and does treatment with dGuo, which has been shown to eliminate the lymphoid components (18), have any effect on the representation of the various stromal cell types?

3.2 *Anatomical distribution of stromal cell types in the embryonic thymus of normal mice*

Serial frozen sections of embryonic thymus of gestational age day 13 till 19 incubated with monoclonal ER-TR antibodies demonstrated that all TR antigens are expressed from day 13 onwards. However, each type of antigen had a distinct localization pattern. Frozen sections of embryonic thymus of gestational age day 13 and 14 incubated with ER-TR4 antibodies show that TR4+ve epithelial cells localize in the outer region of the lobes, the developing cortex, whereas the centre of the lobes is TR4-ve (Fig. 1a). Serial frozen sections incubated with ER-TR5 reveal that ER-TR5+ve epithelial cells are present in more restricted foci (Fig. 1b). ER-TR6 reacts with scattered stromal cells in the centre and with the capsule and trabeculae (Fig. 1c). Serial frozen sections incubated with ER-TR7 demonstrate that ER-TR7+ve reticular fibroblasts can be detected at day 13 and 14, in the thymic capsule and trabeculae (Fig. 1d). A few TR7+ve cells localize in cortex and medulla. Frozen sections incubated with ER-TR3 antibodies show that, at day 13 and 14, expression of TR3 (Ia) is focal (Fig. 2a) with a pattern of localization, resembling that of TR5. A negative control sections, incubated with second stage antibodies only, is shown in Fig. 2b.

At day 16 of gestation, the distribution of TR3+ve cells has markedly changed. For the first time the distribution of TR3+ve cells resembles the distribution of TR3+ve cells in adult thymus, i.e. a reticular meshwork in the cortex and a more confluent pattern in the medulla (15). In contrast, at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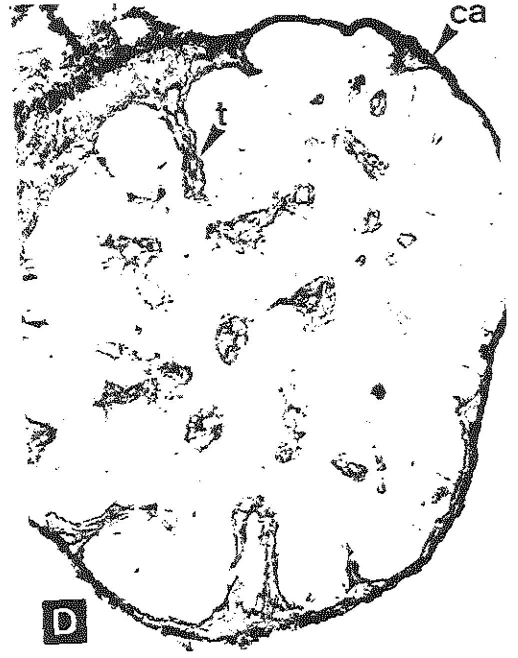
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Figure 2. Immunoperoxidase staining pattern of isolated thymic lobes of CBA mice on day 14 of gestation. (a) and (b) represent frozen sections incubated with ER-TR3 and a negative control section, incubated with 2nd step and DAB only, respectively. Magnification: 150x.

this stage in embryonic development the distribution patterns of TR4,5,6 and 7 positive cells have not changed when compared to day 13. Basically, the distribution patterns are similar to the patterns observed in adult thymus.

With increasing age the thymic lobes increase in size and cellularity. The medulla becomes more branched and extended and trabeculae penetrate deeper into the lobe. But basically the stromal architecture does not change anymore.

Thus, as far as stromal components are concerned the thymus at day 13 in ontogeny is already subdivided into cortex and medulla. Already at day 16 the stromal organization resembles the architecture of the adult thymus.

Figure 1. Immunoperoxidase staining pattern of isolated thymic lobes of CBA mice on day 14 of gestation. (a), (b), (c) and (d) represent serial frozen sections incubated with monoclonal antibodies ER-TR4, 5, 6 and 7, respectively. ca = capsule, c = cortex, m = medulla. Magnification: 150x.

3.3 Anatomical distribution of stromal cell types in the rudimentary thymus of the nude mouse embryo

Frozen sections of the thymic rudiment of the nude mouse embryo at gestational age day 16 incubated with ER-TR3 do not reveal any TR3+ve cells (not shown). Frozen sections incubated with ER-TR7 reveal that the TR7-ve thymic tissue of the embryonic nude thymus is embedded in a thick capsule of TR7+ve reticular fibroblasts (Fig. 3a). Serial frozen sections incubated with ER-TR4

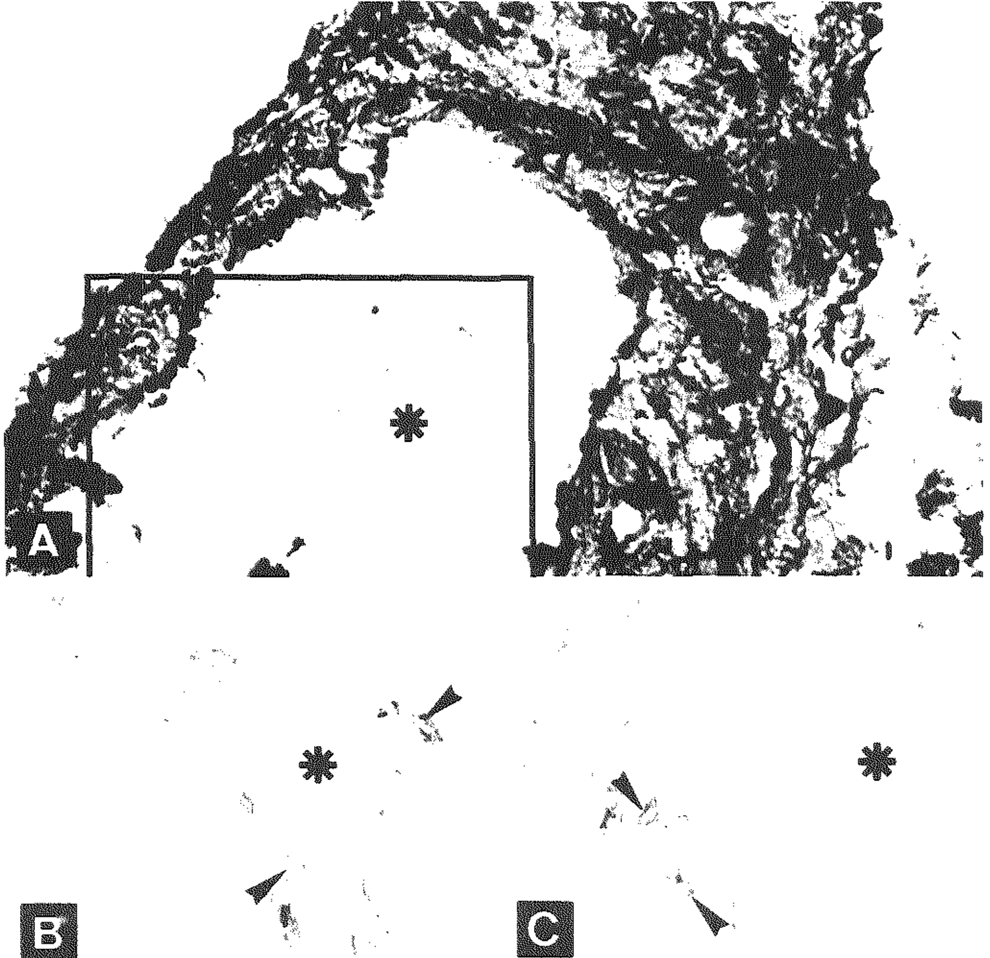
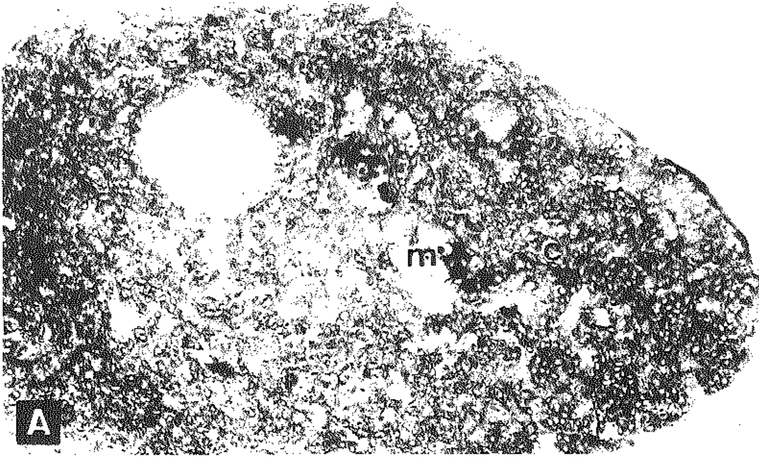


Figure 3. Immunoperoxidase staining pattern of the thymic rudiment of B10.BR nu/nu mice on day 16 of gestation. (a), (b) and (c) represent serial frozen sections incubated with monoclonal antibodies ER-TR7, 4 and 5, respectively. Only the boxed area of (a) is shown in (b) and (c). Note the large cyst indicated by an asterisk. Occasional positive cells are indicated by arrows.
Magnification: 360x.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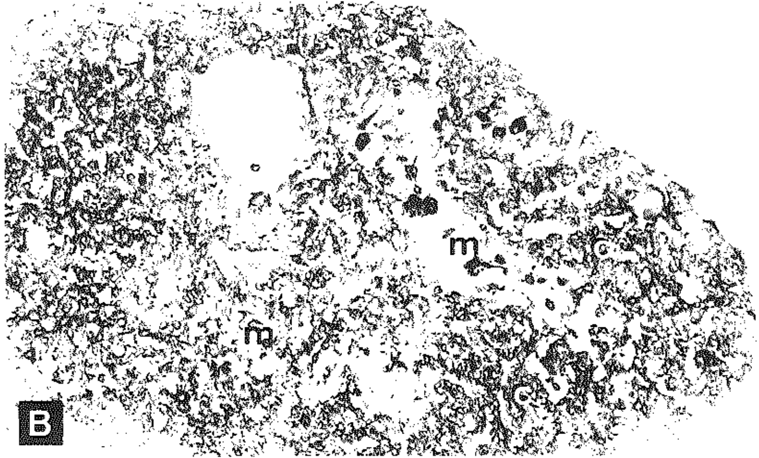
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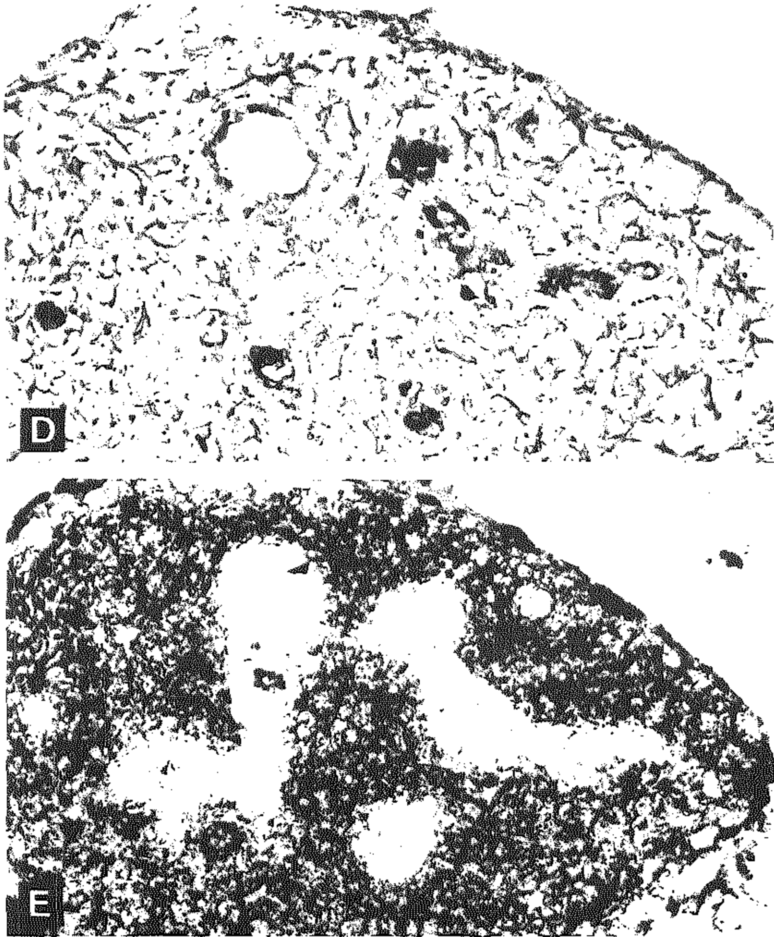

Figure 4. Immunoperoxidase staining pattern of isolated thymic lobes of CBA mice explanted into organ culture on day 14 of gestation and cultured for 5 days in dGuo. (a), (b), (c), (d) and (e) represent serial frozen sections incubated with monoclonal antibodies ER-TR3, 4, 5, 6 and 7, respectively.
c = cortex, m = medulla. Magnification: 150x.

(Fig. 3b) and ER-TR5 (Fig. 3c) reveal that a few TR4 and TR5+ve cells line the cysts of these abnormal thymuses. The serial sections show that these cells express either TR4 or TR5. Serial sections incubated with ER-TR6 reveal that only the capsule is TR6+ve (not shown). In summary, the thymic rudiment of the nude mouse embryo differs markedly from the normal embryonic thymus in its lack of demonstrable Ia antigens. Furthermore, only a very small minority of epithelial cells of the nude rudiment express either TR4 or TR5. The majority of cells of the nude stroma do not express any of the TR antigens.

3.4 *Anatomical distribution of stromal cell types in the embryonic thymus in vitro*

Frozen sections of embryonic thymus cultured with dGuo for 5 days, incubated with monoclonal anti-Thy-1 antibodies do not show any Thy-1+ve lymphoid cells (not shown) in agreement with our previous observations (18). Serial frozen sections incubated with ER-TR3 (Ia), reveal a confluent staining pattern throughout most of the lobe, whereas some foci of cells stand out as negative (Fig. 4a). Serial frozen sections incubated with ER-TR4 (Fig. 4b) show a similar staining pattern, though less intense and TR4+ve cells correspond to TR3+ve cells. Thus dGuo treatment appears not to impair the development of Ia antigen expression on the cortical epithelium. In contrast, serial frozen sections incubated with ER-TR5 (Fig. 4c) demonstrate foci of TR5+ve cells, whereas the majority of stromal cells are TR5-ve. From the serial sections it can be seen that TR5+ve cells are TR4-ve. Serial sections incubated with ER-TR6 demonstrate a meshwork of nonlymphoid cells throughout the lobe which is TR6+ve with occasional accumulations of TR6+ve cells. Whether these cells are fibroblastic or belong the macrophage or dendritic cell lineage (Table 1) cannot be ascertained at the present time. Furthermore the capsule is also TR6+ve (Fig. 4d). Serial sections incubated with ER-TR7 reveal that the thymic lobe contains TR7+ve cells, which localize throughout the more peripheral areas (Fig. 4e).

Frozen sections of embryonic thymus cultured for 11 days without dGuo, and incubated with ER-TR antibodies reveal staining patterns similar to those seen *in vivo*.

In summary, the present results show that organization of the stroma of cultured intact thymic lobes remains intact for a period of at least 11 days. Stromal cells apparently do not become randomly distributed *in vitro*. Embryonic thymi cultured in the presence of dGuo contain cortical and medullary regions, as identifiable by the presence of TR3,4+ve and TR5+ve cells, indicating that neither of these components is impaired by dGuo treatment.

4. *DISCUSSION*

The present study reports on the anatomical distribution of the nonlymphoid components in the normal embryonic thymus *in vivo* and *in vitro* and in the embryonic thymic rudiment of the nude mouse. For the first time, we demonstrate that as early as day 13 of development the thymic stroma of the normal embryo is differentiated into cortical and medullary regions identified by TR4+ve cortical epithelial cells and TR5+ve medullary epithelial cells. Comparison of the labeling pattern with antibodies TR5 and TR3 (anti Ia) also revealed that initial Ia expression at day 13 is localised to the medulla, expanding throughout the thymic lobe by day 16 as reported previously (17). At present it is not clear whether this focal pattern of Ia labeling at day 13 reflects labeling of epithelial cell Ia (17) or labeling of Ia+ve immigrant antigen presenting-type cells of hematogenous origin (19). Recently, however, we have demonstrated the existence of at least three subpopulations of medullary stromal cells in the adult mouse thymus using two-color fluorescence: (1) TR5+ve,Ia-ve; (2) TR5-ve,Ia+ve; (3) TR5+ve,Ia+ve; and it should be possible to extend this analysis to the fetal situation (E. van Vliet, unpublished observations).

A pattern of TR antigen expression similar to that seen during *in vivo* development was also observed in thymus lobes explanted at day 14 and maintained in organ culture for up to eleven days. Even when thymic lobes were rendered alymphoid by organ culture in the presence of dGuo for five days, discrete medullary foci within a matrix of cortical type cells were observed.

Thus organ cultures provide a reasonable representation of the *in vivo* thymic environment with regard to cortical and medullary epithelial areas and this pattern is not grossly disturbed by dGuo treatment. In contrast, the alymphoid thymic rudiment of the nude mouse was found to contain only occasional TR4 or TR5 positive cells associated with epithelial cysts and was not organized into cortical and medullary areas. As indicated by the presence of cortical and medullary cell groupings in alymphoid dGuo-treated normal rudiments, this lack of organization is unlikely to be due to the absence of lymphoid cells, supporting the notion of a defect in one or more epithelial components in the nude (10,12,14). The absence of Ia in the nude thymic rudiment together with the lack of TR6 labeling in the epithelial region also implies that not only is epithelial Ia absent as described previously (14), but that the nude epithelium, unlike the normal epithelium, does not support colonization by Ia+, TR6+ accessory cells of hematogenous origin. In addition to the epithelial defect, this could contribute to the inability of the nude thymic environment to support thymopoiesis.

Overall, our observations indicate that the division of those thymic stromal components essential for thymopoiesis into cortical and medullary regions is evident by day 13 of gestation. This precedes the expression of most T cell markers on the lymphoid population (16) indicating that cortical and medullary stromal environments are established before the development of the two major subpopulations of cortical and medullary thymocytes. Whether this early division of the stroma into cortex and medulla provides the environments needed for the maturation of two lymphoid precursor types separately committed to the cortical and medullary pathways, as suggested by some authors (20), or whether the progeny of a single precursor type move sequentially through cortical and medullary environments remains a matter for speculation.

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CHAPTER 7

THE INFLUENCE OF DEXAMETHASONE TREATMENT ON THE LYMPHOID AND STROMAL COMPOSITION OF THE MOUSE THYMUS; A FLOWCYTOMETRIC AND IMMUNOHISTOLOGICAL ANALYSIS

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SUMMARY

The effect of injection of a range of doses of dexamethasone on the distribution of T cell subpopulations and stromal cells in the thymus of BALB/c mice was investigated with flowcytometry and immunohistology. To this purpose we used (1) monoclonal antibodies directed to the T cell differentiation antigens Thy-1, T200, Lyt-1 Lyt-2, T4, MEL-14, and (2) monoclonal antibodies directed to various classes of stromal cells.

Injection of increasing doses of 5-130 mg/kg body weight dexamethasone gradually leads to a depletion of the cortical thymocyte population, i.e. bright Thy-1+ve, dull T-200+ve, bright Lyt-2+ve and bright T4+ve cells. These cortical cells are very dull MEL-14+ve and express variable numbers of Lyt-1 molecules.

Not only the cortex but also the medulla is affected by dexamethasone although to a lesser extent. Dexamethasone injection at 130 mg/kg selects for a medullary population, which is predominantly dull Thy-1+ve, bright T-200+ve, bright Lyt-1+ve and Lyt-2-ve. These dexamethasone-selected medullary thymocytes are either T4+ve or T4-ve. Under these conditions, MEL-14+ve cells were no longer present in the cortex but accumulated in the medulla. Interestingly, the large majority of cells in the perivascular spaces were brightly MEL-14+ve. Staining of sequential sections showed that this subpopulation has a typical 'helper' phenotype. This observation provides strong evidence that under the present experimental conditions, these perivascular compartments are an exit pathway for emigrating T cells.

The medullary population contains a phenotypically distinct, dexamethasone sensitive subpopulation. This conclusion is based on two findings: (1) 130 mg/kg dexamethasone depletes the thymus of all but 4% of the thymocytes, which form a much smaller subpopulation than the population of dull Thy-1+ve cells (amounting to 15% of the total thymocytes). (2) The medulla contains a subpopulation of dull Lyt-2+ve cells, which are resistant to 20 mg/kg dexamethasone, but depleted by 130 mg/kg.

Dexamethasone does not only affect thymic lymphoid cells, but also has a severe effect on thymic nonlymphoid cells. Even at low doses, dexamethasone induces TR4+ve cortical epithelial-reticular cells to become spherical ('nurse cell like') structures which are depleted of lymphoid cells. These stromal cells do no longer express MHC antigens in a membrane bound fashion. With increasing dexamethasone doses this phenomenon becomes more pronounced. In contrast, the medullary epithelial cells appear morphologically unaffected even

at a dose of 130 mg/kg dexamethasone.

The present data indicate a functional correlation between stromal cells in the cortex and developing cortical thymocytes.

1. *INTRODUCTION*

The study of intrathymic T cell differentiation is facilitated by markers which allow identification and separation of cells at different stages of maturation. Within the normal mouse thymus two subpopulations of thymocytes can be identified: (1) a major population of small immature cortical thymocytes, comprising 85% of the thymocyte population. These cells are brightly Thy-1+ve, Lyt-2+ve; variable in their Lyt-1 expression (1) and express high levels of receptors for peanut agglutinin (PNA) (2,3); (2) a minor population of immunologically competent medullary thymocytes, comprising 15% of thymocytes. The majority of the latter cells are dull Thy-1+ve, very dull PNA+ve ('PNA-ve'), brightly Lyt-1+ve and Lyt-2-ve. There is accumulating evidence that these subpopulations each have their own generative compartment (4-6). One of the classical methods of separating cortical and medullary cells is the treatment of mice with corticosteroids, which depletes all but the medullary functionally mature cells (7,8). In recent immunocytochemical studies from our group the effect of a single dose of dexamethasone on thymocyte subsets was investigated with monoclonal antibodies directed against the T cell differentiation antigens Thy-1, T-200, Lyt-1 and Lyt-2 (1). The major finding was that injection of dexamethasone resulted in selection of dull Thy-1+ve, bright T-200+ve, bright Lyt-1+ve, Lyt-2-ve cells, being the major medullary subpopulation of thymocytes. However, recent studies indicate that part of the medullary cells are dexamethasone sensitive and that the size of the remaining pool is critically dependent on the dose of corticosteroids used (3,9). This prompted us to extend our previous immunocytochemical studies with a range of doses of dexamethasone and a panel of monoclonal antibodies directed to mouse T cell differentiation antigens, including T4 (10) and MEL-14 (11). Furthermore, the present study includes immunohistological studies of the thymus with monoclonal antibodies directed against various classes of stromal cells in the mouse thymus (12). These latter antibodies enable us to approach the question whether the stroma is also affected by dexamethasone treatment and thus responsible for a defective T cell differentiation process in the thymus. The present results indicate that dexamethasone treatment has a profound effect on cortical thymocytes as well as a subpopulation of medullary thymocytes. In addition, the cortical stroma but not the medullary stroma is strongly affected.

2. *MATERIALS AND METHODS*

2.1 *Mice*

Male BALB/c mice (Bomholtgard, Ry, Denmark), age 6-7 weeks, were used in the present study. They were kept under routine laboratory conditions.

2.2 *Dexamethasone treatment*

Groups of 10 mice were injected i.p. with single doses of 5, 10, 15, 20 or 130 mg/kg body weight of dexamethasone-2-phosphate (Organon, Oss, The Netherlands) diluted in phosphate buffered saline (PBS), 48 hours before sacrifice.

2.3 *Cell suspensions*

Lymphoid cell suspensions were prepared from 8 thymi for each dose of dexa-

methasone. The suspensions were fixed in 0.5% paraformaldehyde (c.f. 13,14) and further processed for flowcytometric analysis.

2.4 Tissue preparation
Thymi of 2 mice of each dose of dexamethasone were collectively embedded and frozen sections were cut as reported before (1).

2.5 Antibodies
The antibodies used in the present study are listed in Table 1. Binding of monoclonal antibodies was detected with rabbit-anti-rat-immunoglobulin conjugated to horse radish peroxidase (RaRa-HRP) (Dako, Copenhagen, Denmark). Alternatively, binding of monoclonal antibodies was detected with rabbit-anti-rat-immunoglobulin conjugated to fluorescein-isothiocyanate (RaRa-FITC) (Nordic, Tilburg, The Netherlands). To prevent aspecific binding to Fc receptors, the conjugates were routinely deaggregated before use by centrifugation in a Beckman airfuge at 10^5 g and optimally diluted in PBS containing 0.5% BSA and 1% normal mouse serum. Immunoperoxidase staining and flowcytometric analysis were performed as reported previously (1,12).

3. *RESULTS*

The effect of dexamethasone injection on the distribution of T cell subpopulations and thymic stromal cells has been studied using two approaches. Firstly, we analyzed the distribution of T cell subpopulations in normal and dexamethasone-treated mice by quantitative flowcytometry. Secondly, we localized these T cell subsets and stromal cells in the thymus by immunohistological methods. Table 2 shows the doses of dexamethasone used, the average weights of the thymuses and the average yields of thymocytes from normal mice and mice treated with dexamethasone. These data show a gradual decrease of both the weight of the thymus and the average cell yield with increasing doses of dexamethasone. At the highest dose used, i.e. 130 mg/kg body weight, the cell yield is reduced to 4% of that of the control mice. This indicates that at this dose the maximal depletion of lymphocytes is reached, and the remaining 4% cells are considered dexamethasone resistant (cf. 9).

3.1 Fluorescence analysis
a. Thy-1
Analysis of cell suspensions obtained from the thymus of normal control mice incubated with monoclonal anti-Thy-1 antibody followed by RaRa-FITC shows that 97% of thymocytes are Thy-1+ve (Fig. 1a, Tables 3 and 4). The fluorescence profile of Thy-1+ve control cells shows a wide range of staining intensities and also shows a shoulder at the left side, which indicates a dull Thy-1+ve subpopulation. Dexamethasone treatment results in a reduction of the relative frequency of the bright Thy-1+ve cells, whereas the relative frequency of dull Thy-1+ve cells increases (Table 4). With increasing doses this effect becomes more prominent and at a dose of 130 mg/kg body weight this effect is most pronounced. Table 3 also shows the percentage of Thy-1-ve cells increasing from 3% in control thymi to 15% Thy-1-ve cells at a dose of 130 mg/kg dexamethasone. It should be noted that the fluorescence profile at a dexamethasone dose of 130 mg/kg is essentially similar to the one shown in a previous report, in which we used 60 mg/kg (cf. 1).
b. T-200
The fluorescence profile of T-200+ve cells shows a small shoulder at the right side of the curve (not shown). It also shows that the variation in anti-

Table 1. Antibodies used in the present study

Reagent code	Reacts with	Reference
59-AD-22	Thy-1 antigen on T cells	15
30-G12	T-200 " " " "	15
53-7-313	Lyt-1 " " " "	15
53-6-72	Lyt-2 " " " "	15
H129.19	T4 " " " "	10
MEL-14	Homing receptor for peripheral lymph nodes	11
ER-TR4	cortical epithelial cells	12
ER-TR5	medullary epithelial cells	12
M1/42.3.98	H-2K	16
M5/114.15.2	I-A/E	17

Table 2. Recovery of thymocytes from normal and dexamethasone-treated mice

Dose of dexamethasone (mg/kg body weight)	Thymus weight (mg)	Thymocyte yield $\times 10^{-6}$
0[a]	55.0 ($\pm$ 2.2)	140.0[b] ($\pm$ 7.6)[c]
5	26.6 ($\pm$ 1.2)	35.9 ($\pm$ 2.7)
10	22.3 ($\pm$ 1.3)	19.3 ($\pm$ 1.7)
15	21.4 ($\pm$ 1.1)	15.6 ($\pm$ 1.9)
20	20.8 ($\pm$ 0.8)	14.7 ($\pm$ 1.3)
130	16.2 ($\pm$ 0.8)	5.8 ($\pm$ 0.5)

a. Dexamethasone treatment was performed by ip injection of dexamethasone-21-phosphate 48 hours before sacrifice.
b. Values are expressed as the mean number of viable cells recovered, calculated from one representative experiment.
c. Values in brackets indicate the SEM (n=10).

Table 3. Percentage labeled cells in thymus suspensions of normal and dexamethasone-treated mice[a].

Reagent	Dose of dexamethasone[b]					
	0	5	10	15	20	130
Thy-1	97(±1)[c]	95(±2)	92(±1)	90(±3)	88(±5)	85(±5)
T-200	99(±1)	98(±1)	96(±1)	94(±3)	93(±3)	93(±3)
Lyt-1	92(±1)	90(±1)	87(±3)	88(±4)	84(±2)	87(±1)
Lyt-2	87(±2)	75(±3)	67(±2)	54(±2)	51(±3)	43(±2)
T4	86(±4)	77(±2)	70(±2)	66(±7)	64(±4)	66(±3)
MEL-14	2(±1)	3(±1)	4(±3)	0(±0)	0(±0)	2(±2)

a. Values are expressed as the mean percentage of positive cells, calculated from 3 experiments.
b. Dexamethasone treatment was performed by ip injection of 0, 5, 10, 15, 20 or 130 mg/kg body weight dexamethasone-2-phosphate 48 hours before sacrifice.
c. Values in brackets indicate the SEM.

Table 4. Fluorescence intensity of thymus suspensions of normal and dexamethasone-treated mice[a]

Reagent	Dose of dexamethasone[b]					
	0	5	10	15	20	130
Thy-1	132	132	132	129	129	124
T-200	123	128	127	129	129	127
Lyt-1	112	121	116	113	114	109
Lyt-2	112	112,125	108,116	124	127	123
T4	98	99	97	102	104	102
MEL-14	71	71	73	72	73	74
neg.[c]	69	70	70	70	69	70

a. The average fluorescence intensity of positive cells is given in arbitrary units. Values are given from one representative experiment.
b. Dexamethasone treatment was performed by ip injections of 0, 5, 10, 15, 20 or 130 mg/kg body weight dexamethasone-2-phosphate 48 hours before sacrifice.
c. neg. = negative control, i.e. cells stained with an irrelevant monoclonal antibody and the conjugate.

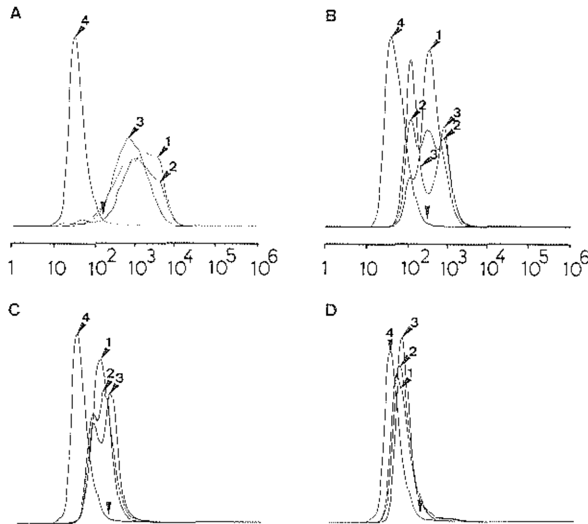


Figure 1. Fluorescence histograms of thymocytes of normal and dexamethasone -treated BALB/c mice, incubated with monoclonal antibodies directed to the antigens (a) Thy-1, (b) Lyt-2, (c) T4, (d) MEL-14. The fluorescence intensity is determined over 256 channels representing 6 decades with a multichannel analyzer and plotted on a logarithmic scale. Fluorescence intensity increases from left to right on the ordinate. For each channel number the relative frequency of cells with the corresponding fluorescence intensity is expressed (abscissa). In Figs. 1a-d 4 profiles are shown: profile 1 represents thymocytes from untreated mice, profile 2 represents thymocytes from mice treated with 10 mg/kg dexamethasone, profile 3 represents thymocytes from mice treated with 130 mg/kg dexamethasone, and profile 4 represents thymocytes from untreated mice, incubated with 2nd step antibodies only. The arrow on the abscissa indicates the cut-off channel used for the calculation of percentages positive cells.

genic density per cell is less when compared with the density variation of Thy-1 molecules. Dexamethasone treatment results in a slight increase in the average fluorescence intensity (Table 4). The frequency of T-200-ve cells increases to 7% at a dose of 130 mg/kg dexamethasone.

c. *Lyt-1*

Analysis of Lyt-1+ve cells in the normal thymus reveals that 92% of thymocytes expresses Lyt-1 (Tables 3 and 4). The fluorescence profile shows a wide variation in antigen density. The shoulder at the right side of the profile indicates a subpopulation of brightly Lyt-1+ve cells. Dexamethasone treatment results in a relative increase in the frequency of the latter cells. This effect is most prominent with 130 mg/kg dexamethasone. However, with increasing dose the percentage of Lyt-1-ve cells also increases, up to 13%.

d. Lyt-2

The fluorescence profile of Lyt-2+ve cells in the thymus of normal mice shows a bimodal fluorescence distribution (Fig. 1b), which indicates a brightly Lyt-2+ve population and a population of cells of which the fluorescence ranges from negative to very dull. In a previous study we tentatively called the latter cells Lyt-2-ve (1). The trimodal profiles which correspond to doses of 5, 10 and 15 mg/kg dexamethasone clearly demonstrate the existence of three populations of cells with regard to the cell surface density of Lyt-2 molecules. At a dose of 10 mg/kg, 29% of cells are brightly Lyt-2+ve, 36% are dull Lyt-2+ve and 35% are Lyt-2-ve. Dot display analysis reveals that the bright Lyt-2+ve subset is slightly larger than the dull subpopulation (data not shown). With a dose of 130 mg/kg the most brightly Lyt-2+ve subset is selected. With increasing dose the percentage of Lyt-2-ve cells increases from 13% in control cells to 57% in mice injected with 130 mg/kg dexamethasone (Table 3). These data indicate that with regard to corticosteroid-sensitivity, three categories of Lyt-2+ve cells can be distinguished in the mouse thymus: (1) the majority of small Lyt-2+ve cells, which are killed at a low dose of 5-20 mg/kg dexamethasone, (2) a minority of medium-sized, dull Lyt-2+ve cells, which are killed in a dose range of 20-130 mg/kg dexamethasone, (3) a minority of medium-sized, bright Lyt-2+ve cells, which are resistant to 130 mg/kg dexamethasone.

e. T4

Analysis of T4+ve cells in the normal thymus shows that 86% of the thymocytes express T4 (Fig. 1c, Table 3). Dexamethasone treatment depletes the thymocytes for T4+ve cells and, upon increasing doses of dexamethasone, the population of T4-ve cells increases gradually to 34% with 130 mg/kg dexamethasone. The T4+ve population shows a shift towards cells with a higher number of molecules T4 expressed per cell (Table 4).

f. MEL-14

Virtually all the thymocytes in the normal thymus express very low levels of the MEL-14 antigen, the receptor for high endothelial venules (HEV) in peripheral lymph nodes (Fig. 1d, Tables 3 and 4), as indicated by the small shift of the fluorescence peak to the right when compared with the negative control. Dexamethasone treatment results in a small change in the fluorescence profiles of all MEL-14+ve cells which indicates a selection for cells with a higher MEL-14 density.

3.2 *Anatomical distribution of T cell subpopulations in the thymus of normal and dexamethasone-treated mice*

a. Thy-1

Frozen thymus sections incubated with anti-Thy-1 antibodies reveal that the major population in the normal thymus consists of bright Thy-1+ve cortical thymocytes, whereas the minor population of medullary cells is dull Thy-1+ve (data not shown; 1). Treatment with 5-130 mg/kg dexamethasone results in a dramatic reduction of the size of the thymic cortex. Most of the remaining cells in the thymus are dull Thy-1+ve; they localize in the medulla. However, we noted small numbers of bright Thy-1+ve cells in the outer cortical zone and in the medulla at all doses of dexamethasone tested. With increasing dose we observed a substantial reduction in the number of bright Thy-1+ve cortical cells. Concomittantly, the numbers of cortical Thy-1-ve cells increased. These cells were predominantly located in the subcapsular cortical area. However, small foci of Thy-1-ve cells were also found in the medulla (not shown).

b. T200

Serial frozen sections incubated with anti-T200 antibodies reveal that virtually all thymocytes in the normal thymus are T200+ve (data not shown; 1). Medullary thymocytes are more brightly labeled than cortical thymocytes. In the dexamethasone-treated thymus, however, at all doses the major population is the brightly labeled medullary population. These cells were also located within perivascular spaces and located around large venules in the medulla. Increasing the dose of dexamethasone results in reduction of the number of T200+ve cells in the cortex and a concomittant increase in the number of T200-ve cells. Comparison of adjacent sections reveals that the frequency of Thy-1-ve cells is slightly larger than the frequency of T-200-ve cells. This observation corresponds to the flowcytometric data (see Table 3) and indicates the presence of a T-200 'only' subpopulation. Occasionally T200-ve cells were noted in the medulla (not shown).

c. Lyt-1

Serial frozen sections incubated with anti-Lyt-1 antibodies reveal a variable expression of Lyt-1 in both cortex and medulla (not shown, 1). The major population of cortical cells is dull Lyt-1+ve. However, a minority of cortical cells is bright Lyt-1+ve. The large majority of medullary thymocytes express high levels of Lyt-1 and it appears that dexamethasone treatment selects for this subpopulation. In the thymic cortex of dexamethasone-treated mice scattered bright Lyt-1+ve cells were noted. By comparison, the frequency of Lyt-1+ve cortical cells is lower than the frequency of cortical Thy-1+ve and T200+ve cells. At 130 mg/kg dexamethasone cortical Lyt-1+ve cells were no longer observed. Depletion among cortical Lyt-1+ve cells as a result of 5-20 mg/kg dexamethasone was random; there was no selection for a particular Lyt-1 subset. In the medulla the distribution of Lyt-1+ve cells was not different from control thymi , however, we noted that the cells in the perivascular spaces were all brightly Lyt-1+ve.

d. Lyt-2

Lyt-2 molecules are brightly expressed on virtually all cortical cells in the normal thymus (not shown, 1). In addition, occasional medullary thymocytes are either very dull or bright Lyt-2+ve. The majority of lymphoid cells in the medulla is Lyt-2-ve. Following treatment with 5-20 mg/kg dexamethasone most of the bright Lyt-2+ve cortical cells disappear. With increasing doses the frequency of the cortical Lyt-2+ve cells further decreases and at 130 mg/kg dexamethasone no Lyt-2+ve cells were observed in the cortex. At doses of 5-20 mg/kg the relative frequency of Lyt-2-ve and Lyt-2+ve cells in the medulla changes: the relative frequency of both dull and bright Lyt-2+ve cells increases. Consequently, the subpopulation of medullary dull Lyt-2+ve cells becomes more prominent. Lyt-2-ve, Lyt-2 dull and Lyt-2 bright cells are randomly distributed in the medulla (Fig. 2). A dose of 130 mg/kg eliminates the dull Lyt-2+ve cells, leaving bright Lyt-2+ve as well as Lyt-2-ve medullary cells behind. Cells in the perivascular spaces were negative for Lyt-2 expression.

e. T4

Serial sections incubated with anti-T4 antibodies demonstrate that the majority of cortical cells are brightly T4+ve (not shown). A subset of medullary thymocytes is also clearly T4+ve. Adjacent sections showed that these medullary cells are Lyt-2-ve. Treatment with increasing doses of dexamethasone results in a gradual decrease in the cortical T4+ve population. At 15-20 mg/kg a few T4+ve cells remain in the cortical area. At 130 mg/kg, however, no T4+ve cells were observed in the cortex. The medulla, in contrast, did not reveal any apparent differences in the T4 staining pattern, irrespective of the dose of dexamethasone. The exception was that the cells in the peri-

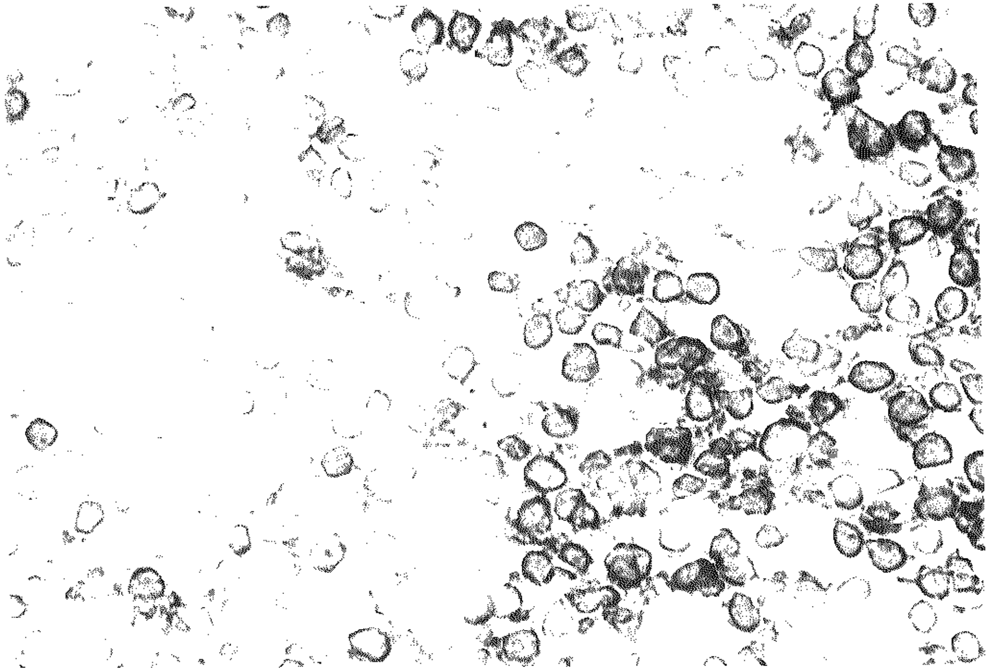


Figure 2. Immunoperoxidase staining pattern of thymus sections of BALB/c mice, treated with 15 mg/kg dexamethasone. Incubation with anti-Lyt-2. Note the variable staining levels of thymocytes. Magnification 875x.

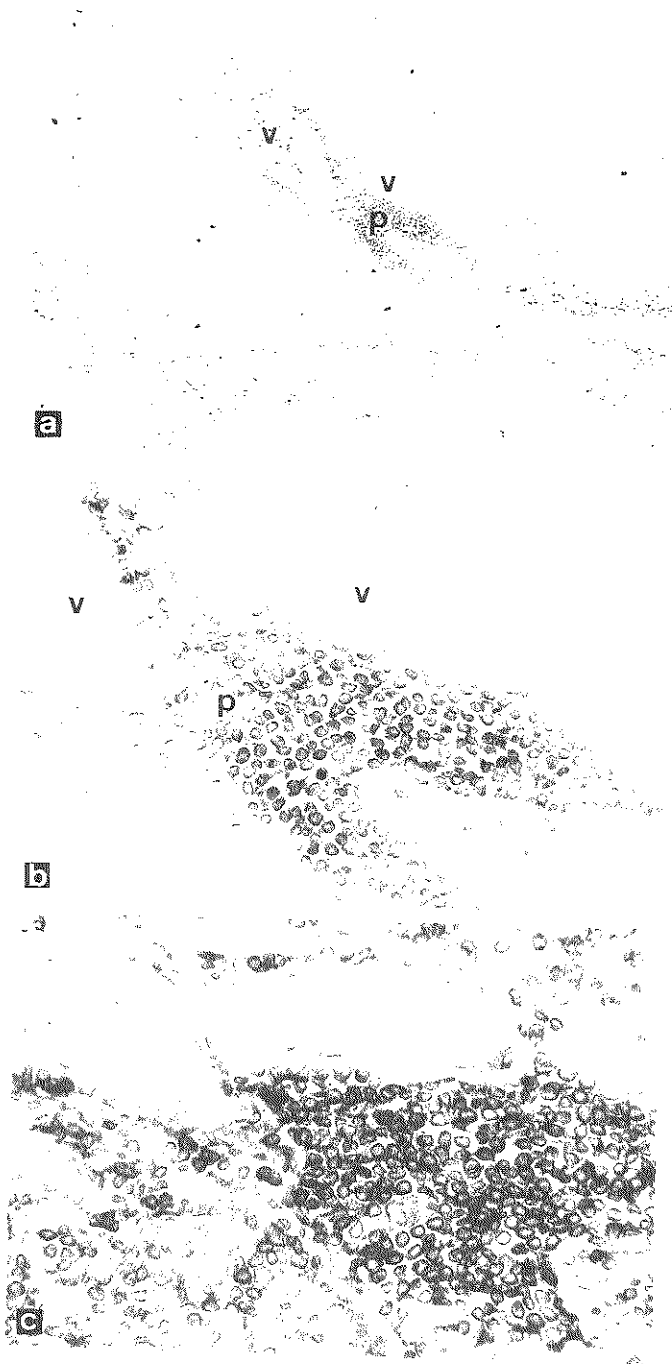
vascular spaces were all brightly T4+ve (Fig. 3).

f. MEL-14

In control thymi of BALB/c mice, MEL-14 is faintly expressed on virtually all cortical thymocytes. Individual cells, however, express MEL-14 at higher density. These cells also occur in the medulla. Even at low doses of dexamethasone, MEL-14 expression in the cortex was not observed, instead we noted foci of MEL-14+ve cells in the medulla. Surprisingly, we then found that virtually all lymphoid cells in perivascular spaces were MEL-14+ve (Fig. 3). This observation indicates that perivascular spaces may be priviliged sites where functionally mature T cells leave the thymus.

Figure 3. Immunoperoxidase staining pattern of frozen thymus sections of BALB/c mice treated with 130 mg/kg dexamethasone. Incubation with (a) and (b) anti-MEL-14, (c) anti-T4. (b) and (c) represent higher magnifications of (a). p = perivascular space; v = vessel. Magnifications: (a) 60x, (b) and (c) 570x.

Figure 4. Immunoperoxidase staining pattern of frozen thymus sections of normal mice and mice treated with 130 mg/kg dexamethasone. Incubation with ER-TR4. (a) and (b) represent the staining pattern of normal and dexamethasone-treated mice, respectively. ca = capsule; c = cortex; m = medulla. Magnification 140x.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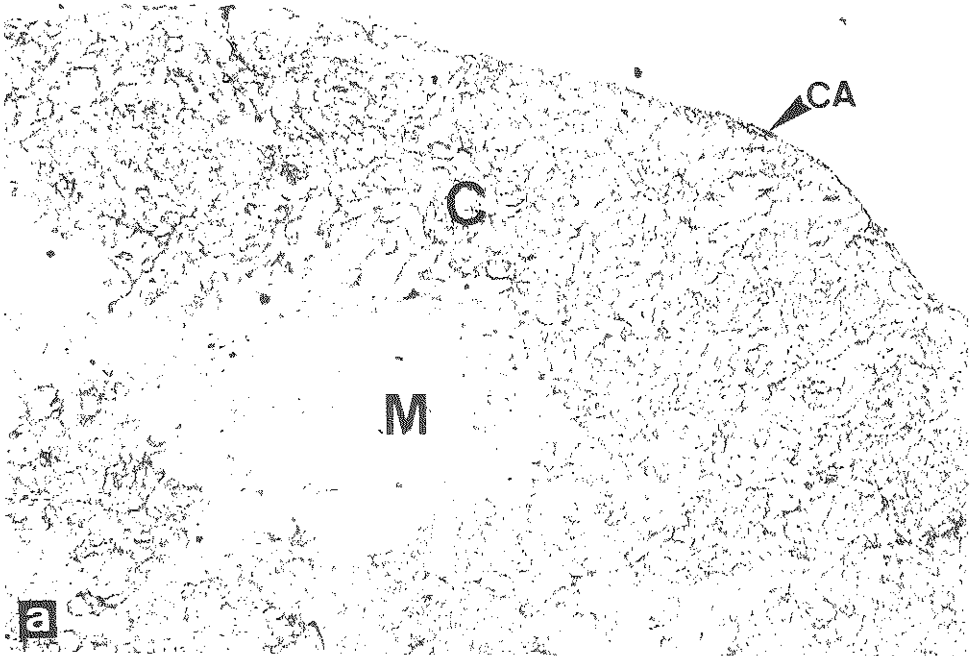
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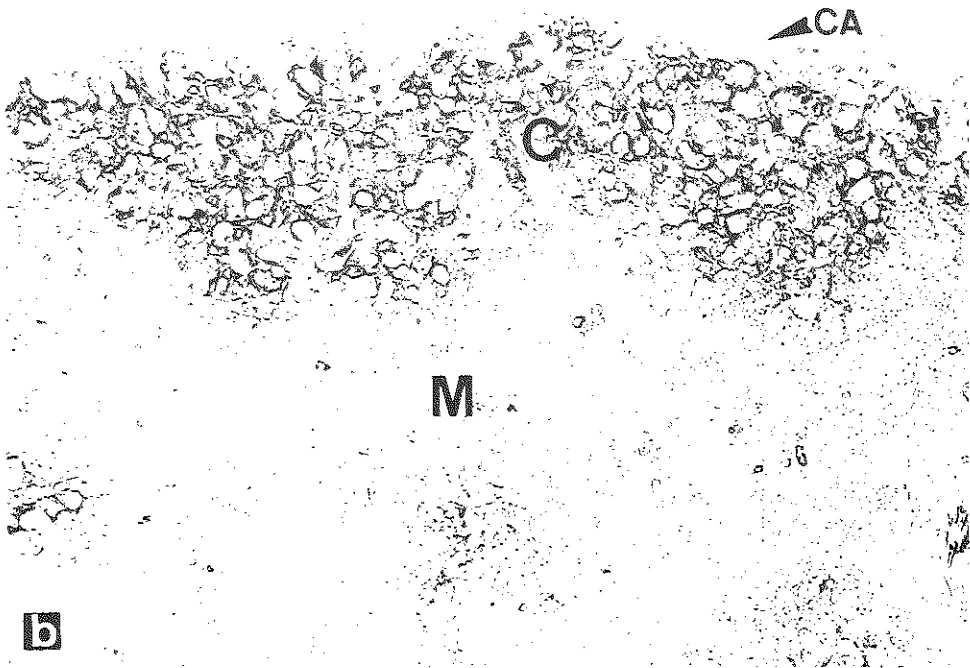
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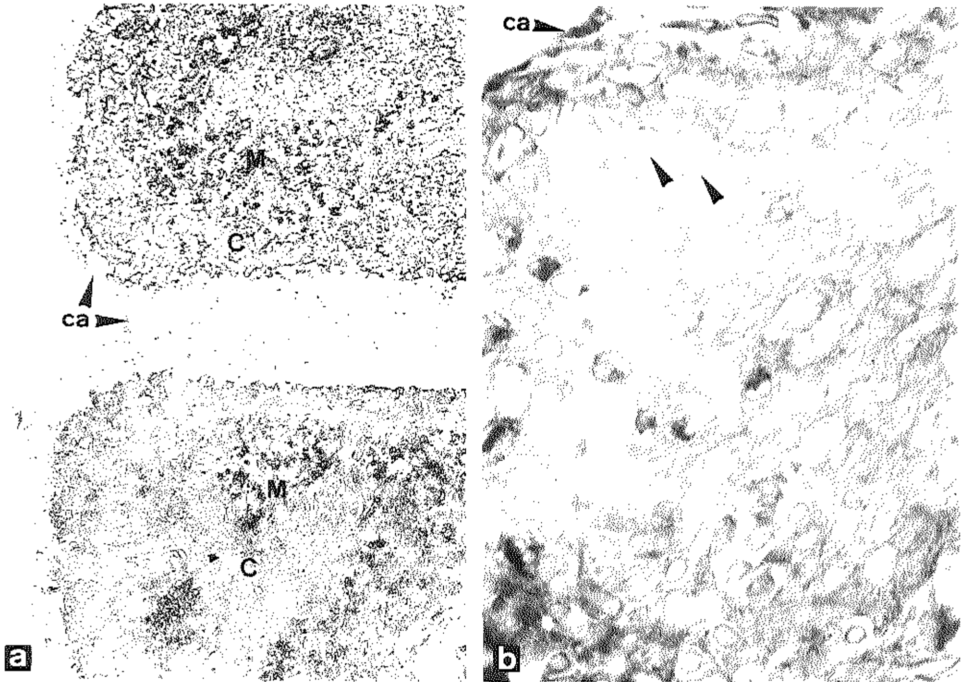

Figure 5. Immunoperoxidase staining pattern of frozen thymus sections of mice treated with 10 mg/kg dexamethasone. Incubation with (a) anti-I-A and (b) anti-H-2K. Note the confluent I-A expression in lower lobe and relatively unaffected upper lobe. Arrows indicate H-2K positive blasts in outer cortex. ca = capsule; c = cortex; m = medulla. Magnifications (a) 60x, (b) 875x.

3.3 *Anatomical distribution of nonlymphoid subpopulations in the thymus of normal and dexamethasone-treated mice*

The stromal composition of the thymus is also affected by dexamethasone treatment. We employed two monoclonal antibodies which react with cortical versus medullary stromal elements. ER-TR4 detects the cortical epithelium. In control thymi these cells form a fine lacework throughout the cortex. ER-TR5 detects medullary epithelial cells which are found in clusters in the medulla. The major finding after dexamethasone treatment is that the cortical stroma is seriously affected, while the medullary stroma remains unaffected. Sections stained with ER-TR4 reveal condensation of the cortical epithelium and epithelial cells tend to form spherical, 'nurse cell' like structures, depleted of thymocytes (Fig. 4). In some places the cortical stroma is even absent from the cortex. The medullary epithelium is not detectably affected by dexamethasone treatment, since treated thymi show distribution patterns which are similar to untreated control thymi. Increasing doses of dexamethasone cause a change in MHC staining patterns. In the cortex I-A antigens are normally expressed in a membrane bound fashion. However, even at a dose of 5 mg dexamethasone, this membrane associated staining pattern is lost (Fig. 5a). In-

creasing doses gradually lead to a confluent I-A staining pattern. H-2K staining changes in a similar way, leading to a strong confluent staining pattern throughout the thymus (Fig. 5b). In addition, these sections show lymphoid cells in the outer cortex which are clearly H-2K positive.

4. *DISCUSSION*

The present study shows that the frequency of thymocyte subpopulations and the anatomical distribution of thymic lymphoid and stromal subpopulations is markedly influenced by dexamethasone treatment. In general the data show that injection of dexamethasone into mice leads to selection for medullary weakly Thy-1+ve, strongly T-200+ve thymocytes. This paper confirms and extends previous immunocytochemical studies from our group (1), in which the effect of a single dose of 60 mg/kg dexamethasone was investigated. We now show that the T cell composition after dexamethasone treatment critically depends on the dose of dexamethasone used.

At increasing doses, a gradual depletion of the cortical population (i.e. brightly Thy-1+ve, dull T-200+ve, Lyt-1+ve, Lyt-2+ve, T4+ve cells) was observed. Concomittantly, cells negative for these T cell markers became prominent in the cortex.

Three minor subpopulations become clearly manifest:

1. 'null' cells, which do not bear any detectable T cell antigens. They are H-2K+ve.
2. cells, which only express T-200 but which do not express Thy-1, Lyt antigens or T4.
3. cells, which express T-200, Thy-1 but which do not expressLyt-antigens or T4.

The frequency of cells from each of these three subpopulations depends on the dose of dexamethasone injected. They cannot easily be identified at doses of 5-15 mg/kg due to the relatively high frequency of cells expressing the 'major' cortical phenotype, i.e. T-200, Thy-1, Lyt, T4+ve. They are however, prominent at a dose of 20-130 mg/kg. Similar subpopulations were observed in the thymus of sublethally irradiated mice (18). The relatively high frequency of these phenotypically distinct thymocyte subsets can be explained in two ways: (1) Although unlikely, it could be argued that dexamethasone injection influences the expression of cell surface antigens. (2) Dexamethasone induces extensive proliferation of 'early' thymocytes, beginning at 48 hours after injection of dexamethasone. The presence of 'null' cells is in line with this hypothesis, since 'null' cells presumably represent intrathymic prothymocytes (19,20). It is assumed that these cells proliferate and differentiate into cortical thymocytes (21). By comparing adjacent sections the following sequential changes in the cortical thymocyte phenotype were observed: 1. H-2K+ve 'null' cells. 2. T-200+ve cells. 3. T200, Thy-1+ve cells. 4. T-200, Thy-1, Lyt, T4, MEL-14+ve cells.

Not only the cortex but also the medulla is affected by dexamethasone. This conclusion is based on two observations: (1) 130 mg/kg dexamethasone depletes the thymus of all but 4% of thymocytes, which clearly is a much smaller subpopulation than the 15% dull Thy-1+ve, PNA-ve medullary cells. This observation is in concordance with the results of others (3,9); (2) the medulla contains a population of dull Lyt-2+ve cells, which are resistant to 20 mg/kg dexamethasone, but which are depleted by 130 mg/kg. This observation provides the first evidence for a differential sensitivity of a phenotypically distinct subpopulation of medullary thymocytes. Thus, dexamethasone resistant thymocytes do not represent the total medullary pool as originally

thought. They lack dull Lyt-2+ve cells. The present study also demonstrates that, whereas the large majority of cortical thymocytes are both Lyt-2+ve and T4+ve, medullary cells express either Lyt-2 or T4. This finding confirms the results of Dialynas et al. (22). We demonstrate here the existence of at least four phenotypically distinct medullary subpopulations: 1. null cells, 2. Lyt-2-ve T4+ve cells, 3. bright Lyt-2+ve, T4-ve cells, 4. dull Lyt-2+ve, T4-ve cells.

Cortical and medullary thymocytes were originally thought to be sequentially related as 'immature' and 'mature' pools on a single developmental pathway (8). Others, however, have demonstrated that the two subclasses develop largely independently (4-6). Our observations are consistent with the latter view. We demonstrated occasional small clusters of 'null' cells in the thymic medulla of dexamethasone-treated mice. Null cells were also found in the medulla by Huiskamp and van Ewijk (18) in sublethally irradiated mice. Furthermore, Jotereau and Le Douarin (23) demonstrated in chick-quail chimeras that during ontogeny thymocyte precursors enter the thymus both at the subcapsular region and at the cortico-medullary junction.

The present study further suggests that MEL-14+ve emigrant T cells use perivascular spaces as their exit pathway. We show here that in the normal thymus bright MEL-14+ve cells are scattered throughout the cortex; they also occur at low frequency in the medulla. Upon dexamethasone treatment these cells accumulate in the medulla (cf. 24). Furthermore, they accumulate in the perivascular spaces of the larger venules. From serial frozen sections it is obvious that these MEL-14+ve cells have a mature T helper phenotype (Thy-1+, T-200+, Lyt-1+, T4+, Lyt-2-). This observation extends our previous scanning electronmicroscopic study, in which we observed many lymphoid cells in contact with the peripheral wall of large venules (25). It is tempting to speculate that, also in the thymus, the migrating cells use MEL-14 or MEL-14-like receptors which recognize the endothelial lining during their exit transportation process. Thus, the presence of such receptors could be of crucial importance in the decision concerning which cells are going to leave the thymus.

The present study further demonstrates that dexamethasone not only affects thymic lymphoid cells, but also has an effect on thymic stromal cells. It appears that dexamethasone induces striking changes in the cortical reticular epithelial stroma but leaves the medullary stroma intact, at least as far as can be detected with the present markers. The TR4+ve cortical epithelial cells change into compact 'nurse cell like' structures (26) and some cortical areas are even devoid of stromal cells. The latter regions could represent similar regions observed in the normal thymus with the light and electron microscope (25,27). The confluent staining pattern observed after staining with anti-MHC antibodies indicates that epithelial-reticular cells no longer express MHC antigens in a membrane bound fashion. This was also observed by Huiskamp et al. (28) after sublethal irradiation. This phenomenon could indicate that structural disorganization of the thymic stroma leads to defective T cell differentiation. Indeed, a correlation between the regeneration of the thymic stroma and the onset of T cell differentiation has been observed (18,28). Similarly, in ontogeny T cell proliferation follows MHC expression in the thymus (29). Furthermore, Kruisbeek et al. (30) have shown that T helper cell differentiation can be blocked by administration of anti I-A antibodies. These experiments all point to the phenomenon that an intact thymic stroma expressing MHC antigens in a membrane bound fashion is a prerequisite for normal T cell development.

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CHAPTER 8

RETICULAR FIBROBLASTS IN PERIPHERAL LYMPHOID ORGANS IDENTIFIED BY A MONOCLONAL ANTIBODY

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SUMMARY

We have produced a panel of monoclonal antibodies directed against nonlymphoid cells in central and peripheral lymphoid organs. In the present paper we present the reactivity of one of these antibodies: ER-TR7. This antibody detects reticular fibroblasts, constituting the cellular framework of lymphoid and nonlymphoid organs and their products. Frozen sections of the spleen incubated with this antibody show a marked delineation of the red pulp and white pulp. Furthermore, the major white pulp compartments, i.e., the follicles and periarteriolar lymphoid sheath as well as the marginal zone are recognized by their characteristic labeling patterns. In lymph nodes, the capsule, sinuses, follicles, paracortex and medullary cords are clearly delineated. In the thymus and bone marrow no such specialized compartments were demonstrated. ER-TR7 reacts with an intracellular component of fibroblasts. Since ER-TR7 does not react with purified laminin, collagen types I-V, fibronectin, heparan sulphate proteoglycan, entactin or nidogen, it detects a hitherto uncharacterized antigen. The possible role of the ER-TR7 positive reticular fibroblasts in the cellular organization of peripheral lymphoid organs will be discussed.

INTRODUCTION

Peripheral lymphoid organs, such as the spleen and lymph nodes are highly compartmentalized. Within these organs T and B lymphocytes each have their own domains. In the spleen B cells localize in follicles in the peripheral part of the white pulp (de Sousa, 1971; Gutman and Weissman, 1973; Nieuwenhuis and Ford, 1976) and in the marginal zone (MZ) (Kumararatne et al., 1981), which separates the white pulp from the red pulp. T cells on the other hand, occupy the central area of the periarteriolar lymphoid sheath (PALS) (Mitchell, 1972; van Ewijk et al., 1974). In lymph nodes, B cells localize in the follicles in the outer cortex, whereas T cells occupy the paracortical area (Parrott et al., 1966; van Ewijk and van der Kwast, 1980). Medullary cords predominantly contain plasma cells which migrate during differentiation from the outer cortex into this region. T and B cells both enter the splenic white pulp via the MZ (Ford, 1969; Nieuwenhuis and Ford, 1976; Brelinska and Pilgrim, 1982). In the lymph nodes they enter through high endothelial postcapillary venules (HEV) located in the paracortex (Butcher et al., 1980). Upon entry T and B

cells segregate and migrate into their respective domains.

The factors which direct the migration and specific homing of B and T cells into their respective domains are still unknown. From studies with the light and electronmicroscope it is known that both spleen and lymph nodes contain several nonlymphoid cell types, such as interdigitating cells (IDC), follicular dendritic cells (FDC), macrophages and reticular fibroblasts (Veerman and van Ewijk, 1975; Humphrey and Grennan, 1982). Morphological observations indicate that FDC and IDC possibly play a role in the homing of B and T cells, respectively (van Ewijk et al., 1974, Dijkstra and Döpp, 1983). Little is known about the function of reticular fibroblasts in the lymphoid microenvironments. These cells constitute a supportive cellular framework and they may also have a directive role in the migration and localization of lymphocytes (De Sousa, 1969; Barclay, 1981).

We recently produced a panel of monoclonal antibodies directed against nonlymphoid cells of the mouse thymus (van Vliet et al., 1984a). These antibodies provide a new approach for a detailed structural analysis of the nonlymphoid constituents of the thymus. In the present paper we present an extensive study of the reactivity of one of these antibodies, ER-TR7, which also reacts with the stroma of peripheral lymphoid organs.

The purpose of the present study is threefold: (1) a detailed anatomical analysis of peripheral versus central lymphoid organs with the use of the monoclonal antibody ER-TR7, (2) a description of the reactivity of ER-TR7 in a variety of other tissues, (3) analysis of the nature of the antigen detected by ER-TR7. The tissue distribution of the antigen expressed on stromal cells detected by monoclonal antibody ER-TR7 was studied with the use of the immunoperoxidase technique on frozen sections.

MATERIALS AND METHODS

Mice

Male and female C3H/HeJ and (CBA x C57BL/6)F1 mice, aged 6-12 weeks, were used for the present study. They were kept in our animal colony under routine laboratory conditions.

Monoclonal antibody

Details of the production of rat monoclonal antibodies directed against stromal cells of the mouse thymus have been published elsewhere (van Vliet et al., 1984a). We obtained seven hybrid cell lines which produce antibodies directed against various stromal cell types in the thymus. In the present study we describe the reactivity of one of these antibodies: ER-TR7, an IgG2a antibody, which also reacts with antigens of the reticular framework of lymphoid organs of the mouse.

Conjugate

Rabbit-anti-rat immunoglobulin coupled to horse radish peroxidase (RαRa-Ig-HRP) (Dako, Copenhagen, Denmark) was used. To prevent aspecific binding of the conjugate, it was deaggregated by centrifugation in a Beckman airfuge at 10^5 g. The conjugate was optimally diluted in PBS containing 0.5% bovine serum albumin (BSA) and 1% normal mouse serum (NMS).

Preparation and incubation of frozen sections

Frozen sections were prepared and incubated with monoclonal antibodies and photography was performed as described elsewhere (van Ewijk et al., 1981).

Mouse fibroblast cell lines

Mouse fibroblast cell line 129 was initiated in our laboratory as a primary culture of strain 129 skin fibroblasts. A9 is a mouse L-cell derivative (Littlefield, 1964).

Preparation and incubation of fibroblast cell lines

Cells were isolated with a rubber policeman, fixed, embedded in agar, and frozen sections were cut as reported before (van Vliet et al., 1984b). Sections were then incubated with ER-TR7 as described above.

Reticulin stain

Frozen tissue sections were stained for reticulin with routine silver impregnation according to Gomörri.

Further characterization of the antigen

The possible antigenic relationship of the matrix component detected by ER-TR7 with laminin, types I-V collagens, nidogen, entactin, fibronectin and heparan sulfate rich basement membrane proteoglycan was tested in several ways:

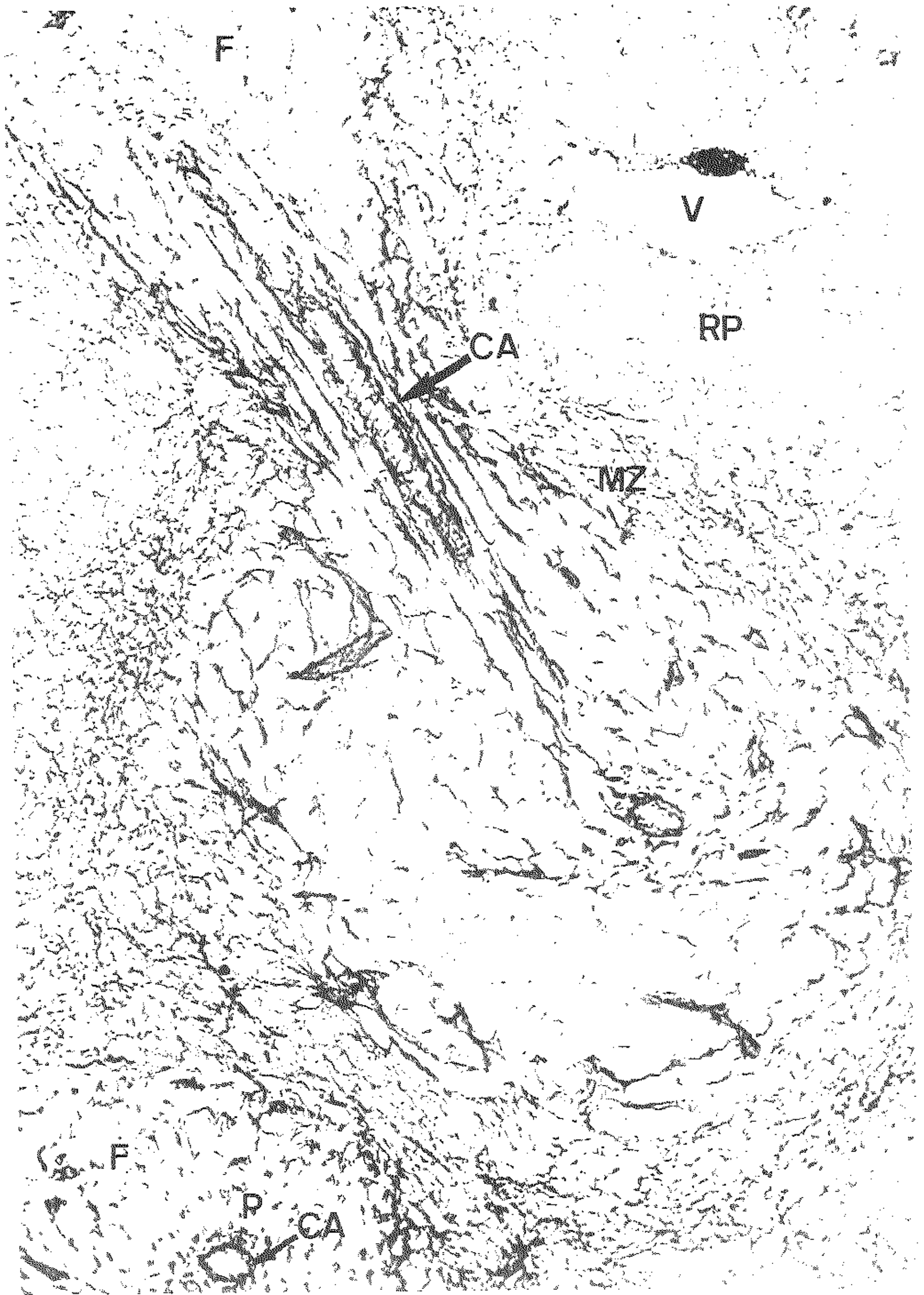
Ouchterlony immunodiffusion. Double radial immunodiffusion in 1 per cent agarose was performed in Immuno-Tek II OT Agarose plates (Behringwerke, Marburg, West Germany). ER-TR7 antibody was put into the central well and the connective tissue antigens were put in the peripheral wells. The proteins were allowed to diffuse overnight at room temperature in a moist chamber.

Immunoelectrophoresis. Immunoelectrophoresis in 1 per cent agarose was performed in electrophoresis base and agar gel plates (Hyland Laboratories, Casta Mesa, California). Laminin, fibronectin or entactin was placed in the well and electrophoresed for 45 min at 30 mA in a barbital buffer system, pH 8.6. Either antibodies to these proteins or ER-TR7 were then placed in the trough. The proteins were allowed to diffuse overnight at room temperature in a moist chamber.

Radioimmunoassay. Interstitial and basement membrane connective tissue antigens were iodinated with ^{125}I by the Chloramine-T method (McConahey and Dixon, 1966). Radioimmunoassay (RIA) was performed as described (Rohde et al., 1976), using purified antibodies to laminin, entactin, types I-IV collagens, fibronectin, heparan sulfate proteoglycan or ER-TR7 antibody.

Enzyme-linked immunosorbent assay (ELISA). ELISA was performed as described previously (Voller et al., 1976). Serial dilutions of purified rabbit antibody to the connective tissue macromolecules were applied to microtiter wells (Cooke Laboratory Products Division, Alexandria, Virginia) coated with fibronectin, laminin or the other biochemically characterized tested antigens.

Immunofluorescence. In order to determine whether the ER-TR7 antibody bound to the same antigenic moieties as did the antibodies to the matrix macromolecules, blocking studies were performed using mouse skin sections as substrate according to a previously described protocol (Yaoita et al., 1978). For example, purified antibody to laminin was conjugated with fluorescein-isothiocyanate (Goldman, 1968). Unlabelled ER-TR7 antibody was reacted for 3 min. Unbound reagents were washed away and fluoresceinated antibody to laminin was applied for another 30 min. The sections were then extensively washed and mounted. Conversely, in other studies, unlabelled rabbit an matrix proteins was first applied to tissue sections for 30 min., unbound antibody was washed away and ER-TR7 antibody was applied for another 30 min. After extensive washings, bound rabbit and rat antibodies were detected using fluorescein-conjugated antibody to rabbit or rat immunoglobulin. If the first antibody 'blocked' binding of the second antibody, this would indicate either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that they were binding to the same antigenic moieties or that there was steric hindrance by the first antibody. The specificity of the ER-TR7 antibody was also assessed by measuring its binding to agarose beads coated with connective tissue proteins as described (Yaoita et al., 1978). Finally, in other studies ER-TR7 antibody was preincubated overnight at 4°C with 100 µg of each tested antigen in a test tube prior to immunofluorescence in order to block antibody binding to the tissue section.

Immunoblotting. The reactivity of ER-TR7 with laminin, fibronectin, entactin, nidogen and types I-V collagens was tested by immunoperoxidase staining of their polypeptides, after electrophoretic transfer from polyacrylamide gels to nitrocellulose sheets as described (Towbin et al., 1979).

RESULTS

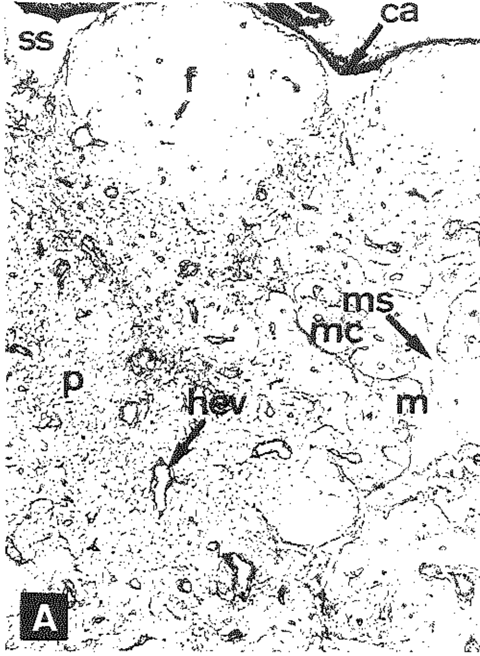
In the first part of this section we describe the localization of cells, expressing the antigen detected by ER-TR7, in peripheral lymphoid organs. In the second part of this section we describe the distribution patterns observed with ER-TR7 in central lymphoid organs such as thymus and bone marrow. To obtain further information on the nature of the antigen detected by ER-TR7, we studied the reactivity of ER-TR7 with various nonlymphoid tissues, with fibroblast cell lines and we compared the reactivity pattern of ER-TR7 with a conventional reticulin stain. We also analyzed the possible antigenic relationship of the antigen detected by ER-TR7 with various purified connective tissue components.

1. *Anatomical distribution of ER-TR7 positive cells in spleen and lymph node*

Frozen sections incubated with ER-TR7 followed by RαRa-Ig-HRP and DAB clearly demonstrate the two major compartments in the spleen, the white pulp, and the red pulp. Fig. 1 shows that the white pulp is located around central arterioles which branch from the splenic artery. This area can be easily distinguished from the red pulp, which is characterised by a randomly distributed meshwork. Within the white pulp, three distinct areas can be delineated by their characteristic labeling patterns. In a longitudinal section of the spleen the PALS contains a network of fibres, concentrically arranged in sheaths, parallel to the central arteriole. The central arteriole is outlined by a brightly stained wall. Follicles at the periphery of the PALS are virtually unstained by the antibody, except for the outer boundary of the follicle with the MZ. The staining pattern in the MZ is a reticular meshwork, which is far more dense than in the red and white pulp. The marginal zone gradually merges into the reticulum of the cords in the red pulp. Red pulp sinuses clearly stand out as negative areas. Trabeculae and the splenic capsule are strongly positive.

In frozen sections of the lymph node the cortex and the medulla can be delineated with ER-TR7 (Fig. 2a). As in splenic sections, follicles located in the outer cortex are unstained, except for the outer boundary. The interfollicular areas of the outer cortex and the paracortex show a characteristic

Figure 1. Immunoperoxidase staining pattern of frozen section of spleen. Incubation with monoclonal antibody ER-TR7, followed by RaRa-Ig-HRP and DAB. Note both longitudinal and transverse sections of the PALS. ca = central arteriole; f = follicle; p = periarteriolar lymphoid sheath; mz = marginal zone; rp = red pulp; v = venule. Magnification: 280x.



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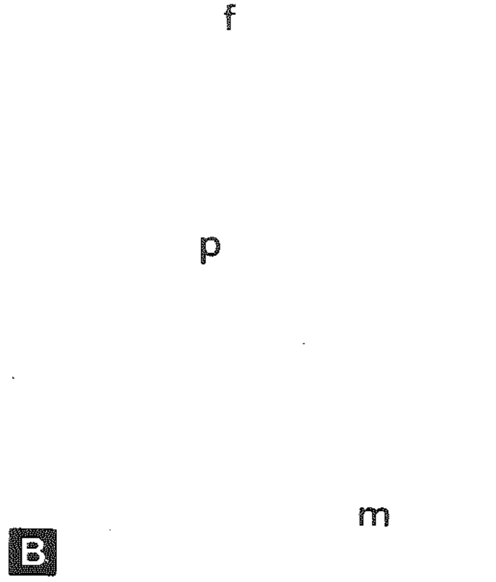

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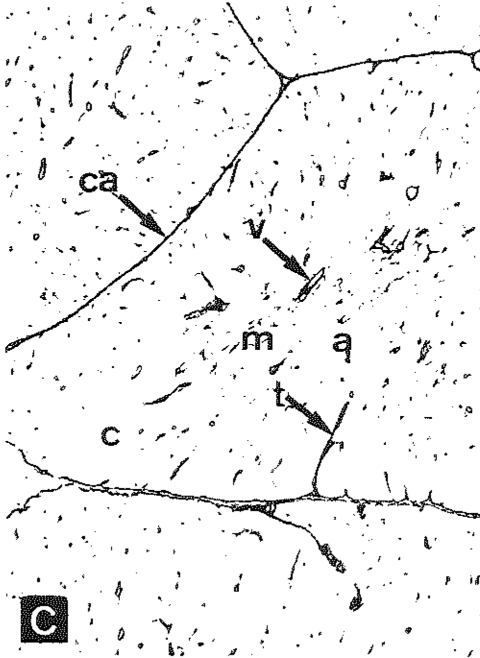

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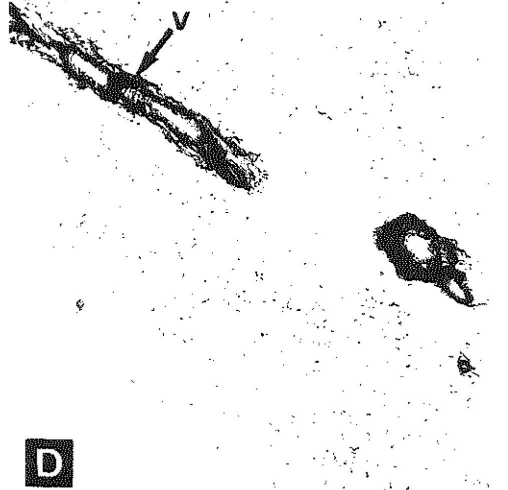

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fine reticular staining pattern. The walls of HEV located in the paracortical area also react strongly with this antibody. The capsule stains intensly with ER-TR7, whereas the subcapsular sinus is negative. In the medulla, strongly stained cords can be distinguished from negative sinuses. This staining pattern is specific since no such pattern was observed in negative control sections, sections incubated with RαRa-Ig-HRP and DAB only (Fig. 2b).

Taken together, labeling of frozen sections of peripheral lymphoid organs with ER-TR7 demonstrates the various domains, where T and B lymphocytes localize.

2. *Anatomical distribution of ER-TR7 positive cells in thymus and bone marrow*

To study a possible compartmentalization of thymus and bone marrow, we incubated frozen sections of these organs with ER-TR7.

Frozen thymus sections, incubated with ER-TR7, show staining mainly of the capsule, blood vessels, and trabeculae (Fig. 2c). The border between cortex and medulla is not clearly outlined although these regions can be distinguished.

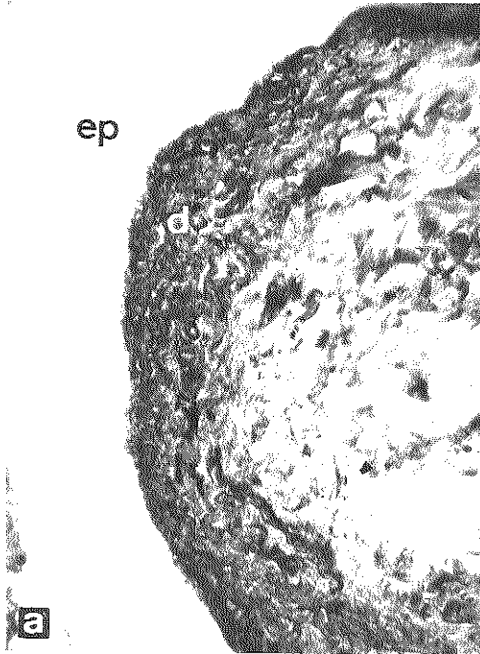
In frozen sections of bone marrow plugs labeled with ER-TR7 (Fig. 2d), a slight reticular pattern can be noted, together with strong staining of the walls of blood vessels. However, in contrast with the spleen and lymph node this labeling pattern does not demonstrate any compartmentalization within the bone marrow.

3. *Anatomical distribution of ER-TR7 positive cells in nonlymphoid organs*

We incubated frozen sections of a variety of nonlymphoid organs and tissues with ER-TR7 in order to study reactivity patterns in these organs. The results are summarized in Table 1. A few examples are shown below to illustrate the specificity of ER-TR7.

In frozen sections of skin incubated with ER-TR7 (Fig. 3a) the dermis was confluently labeled, whereas the epidermis was negative. Sections of the small intestine incubated with ER-TR7 show a similar confluent staining of the lamina propria (Fig. 3b). No staining of the intestinal epithelium was observed. Similarly, in sections of the stomach a positive lamina propria and a negative epithelium were observed (not shown). The ovarian stroma was positive, whereas the follicles were negative (not shown). ER-TR7 stains the connective tissue which forms a supporting network between parenchymal cells in various organs, such as salivary gland, kidney, testis, liver and pancreas. Cardiac and striated muscle also contain such an ER-TR7 positive connective tissue network. Examples of this staining pattern are demonstrated in sections of salivary gland and kidney (Fig. 3c, d). In the salivary gland the epithelial cells of the acini and collecting ducts are negative, whereas the reticular connective tissue around the acini and ducts can be seen as thin lines (Fig. 3c). A similar staining of connective tissue elements, but no staining of muscle fibers, was observed in striated muscle sections. In the testis the interstitial tissue between seminiferous tubuli is positive. Both glomeruli and interstitial connective tissue between tubuli of the kidney are strongly labeled with ER-TR7 (Fig. 3d). In the liver the lining of sinusoids are posi-

Figure 2. Immunoperoxidase staining pattern of (a)(b) mesenteric lymph node, (c) thymus and (d) bone marrow. Incubation with ER-TR7 (a), (c), (d) or negative control (b). ca = capsule; c = cortex; f = follicle; hev = high endothelial venule; m = medulla; mc = medullary cord; ms = medullary sinus; p = paracortex; ss = subcapsular sinus; t = trabeculae; v = blood vessel.
Magnification: (a),(c) 60x; (b),(d) 140x.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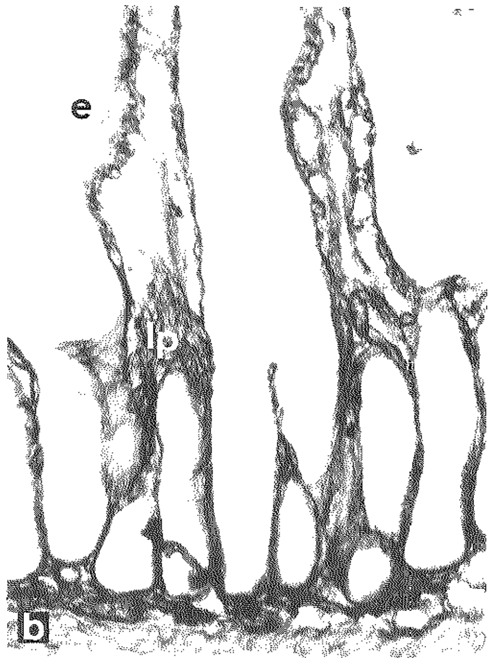
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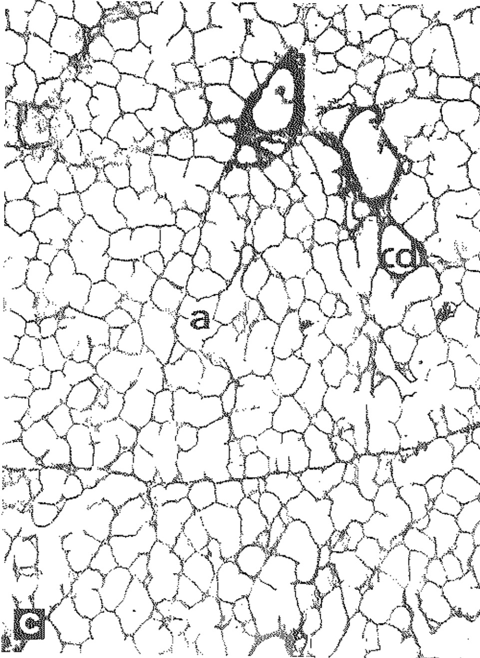
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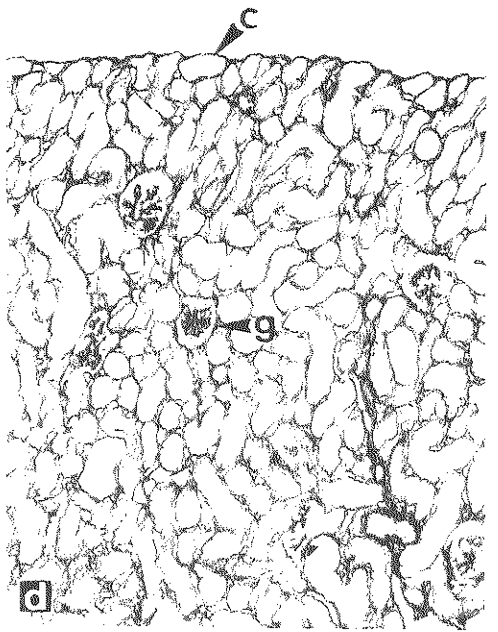
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Table 1 Reactivity of ER-TR7 with various non-lymphoid organs of the mouse

Organ	Reactivity with
Submandibular salivary gland	Interstitial CT[1) 2)] between acini
Stomach	Lamina propria, CT of muscularis, serosa
Small intestine	Lamina propria, CT of muscularis, serosa
Pancreas	Interstitial CT between acini
Liver	Lining of liver cords
Skin	Dermis
Ear	Extracellular matrix of cartilage, dermis
Striated muscle	Interstitial CT between muscle fibers
Cardiac muscle	Interstitial CT between muscle fibers
Tendon	Fibers
Ovary	Connective tissue stroma, tunica albuginea
Testis	Interstitial CT between seminiferous tubuli
Kidney	Glomeruli and interstitial CT between tubuli
Brain	Blood vessels, meninges

1) CT = connective tissue.
2) In tissues tested ER-TR7 reacts with blood vessel walls and capsule.

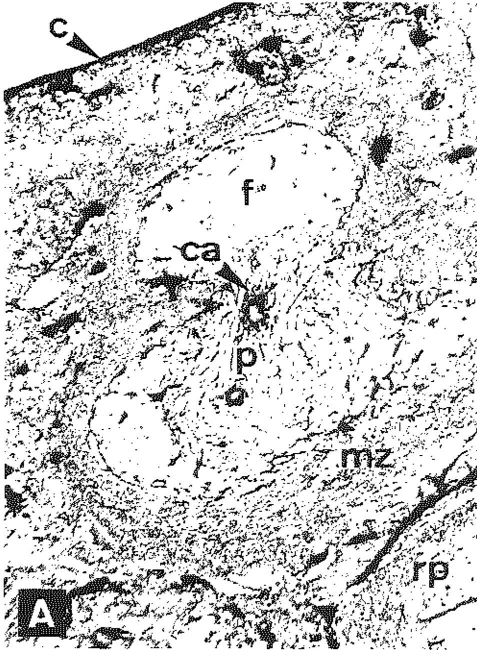
tive, whereas parenchymal cells are not labeled. ER-TR7 reacts with the extracellular matrix of cartilage and with the dermis in sections of ear (not shown). Blood vessels always showed a strongly positive wall.

Taken together, in all the organs mentioned above, connective tissue compartments can be identified with the present monoclonal antibody.

4. Comparison of the reactivity pattern of ER-TR7 with a convential reticulin stain

Further study of the nature of the antigen detected by ER-TR7 involved conventional silver impregnation of spleen sections. This method is known to detect reticulin, which is defined as the connective tissue which stains with silver (Hay et al., 1978). Fig. 4a and 4b show serial frozen spleen sections which were stained with ER-TR7 or by conventional silver impregnation, respectively. Silver impregnation resulted in a reticular labeling pattern within the splenic white pulp which was in general similar to the staining pattern observed with ER-TR7, although less dense. However, no labeling of the ER-TR7

Figure 3. Immunoperoxidase staining pattern of (a) skin, (b) small intestine, (c) salivary gland, (d) kidney. a = acinus; c = capsule; cs = collecting duct; d = dermis; e = epithelium; ep = epidermis; g = glomerulus; lp = lamina propria; t = tubuli.
Magnification: (a), (b), 350x; (c), (d), 140x.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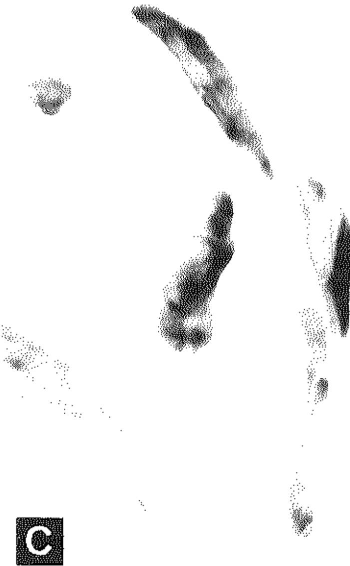
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+ve meshwork of the splenic red pulp and MZ was seen after silver impregnation. From this observation we tentatively conclude that ER-TR7 reacts with reticulin but also with other connective tissue components yet to be determined.

5. *Reactivity of ER-TR7 with fibroblast cell lines*

To study the reactivity of ER-TR7 with fibroblasts we incubated frozen sections of cell pellets of mouse fibroblast cell lines 129 and A9 with ER-TR7. ER-TR7 was shown to react with the cytoplasm of fibroblasts of each of these cell lines (Fig. 4c). 80-90% of the cells are ER-TR7 positive. A negative control section is shown in Fig. 4d.

6. *Characterization of the antigen detected by ER-TR7*

The reactivity of ER-TR7 with purified laminin, fibronectin, types I-V collagens, heparan sulfate proteoglycan, entactin and nidogen was investigated by Ouchterlony technique, immunoelectrophoresis, RIA, ELISA, immunoelectroblotting and indirect immunofluorescence blocking and inhibition studies. ER-TR7 reacted with none of the tested matrix components in any of the test systems. These results exclude that ER-TR7 detects a strict basement membrane component or any major collagen type or fibronectin. In addition, ER-TR7 reacts in immunofluorescence studies with interstitial stroma and matrix cartilage but not with the basement membrane matrix deposited by EHS sarcoma or L2 tumors, two transplantable murine and rat tumors that synthesize a matrix of basement membrane (Timpl et al., 1979; Wever et al., 1981). In culture, ER-TR7 reacts with murine fibroblasts but not with L2 cells, vascular endothelial cells, nor with glomerular epithelial cells, suggesting that the antigen detected by ER-TR7 is synthesized by mesenchymal cells rather than by epithelial or endothelial cells.

DISCUSSION

In the present study we have analyzed the anatomical distribution of the cellular framework of lymphoid and nonlymphoid organs, detected by monoclonal antibody ER-TR7 and the nature of the antigen detected by ER-TR7. Our results clearly demonstrate that ER-TR7 can be used to study the micro-anatomy of various organs. In summary, we have demonstrated that (1) ER-TR7 outlines the various compartments of peripheral lymphoid organs by characteristic labeling patterns; (2) no such compartments are found in central lymphoid organs; (3) ER-TR7 delineates various types of connective tissue compartments in nonlymphoid organs; (4) The antigen detected is not a basement membrane component, nor any major collagen type or fibronectin.

Our results show furthermore that ER-TR7 reacts with the basic cellular framework in peripheral lymphoid organs. This cellular framework consists of the reticular fibroblasts, described by Müller-Hermelink et al. (1974), Veerman and van Ewijk (1975), Villena et al. (1983) and their products. The intracellular reactivity of ER-TR7 with fibroblast cell lines and the confluent

Figure 4. (a) Immunoperoxidase staining pattern of spleen with ER-TR7. (b) Silver impregnation pattern of spleen. Immunoperoxidase staining pattern of frozen sections of a pellet of A9 cells, incubated with ER-TR7 (c) and a negative control section (d). c = capsule; ca = central arteriole; f = follicle; p = periarteriolar lymphoid sheath; mz = marginal zone; rp = red pulp; t = trabeculae. Magnification: (a), (b) 60x; (c), (d) 875x.

staining in the dermis of the skin and the lamina propria of the intestine shows that ER-TR7 does not only detect intracellular components of fibroblasts, but also reacts with extracellular products. The spleen sections incubated with ER-TR7 and stained by conventional silver impregnation clearly demonstrate that the antigen detected by ER-TR7 has a tissue distribution which is wider than that of reticulin. The major difference between these two staining procedures is that reticular components in the marginal zone and in the red pulp are not detected by silver impregnation, but are detected by ER-TR7 antibodies. Although the tissue distribution suggests that this antibody detects a major component of the extracellular matrix such as collagen, the fact that ER-TR7 does not react with a variety of collagens, glycoproteins or basement membrane proteoglycan with comparable tissue distributions excludes that ER-TR7 detects a strict basement membrane component or any major collagen type or the biochemically characterized glycoprotein - fibronectin. The tissue distribution of the antigen recognized by ER-TR7 is clearly distinct from that reported for all other biochemically characterized connective tissue macromolecules since it is an ubiquitous component of stromal (interstitial) matrix cartilage and of at least some basement membrane zones. ER-TR7 is particularly useful as a tool for dissecting various lymphoid compartments in central and peripheral lymphoid organs. Thus, based on the distribution pattern of ER-TR7 positive fibroblasts, the various compartments in the spleen, i.e. red pulp, MZ, PALS and follicles and lymph nodes, i.e. paracortex, follicles and medulla, can be clearly distinguished. This antibody also shows that the thymus, a lympho-epithelial organ only shows mesenchymal components in the medulla and in the capsule. As can be judged from the staining pattern these fibroblasts are not only present as components in a general framework structure, they also create various microenvironments in the different lymphoid compartments. In the spleen this is most obvious in the marginal zone and in the central part of the white pulp (T zone). We speculate that this arrangement of fibroblasts is involved in two major functions of the spleen, i.e. phagocytosis of blood borne substances (e.g. erythrocytes, antigens) and initiation of the immune response. The dense meshwork in the marginal zone might then function as a filter slowing entry of the blood flow in this particular area (see also Veerman and van Ewijk, 1975). By this mechanism, mononuclear phagocytes associated with this reticular meshwork can optimally clear the in-flowing blood (Van Vliet et al., 1985). The typical arrangement of fibroblasts in lymphoid organs might also guide migration of lymphocytes after their entry into the splenic white pulp or into the lymph node paracortex (de Sousa, 1969; Barclay, 1981) and promote the intercellular contact between lymphocytes and those cell types which regulate the ultimate homing of lymphocytes into their respective domains.

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CHAPTER 9

MARGINAL ZONE MACROPHAGES IN THE MOUSE SPLEEN IDENTIFIED BY A MONOCLONAL ANTIBODY. ANATOMICAL CORRELATION WITH A B CELL SUBPOPULATION

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SUMMARY

The reactivity of a monoclonal antibody, ER-TR9, that demonstrates heterogeneity among mononuclear phagocytes is described. In the spleen ER-TR9 exclusively reacts with a population of macrophages located in the marginal zone. ER-TR9 does not react with macrophage antigen 1 positive red pulp macrophages or any other types of splenic stromal cells.

ER-TR9+ve cells localize in anatomical proximity of a subpopulation of B cells, i.e., B cells that are immunoglobulin M positive and weakly positive to negative for immunoglobulin D. The possible significance of this particular interaction between both cell types during the immune response is discussed.

INTRODUCTION

It is generally accepted that macrophages play an important role in phagocytosis, processing and presentation of antigenic material to B and T lymphocytes (Unanue, 1978; Erb et al., 1980; van Furth et al., 1980). To provide a means for studying macrophage differentiation and subsets, monoclonal antibodies to cell surface antigens of macrophages have been prepared by various investigators (Springer et al., 1979; Unkeless, 1979; Austyn and Gordon, 1981; Ho and Springer, 1982a, b, 1983). These antibodies react with cell surface antigens, which are considered to be general macrophage markers.

Recently, evidence has been presented that indicates functional heterogeneity among splenic macrophages. Humphrey (1980) and Humphrey and Grennan (1981) demonstrated, that neutral polysaccharides, when injected into mice, were selectively taken up by macrophages in the marginal zone (MZ) of the spleen, whereas acidic polysaccharides were detectable in red pulp macrophages. Thus, the MZ may be a specialized compartment in the spleen.

The MZ of the rat spleen was also shown to be the site of a distinct subpopulation of B lymphocytes (Kumararatne et al., 1981; MacLennan et al., 1982). MZ B cells do not recirculate and they express cell surface immunoglobulin M (μ), but do not express cell surface immunoglobulin D (δ), i.e., they are μ positive (μ+ve), δ negative (δ-ve). In contrast, follicular B cells are both μ+ve and δ+ve.

In the present article we report the reactivity of a monoclonal antibody, ER-TR9, that exclusively reacts with MZ macrophages in the mouse spleen. We demonstrate that the localization of ER-TR9+ve cells correlates with the localization of μ+ and δ-ve to weakly δ+ve B cells. We discuss the findings in relation to the humoral immune response.

MATERIALS AND METHODS

Mice
Male and female C3H/He and C3H/law mice, age 4-8 weeks, were used for the present study. They were kept in our animal colony under routine laboratory conditions.

Antisera and conjugates
Details of the production of monoclonal antisera directed to stromal cells of lymphoid organs have been published elsewhere (van Vliet et al., 1984). In the present study we describe the reactivity of one of these antibodies, ER-TR9.

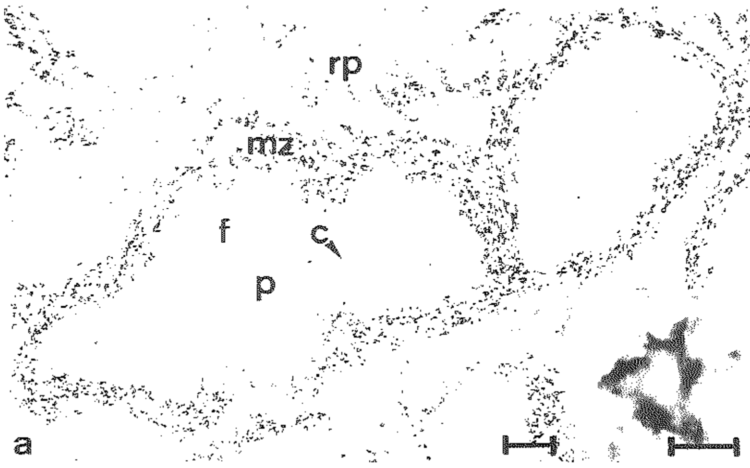
To detect binding of monoclonal antibody ER-TR9, 151-119-14 (anti immunoglobulin M (IgM), Oi et al., 1978) and M1/70 (directed to the macrophage antigen 1 (Mac-1) on macrophages and granulocytes, Springer et al., 1979) we used rabbit-anti-rat immunoglobulin coupled to horseradish peroxidase (RαRa-Ig-HRP) (Dako, Copenhagen, Denmark). Rabbit-anti-mouse IgD conjugated to biotin, and horse radish peroxidase conjugated to avidin were a kind gift of Dr. G. Kraal.

Preparation and incubation of frozen sections
Frozen sections were prepared and incubated with monoclonal antibodies and photography was performed as described elsewhere (van Ewijk et al., 1981). To block endogenous peroxidase, we preincubated frozen sections in 0.1 mg/ml phenylhydraziniumchloride in phosphate buffered saline (PBS) for 60 min at 37°C. We then incubated frozen tissue sections with ER-TR9, anti-IgM or anti-Mac-1, followed by RaRa-Ig-HRP and diaminobenzidine tetrahydrochloride (DAB). Alternatively, we incubated frozen sections with biotin-conjugated anti-IgD, avidin-conjugated HRP and DAB.

RESULTS

Anatomical distribution of ER-TR9+ve cells in the spleen
In frozen sections of the spleen the monoclonal antibody ER-TR9 strongly reacts with a subpopulation of large irregularly shaped non-lymphoid cells that localize in a rim around the white pulp of the spleen (Fig. 1a). ER-TR9 does not react with any other nonlymphoid cells, such as interdigitating cells (IDC), follicular dendritic cells (FDC), or macrophages that localize in the red pulp.

Figure 1. Immunoperoxidase staining pattern of frozen spleen sections, incubated with (a) ER-TR9, (b) anti-IgM, (c) anti-IgD. (a) and (b) represent serial sections. c = central arteriole, f = follicle, mz = marginal zone, p = central peri-arteriolar lymphoid sheath, rp = red pulp. Magnification: (a), (b) 60x (bar = 0.1 mm); (c) 140x (bar = 0.05 mm); inset 875x (bar = 0.01 mm) (higher magnification of (a)).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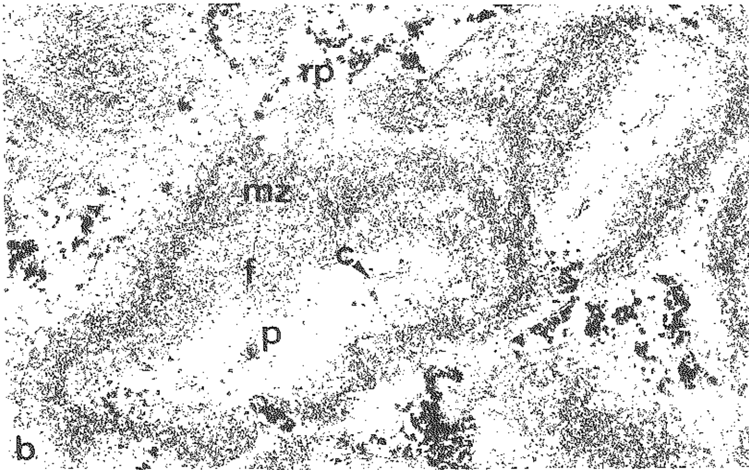
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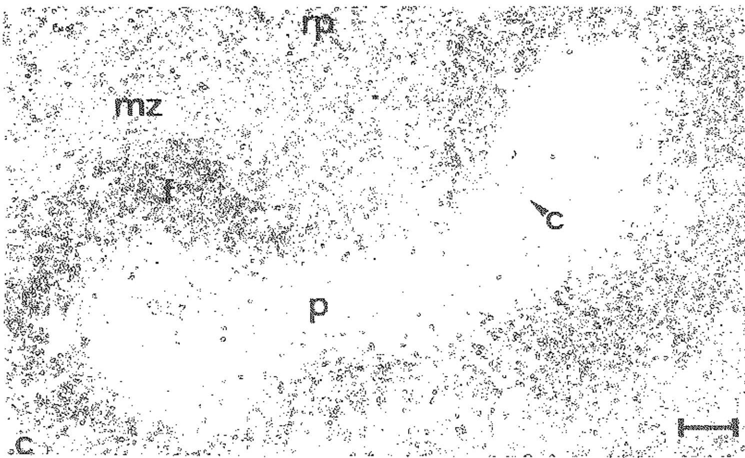
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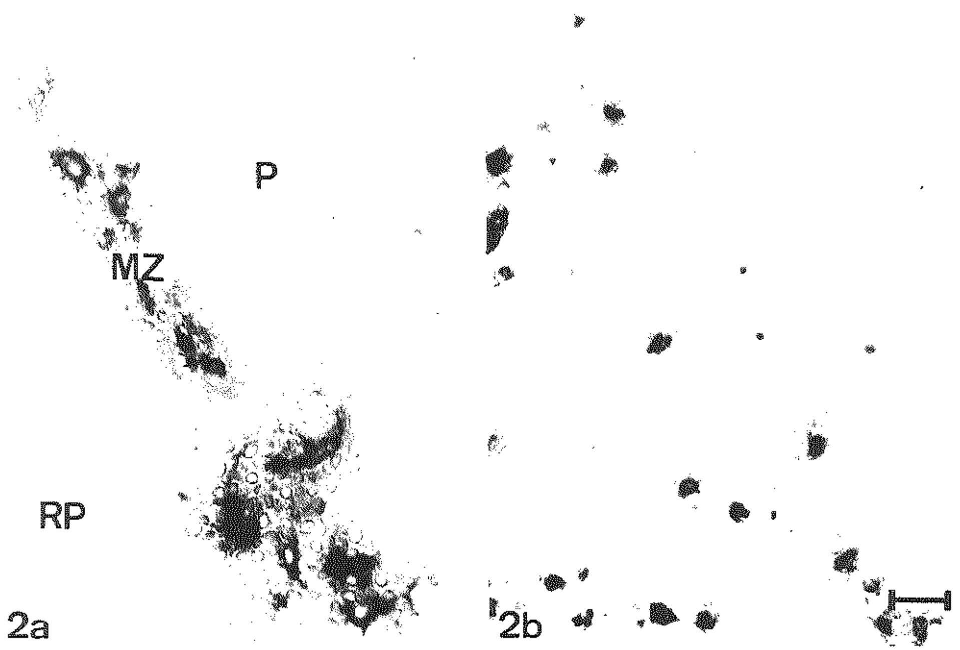
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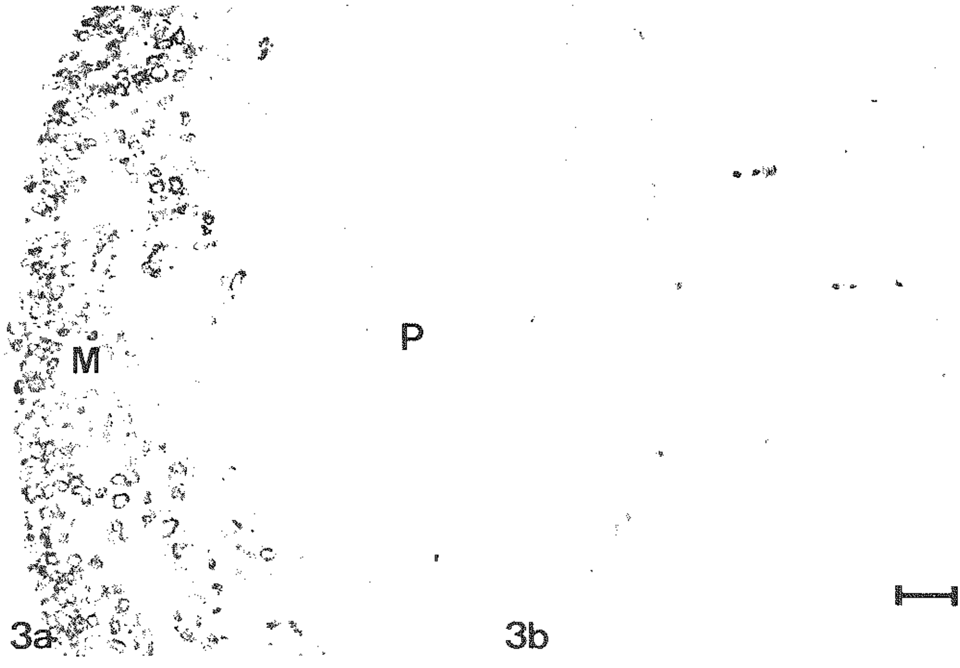
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Anatomical distribution of μ+ve and δ+ve B cells in the spleen
In serial frozen sections, incubated with anti-IgM, μ+ve B cells are present throughout both follicles and MZ (Fig. 1b). Follicular B cells are moderately μ+ve, whereas MZ B cells are strongly μ+ve. The serial sections clearly demonstrate that the localization of the latter cells corresponds to the localization of TR9+ve cells. In contrast, the majority of strongly δ+ve B cells localize in follicles (Fig. 1c). The MZ is the site of a population of B cells with expression of IgD varying from negative to weakly positive. Thus, the MZ contains a population of B cells, which is strongly μ+ve and δ-ve to weakly δ+ve.

Anatomical distribution of Mac-1+ve cells in the spleen
Serial sections incubated with anti-Mac-1 antibodies show that strongly Mac-1+ve cells predominantly localize in the red pulp (Fig. 2). Only occasional Mac-1+ve cells occur in the MZ. Springer et al. (1979) demonstrated that Mac-1+ve cells include macrophages, monocytes and granulocytes. We observed that the majority of Mac-1+ve cells in the spleen were monocytes and granulocytes, scattered throughout the red pulp. As can be seen from the serial sections Mac-1+ve cells were smaller and less irregularly shaped than ER-TR9+ve cells. A minority of Mac-1+ve cells are macrophages, which predominantly localize around trabeculae in the red pulp (not shown). In addition, anti-Mac-1 antibodies faintly but reproducibly react with cells in the MZ. This reactivity pattern is specific since negative control sections, incubated with RaRa-Ig-HRP and DAB only, did not reveal such a pattern. Neither the peri-arteriolar lymphoid sheath (PALS), nor the follicles contain any Mac-1+ve cells. Thus, ER-TR9+ve cells are Mac-1-ve or weakly Mac-1+ve.

Anatomical distribution of ER-TR9+ve cells in the mesenteric lymph node
In frozen sections of the mesenteric lymph node ER-TR9 strongly reacts with nonlymphoid cells in medullary sinuses and a rim of cells lining the subcapsular sinus of the mesenteric lymph node (Fig. 3a). No ER-TR9+ve cells were detected in follicles or paracortex.

Anatomical distribution of Mac-1+ve cells in the mesenteric lymph node
Only a few strongly Mac-1+ve cells were noted in the medulla of the mesenteric lymph node (Fig. 3b). PALS and follicles did not contain any Mac-1+ve cells. However, similarly to the MZ of the spleen, the medulla of the lymph node contains faintly positive cells. These cells show morphological similarity to the ER-TR9+ve cells (compare Figs. 3a and b).

Figure 2. Immunoperoxidase staining pattern of serial frozen sections of spleen, incubated with (a) ER-TR9, (b) anti-Mac-1. c = central arteriole, mz = marginal zone, rp = red pulp, p = peri-arteriolar lymphoid sheath. Magnification: 320x (bar = 0.02 mm).

Figure 3. Immunoperoxidase staining pattern of frozen sections of mesenteric lymph node, incubated with (a) ER-TR9, (b) anti-Mac-1. m = medulla, p = paracortex. Magnification: 140x (bar = 0.05 mm).

Anatomical distribution of μ+ve and δ+ve B cells in the mesenteric lymph node (not shown).

In frozen sections of the mesenteric lymph node μ+ve B cells are predominantly found in the follicles in the outer cortex. However, a minority of scattered μ+ve B cells localize in the medulla. No distinct subpopulations expressing different levels of μ are present. All μ+ve B cells are strongly μ+ve. The majority of δ+ve B cells also localize in the follicles, whereas a minority of δ+ve B cells localize in the medulla. All δ+ve B cells in the mesenteric lymph node are strongly δ+ve. In contrast to the δ+ve corona the germinal center of follicle was δ-ve. Thus, in contrast to the spleen, the mesenteric lymph node was not shown to contain phenotypically distinct B cell subpopulations.

DISCUSSION

In the present study we have analysed the anatomical distribution of the cells, detected by monoclonal antibody ER-TR9, in frozen sections of spleen and lymph node of the mouse. Furthermore, we have investigated a possible correlation among the anatomical distribution of these cells and the distributions of Mac-1+ve macrophages and subpopulations of B lymphocytes.

The present study demonstrates that ER-TR9 exclusively reacts with a subpopulation of macrophages, which reside in the MZ of the spleen and in the medullary sinuses of lymph nodes. ER-TR9+ve cells were also found lining the subcapsular sinus of lymph nodes.

The nonlymphoid cells detected by ER-TR9 in the spleen are macrophages, since they were shown to have phagocytic capacity: they ingest latex, India ink and fluorescein-conjugated Ficoll (Dijkstra et al., submitted for publication). Furthermore, ER-TR9+ve cells are moderately acid phosphatase and nonspecific esterase positive. These cells are not the so-called marginal metallophyls. The latter cells exclusively localize at the inner border of the marginal sinus (Satodate et al., 1971; Eikelenboom et al., 1978; Dijkstra et al., submitted for publication). whereas ER-TR9+ve cells localize throughout the MZ. Together these data indicate that the ER-TR9+ve macrophages in the spleen are the cells described by Humphrey and Grennan (1981). They were the first to identify in the spleen a subpopulation of macrophages, which are morphologically and functionally distinct from the majority of splenic macrophages. In contrast to the vast majority of splenic macrophages, which ingest acidic polysaccharides, these 'marginal zone macrophages' selectively ingest neutral polysaccharides. Humphrey and Grennan (1981) also identified such a functionally distinct subpopulation lining the subcapsular sinus in lymph nodes. However, in lymph nodes ER-TR9 expression is not limited to this subpopulation, since ER-TR9 also reacts with macrophages in the medulla. Thus, expression of ER-TR9 and ingestion of neutral polysaccharides might identify overlapping but not identical subpopulations in lymph nodes.

Do ER-TR9+ve macrophages in spleen and lymph node express the general macrophage marker Mac-1, which is associated with the type III complement receptor (Beller et al., 1982)? The present study demonstrates that only few Mac-1+ve cells localize in the MZ. These cells predominantly localize in the splenic red pulp. This observation is consistent with the results of Ho and Springer (1982b). In addition we demonstrate weak Mac-1 expression in the MZ. Thus, the present study demonstrates the existence of at least two macrophage subpopulations in the spleen: ER-TR9+ve, Mac-1-ve or weakly Mac-1+ve macrophages that localize in the MZ and ER-TR9-ve, Mac-1+ve macrophages that localize in the red pulp. We also demonstrate that lymph nodes contain similar

subpopulations of TR9+ve, Mac-1-ve or weakly Mac-1+ve macrophages in the medullary and subcapsular sinuses, and ER-TR9-ve, Mac-1+ve cells.

Does the localisation of ER-TR9+ve macrophages correlate with the localization of a distinct B cell subpopulation in spleen and lymph nodes? The present study demonstrates that ER-TR9+ve macrophages in the spleen localize in anatomical proximity of a major population of strongly μ+ve, δ-ve to weakly δ+ve B cells in the MZ. Our findings with regard to μ+ve and δ+ve B cells in the mouse spleen are in concordance with the findings of others in the rat spleen (MacLennan et al., 1982) and human spleen (Stein et al., 1980). The present results also demonstrate that the phenotypical difference between MZ and follicular B cells in the mouse spleen is not as clear-cut as in the rat spleen, since the MZ B cell population of the mouse spleen is not totally δ-ve. The MZ of the rat spleen is the site of a subpopulation of predominantly μ+ve, δ-ve B cells that do not recirculate (Kumararatne et al., 1983). The small number of μ+ve, δ+ve B cells in the MZ presumably represent recirculating δ+ve B cells as they are depleted when recirculating B cells are depleted. Upon stimulation with T cell independent (T-I) antigens MZ B cells give rise to longlasting IgM responses (Humphrey, 1980; Humphrey and Grennan, 1981).

In contrast to the findings in the spleen, the present study does not demonstrate any correlation between ER-TR9+ve macrophages and specific subpopulations of μ+ve or δ+ve B cells in the mesenteric lymph node. These observations suggest that the MZ is a unique compartment in peripheral lymphoid organs.

What is the function of the population of ER-TR9+ve MZ macrophages? This paper and the results of Humphrey and Grennan (1981) indicate that the MZ macrophages may have a special role in presenting T cell independent antigens to the B cell population in the MZ. Currently studies are underway to investigate whether in vivo administered ER-TR9 can interfere with the uptake of T cell independent antigens by MZ macrophages.

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10. GENERAL DISCUSSION

The complex process of T lymphocyte differentiation takes place mainly in the thymus. During this process BM-derived prothymocytes enter the thymus, proliferate extensively and give rise to thymocytes. These thymocytes undergo differentiation and selection within the thymic microenvironment. It has often been claimed that about 99% of the daily produced thymocytes die intrathymically. Eventually, 1% of the thymocytes emigrate from the thymus to peripheral lymphoid organs as mature immunocompetent T cells where they can undergo antigen-induced activation and become effector T cells. During the intrathymic differentiation process thymocytes are selected on the basis of tolerance to self-MHC gene products and they obtain the capacity for recognition of foreign antigens in the context of self-MHC antigens. Also, they acquire various differentiation markers on the cell surface.

The thymus is a complex lympho-epithelial organ consisting of 'fixed' stromal cells and 'free' lymphoid cells. The process of T cell differentiation is apparently only possible within this specific microenvironment and it is a generally accepted concept that the interaction between differentiating T cells and stromal cells plays a crucial inducing role in the differentiation process. The functional and morphological evidence in support of this notion has been discussed in Chapter 2.2.5. However, to date, our knowledge of this complex process is still very incomplete. Though thymocyte subpopulations have been well characterized on the basis of differentiation markers, the thymic stroma is still poorly characterized. This thesis provides a contribution to an inventory of subpopulations of thymic stromal cells. Its primary aim is the characterization of the stromal cells in the microenvironments of the two major intrathymic compartments, cortex and medulla, where the most immature and mature thymocytes reside, respectively.

It appears that cortex and medulla are clearly different compartments in terms of stromal cells (Chapter 4). The following subpopulations have been identified in the murine thymic stroma (Chapters 4, 5):

1. TR4+, Ia+, TR5- cortical epithelial cells, including TNC,
2. TR4-, Ia-, TR5+ medullary epithelial cells,
3. TR4-, Ia+, TR5+ medullary epithelial cells,
4. TR4-, Ia+, TR5-, TR6+ medullary IDC,
5. TR7+ reticular fibroblasts in cortex and medulla,
6. TR6+ macrophages.

Additional evidence demonstrates the localization of TR6+ macrophages (subpopulation 6). Combination of ER-TR6 staining with staining for acid phosphatase, which labels macrophages brightly positive, shows that the majority of acid phosphatase bright macrophages localize in the cortex and that they are all TR6+ (unpublished observations). Combination of staining for Ia and acid phosphatase shows these cortical macrophages to be generally Ia-. Therefore, macrophages in the cortex are TR6+, Ia-. Thus, it can be concluded that the thymic stroma can be subdivided into two distinct microenvironments, a cortical and a medullary one, each consisting of characteristic sets of stromal cells.

During embryonic development, at the time when the first prothymocytes enter the thymic anlage, this subdivision of the thymic stroma into cortical and medullary stromal microenvironments is already apparent (Chapter 6). We speculate that this early subdivision into cortical and medullary stroma may provide the necessary microenvironments for the development of two precursors

destined to give rise to cortical and medullary thymocytes, respectively.

The intimate contact between stromal cells and differentiating thymocytes can be demonstrated *in vitro*. TNC, lympho-stromal complexes, can be isolated from the thymus (Wekerle and Ketelsen, 1980). TNC appear to represent the close interaction between thymocytes and epithelial cells in the most immature thymic compartment , the cortex (Chapter 5). Similarly, thymocyte rosettes, complexes of thymocytes and BM-derived thymic stromal cells *in vitro*, have been demonstrated to represent lympho-stromal interaction in the thymic medulla (Kyewski et al., 1982).

We have furthermore shown that the alymphoid thymic rudiment of the nude mouse embryo has an abnormal stroma (Chapter 6). The nature of the defect has been shown to be a deficiency of TR4+, Ia+, TR5- epithelial cells and Ia+, TR6+ IDC and macrophages, and prominence of a subpopulation of TR4-, Ia-, TR5- stromal cells. From these studies it can be concluded that normal organization of cortical and medullary epithelial cells as well as expression of MHC antigens is a prerequisite for normal T cell development. A similar conclusion can be drawn from the data concerning the composition of the thymic stromal and lymphoid populations of mice injected with dexamethasone (Chapter 7). Dexamethasone-induced death of thymocyte subpopulations correlates with morphological changes in the stroma. Dexamethasone induces 'nurse cell like' conformation of cortical epithelial cells and loss of membrane bound expression of MHC antigens. Similar effects were also noted after sublethal irradiation of mice. Moreover, during regeneration of the thymus following sublethal irradiation, the normal architecture of the thymic stroma and its expression of MHC antigens are restored prior to re-expression of the normal pattern of T cell antigens on the cell surface of the regenerating lymphoid population (Huiskamp and van Ewijk, 1985; Huiskamp et al., 1985). Another indication for the relevance of the thymic stroma in T cell differentiation is the abnormal stromal and lymphoid composition of thymomas. Van der Kwast et al. (in press) have classified thymomas as cortical or medullary epithelial neoplasms with abnormal thymocyte populations. Thus, stromal abnormalities correlate with changes in the thymocyte population. Collectively, all these morphological studies demonstrate elements of lympho-stromal interaction. However, there is also functional evidence, at least for the differentiation of Th cells, that the thymic stroma is involved in the T cell differentiation process. Neonatal mice injected with anti-I-A antibodies lack thymic and peripheral L3T4+, Lyt2- Th cells (Kruisbeek et al., 1983; Kruisbeek et al., in preparation), suggesting that the generation of Th cells is blocked by interference of the anti-I-A antibodies with their binding to the I-A+ stromal cells. Immunohistological investigation of the thymi of these mice showed no gross changes in the stromal architecture but surprisingly revealed the intrathymic presence of the injected anti-I-A antibodies (van Vliet and Kruisbeek, unpublished observations). The injected antibodies were found to bind to cortical I-A bearing epithelial cells as well as to the medullary stroma. Presumably, they blocked the generation of Th cells by interference with their binding to the I-A+ stroma of cortex and/or medulla. Further studies may show whether the monoclonal anti-stroma antibodies presented in Chapter 4 detect stromal determinants relevant for lympho-stromal interaction and upon injection block certain steps in the T cell differentiation process. An *in vitro* approach to the identification of the respective contributions of stromal populations to T cell differentiation would be to dissect embryonic thymus in very early stages of development, i.e. before day 13 in ontogeny, into cortical and medullary stroma. Recolonization of the dissected cortical or medullary stroma *in vitro* with

fetal liver prothymocytes could then be used to assess the role of dissected stromal cells in T cell development.

Stromal cells are an essential component of the thymus. However, also in the peripheral lymphoid organs stromal cells constitute lymphoid microenvironments. In peripheral lymphoid organs such microenvironments play a role in the antigen-dependent phase of lymphoid differentiation, the generation of effector cells. This thesis deals with two monoclonal antibodies which define certain microenvironments in the spleen and lymph nodes. The monoclonal antibody ER-TR7 outlines the various splenic compartments and domains, i.e. red pulp, marginal zone, peri-arteriolar lymphoid sheath and follicles (Chapter 8). It also delineates T and B cell domains in lymph nodes. We show that ER-TR7 reacts with fibroblasts and their products. We presume that fibroblasts constitute a structural framework for the localization and antigen-dependent differentiation of both T and B cells in peripheral lymphoid organs. The monoclonal antibody ER-TR9 delineates the marginal zone of the spleen. It identifies a subpopulation of stromal cells, the so-called 'marginal zone macrophages' (Chapter 9). It also detects macrophages in the medulla and sinuses of lymph nodes. Dijkstra et al. (in press) have further characterized these marginal zone macrophages and have shown that they are unique in phenotype and antigen-presenting function. Due to their strategic localization these cells may have a dual role, i.e. antigen trapping and presentation of T cell independent antigens to a subpopulation of B lymphocytes that occupy the marginal zone.

In summary, this thesis provides morphological evidence to support the notion that lymphocytes interact with stromal cells during their antigen-independent differentiation in central lymphoid organs as well as during antigen-driven generation of effector cells in peripheral lymphoid organs. This thesis provides insight into the complex architecture of these lymphoid microenvironments.

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SUMMARY

The stromal cells in the thymus play a crucial role in the complex process of intrathymic T cell differentiation (documented in Chapter 2). However, several aspects of this process still remain elusive. These are for instance identification of stromal cells by means of specific cell surface antigens and the contribution of each of these stromal cell types to the generation of functionally different T cell subpopulations, e.g., Th cells and CTL. Therefore we have produced a panel of monoclonal antibodies directed to various types of stromal cells of the murine thymus (Chapter 4). Using the immunoperoxidase technique on frozen thymus sections, these antibodies have been subdivided into the following four categories: (1) ER-TR1, 2 and 3 detect class II MHC antigens on cortical and medullary stromal cells as well as on medullary lymphoid cells, (2) ER-TR4 antibodies detect cortical epithelial cells, (3) ER-TR5 and 6 antibodies both react with medullary stromal cells. ER-TR5 reacts exclusively with medullary epithelial cells, whereas ER-TR6 detects IDC and macrophages, (4) ER-TR7 reacts with reticular fibroblasts. Thus, these antibodies provide a means to distinguish four categories of thymic stromal cells. They have been used to investigate (1) the relationship of TNC *in vitro* to stromal cell types defined *in situ* (Chapter 5), (2) the embryonic development of the thymic stroma (Chapter 6), (3) the effects of dexamethasone on the thymic stroma (Chapter 7). Furthermore, ER-TR7 and 9 have been employed to study the stroma of peripheral lymphoid organs (Chapters 8 and 9, respectively).

In Chapter 5 both stromal and lymphoid constituents of TNC were analyzed, with monoclonal antibodies directed to stromal cells (Chapter 4) and T cell antigens, respectively. The purpose of the experiments described in Chapter 5 was to investigate the relationship between isolated TNC and thymic stromal cells defined *in situ*. The results indicate that the stromal component of TNC is an ER-TR4+, Ia+, H-2K+ cortical epithelial-reticular cell. They also show that the thymocytes enclosed by TNC bear the phenotype of cortical thymocytes, i.e., they are bright Thy1+, dull T200+, PNA+ and variable in Lyt1 expression. Therefore, TNC originate in the cortex. We assume that TNC round off during isolation, because: (1) the lack of demonstrable Lyt2 antigens on the enclosed thymocytes suggests that Lyt2 was removed by the enzymatic degradation procedure used to isolate TNC; (2) the ER-TR4 staining pattern in the cortex, reported in chapter 4, demonstrates that ER-TR4+ cortical epithelial cells have a reticular shape, not a spherical one. Nevertheless TNC are the *in vitro* representative of cortical epithelial-reticular cells *in vivo*. As such they represent a tight contact between the cortical stroma and differentiating cortical thymocytes.

In Chapter 6 the *in vivo* development of the stroma of the embryonic thymus is described. The purpose of the research reported in this chapter was to investigate the initial development of the stroma during thymic ontogeny when the first prothymocytes enter the thymic anlage. It was shown that embryonic thymus of gestational age day 13 already shows a cortex and a medulla, identified with TR4+ and TR5+ epithelial cells, respectively. This subdivision into cortical and medullary stroma precedes expression of T cell markers on the lymphoid cells. It was furthermore shown that Ia is present as foci on day 13, extending throughout the lobe by day 16. Thus, as far as the stromal compartment is concerned, embryonic thymus of gestational age day 16 resembles adult thymus. The architecture of the nude's rudimentary thymus was shown

to differ markedly from that of its normal counterpart. The nude's thymic rudiment is surrounded by a large TR7+ capsule. No TR3 (Ia) or TR6 antigens were present on the thymic tissue proper. Only rare TR4+,5- cortical or TR4-, 5+ medullary epithelial cells localize around cysts of these thymic rudiments. So both cortical and medullary epithelial cells are represented in the nude's thymic remnant, although at low incidence. The large majority of cells were negative with all antibodies tested. The relationship of these latter cells to cells of the normal embryonic thymus remains to be established. The stroma of the normal embryonic thymus of day 14, maintained in organ culture for up to 11 days, resembles that of the normal embryonic thymus of day 16 or older. These organ cultured lobes become depleted of Thy1+ cells when cultured in the presence of deoxyguanosine. Nevertheless the composition of the stroma of these deoxyguanosine-treated thymic lobes is identical to that of the embryonic thymus *in vivo*. This demonstrates that depletion of lymphoid cells alone does not cause the abnormal stromal development in the nude.

Chapter 7 describes the effect of injection of a range of doses of dexamethasone on thymocyte subpopulations and thymic stroma. This chapter reports on analysis of the influence of dexamethasone treatment on thymocyte subpopulations and correlates changes in the phenotype of the thymocytes with changes in the thymic stroma due to dexamethasone treatment. Dexamethasone injection was shown to result in depletion of the cortical population of bright Thy1+, dull T200+, T4+, Lyt2+, MEL14- thymocytes, with variable Lyt1 expression (85% of total thymocytes). Dexamethasone also affects the medullary population of thymocytes (15% of total thymocytes), though to a lesser extent. 130 mg/kg body weight of dexamethasone leaves a population of 4% dexamethasone resistant cells, which are dull Thy1+, bright T200+, T4+, Lyt2- and MEL14+. Furthermore, we showed that dexamethasone resistant thymocytes are not representative of the total pool of medullary thymocytes, as originally thought; they lack a subpopulation of dull Lyt2+ cells. Dexamethasone treatment appeared to select for a population of MEL14+ cells in the perivascular spaces of the medulla. Serial sections reveal that these cells have a 'helper' phenotype, i.e. T4+, Lyt2-. Dexamethasone also affects the thymic stroma. The TR4+ cortical epithelium, but not the TR5+ medullary epithelium, appears affected by dexamethasone, as judged by morphological criteria. The TR4+ cells display a 'TNC-like' shape. Dexamethasone treatment resulted in loss of membrane-associated expression of Ia and H-2K antigens in the cortex. Thus, both stromal and lymphoid components of the thymus are affected by dexamethasone.

Chapter 8 describes the reactivity of the monoclonal antibody ER-TR7 (Chapter 4), which detects reticular fibroblasts not only in the thymus but also in peripheral lymphoid organs. Frozen sections of spleen and lymph nodes, incubated with ER-TR7 by the immunoperoxidase method, reveal that the distribution of TR7+ cells characteristically outlines the various compartments of these organs. In the spleen red pulp and white pulp clearly stand out, the latter being subdivided into marginal zone, follicles (B cell domains) and peri-arteriolar lymphoid sheath (T cell domains). In the lymph node paracortex, T cell domains, follicles and medulla can be distinguished. ER-TR7 was shown to react with a cytoplasmic component of fibroblasts, and with an extra-cellular component of connective tissue. We were unable to demonstrate any reaction of ER-TR7 with collagen types I, II, III, IV, V, fibronectin, laminin, heparan sulphate proteoglycan, entactin or nidogen. Therefore, this antibody reacts with a hitherto unknown component. It may provide a useful tool in future studies of connective tissue.

Chapter 9 shows that monoclonal antibody ER-TR9, which was generated by its reaction with a very small number of thymic stromal cells, reacts with irregularly shaped macrophages in the marginal zone of the spleen. We demonstrated that the marginal zone of the spleen is a unique compartment in terms of both lymphoid and nonlymphoid cells: it contains the characteristic TR9+ marginal zone macrophages and a subpopulation of non-recirculating IgM+, IgD- to dull IgD+ B cells, which are functionally distinct from IgM+, IgG+ recirculating B cells.

SAMENVATTING

Het stroma van de thymus speelt een essentiële rol in de differentiatie van T lymfocyten. In dit proefschrift wordt de samenstelling van het heterogene stroma van de thymus van de muis geanalyseerd met behulp van een panel monoclonale antistoffen. Dit panel wordt in Hoofdstuk 4 beschreven. Het panel monoclonale antistoffen is gericht tegen verscheidene typen niet-lymfoïde (stromale) cellen in de thymus van de muis. Met behulp van de immunoperoxidase techniek op vriescoupes werden de antistoffen op grond van hun reaktiepatroon onderverdeeld in de volgende vier categorieën: (1) ER-TR1, 2 en 3 reageren met klasse II MHC antigenen, welke worden aangetroffen op corticale en medullaire stromale cellen, alsmede op medullaire thymocyten, (2) ER-TR4 reageert met reticulo-epitheliale cellen in de cortex, (3) ER-TR5 en 6 reageren met stromale cellen in de medulla. ER-TR5 reageert alleen met epitheelcellen in de medulla. ER-TR6 reageert met interdigiterende cellen en macrofagen. (4) ER-TR7 reageert met fibroblasten. Met deze antistoffen kunnen dus vier categorieën stromale cellen onderscheiden worden. De antistoffen zijn vervolgens gebruikt voor onderzoek naar: (1) de relatie tussen Thymic Nurse Cells (TNC) *in vitro* en de stromale cellen gedefinieerd *in vivo* (Hoofdstuk 5), (2) de embryonale ontwikkeling van het thymus stroma (Hoofdstuk 6), (3) het effect van dexamethason op het thymus stroma (Hoofdstuk 7). De monoclonale antistoffen ER-TR7 en 9 werden gebruikt voor onderzoek naar de architectuur van het stroma van perifere lymfoïde organen (respectievelijk Hoofdstuk 8 en 9).

In Hoofdstuk 5 wordt het fenotype van de stromale, alsmede van de lymfoïde component van TNC geanalyseerd met monoclonale antistoffen gericht tegen respectievelijk stromale cellen en subpopulaties T cellen. Het doel van de in dit hoofdstuk beschreven experimenten is het verrichten van een analyse van de relatie tussen TNC geisoleerd uit de thymus en verscheidene *in situ* gedefiniëerde typen stromale cellen. De resultaten tonen aan, dat het stromale deel van TNC een TR4+, Ia+, H-2K+ corticale reticulo-epitheliale cel is. Ze tonen tevens aan dat de door TNC omsloten thymocyten het fenotype van corticale thymocyten hebben: ze zijn sterk Thy1+, zwak T200+, PNA+ en vertonen een variabele expressie van Lyt1. Twee waarnemingen geven een aanwijzing over de vorm van TNC *in vivo:* 1. het ontbreken van Lyt2 op het oppervlak van de thymocyten is waarschijnlijk het gevolg van enzymatische degradatie tijdens de isolatie, en duidt op bereikbaarheid van de thymocyten voor enzym tijdens de isolatie van TNC; 2. het patroon van TR4 kleuring van de reticulo-epitheliale cellen in de cortex toont aan, dat deze cellen niet bolvormig zijn, maar een dendritische vorm hebben. Hieruit concluderen we, dat TNC zich afronden tijdens de isolatie. TNC zijn dus de *in vitro* vertegenwoordigers van corticale epitheelcellen *in vivo*. Het feit dat TNC als lymfo-stromale complexen *in vitro* geïsoleerd kunnen worden duidt op een nauw contact tussen corticaal stroma en differentiërende corticale thymocyten.

In Hoofdstuk 6 wordt de *in vivo* ontwikkeling van het stroma van de embryonale thymus beschreven. In dit hoofdstuk wordt onderzoek beschreven, dat verricht werd naar het verschijnen van verscheidene typen stromale cellen tijdens de ontogenie van de thymus, de periode waarin de eerste prothymocyten naar de thymus migreren en uitrijpen tot thymocyten. Op dag 13 van de embryonale ontwikkeling bestaat de thymus van de muis al uit een cortex en een medulla, die te onderscheiden zijn aan de hand van respectievelijk TR4+ corticale en TR5+ medullaire epitheelcellen. Het stroma van cortex en medulla ontwikkelt zich dus voordat de eerste T cel markers op thymocyten in de embryonale thymus verschijnen. De Ia expressie van het stroma komt op dag 13 slechts

als foci tot uiting. Op dag 16 komt Ia tot expressie in de gehele thymus-lob. Wat betreft het stroma is de thymus dus al op dag 16 van de embryonale ontwikkeling identiek aan de volwassen thymus. 'Nude' muizen hebben een gestoorde ontwikkeling van de thymus, met als gevolg dat ze geen T cellen hebben. De bouw van het thymusrudiment van het embryo van de nude muis verschilt zeer sterk van dat van de embryonale thymus van de normale muis. Het thymusrudiment wordt omgeven door een dik TR7+ kapsel. Noch TR3 (Ia+) cellen, noch TR6+ cellen zijn aantoonbaar. TR4+,5- corticale of TR4-,5+ medullaire epitheelcellen komen slechts sporadisch voor, met name rondom cysten. De meeste cellen zijn echter negatief met alle geteste antisera. De relatie van deze cellen tot het stroma van de normale embryonale thymus is nog onduidelijk. In orgaankweken van de normale embryonale thymus van dag 14 blijven alle typen stromale cellen behouden gedurende een periode van 11 dagen. De bouw van deze gekweekte thymuslobben komt overeen met de *in vivo* bouw en er zijn Thy1+ thymocyten in aantoonbaar. In de embryonale thymus, welke gedurende 5 dagen gekweekt werd in aanwezigheid van deoxyguanosine, zijn geen lymfoïde cellen meer aantoonbaar. De architectuur van het stroma en de verdeling in cortex en medulla is echter nog volledig vergelijkbaar met de *in vivo* architectuur. Orgaankweken van de thymus, die voor recolonisatie studies *in vitro* met prothymocyten gebruikt worden, zijn dus een goede afspiegeling van de thymus *in vivo*.

Hoofdstuk 7 beschrijft het effekt van injektie van verscheidene doses dexamethason op subpopulaties thymocyten en stromale cellen. In dit hoofdstuk wordt beschreven wat het effect is van dexamethason op de samenstelling van T cel subpopulaties en wordt een verband gelegd tussen de veranderingen in de samenstelling hiervan en veranderingen in het thymus stroma ten gevolge van een injektie met dexamethason. Als gevolg van dexamethason injectie wordt de subpopulatie van sterk Thy1+, zwak T200+, T4+, Lyt2+, MEL14+ corticale thymocyten met variabele expressie van Lyt1 gedood (85% van de thymocyten). Medullaire thymocyten (15% van de thymocyten) zijn deels ook gevoelig voor dexamethason. 130 mg/kg lichaamsgewicht dexamethason doodt alle thymocyten met uitzondering van een kleine subpopulatie (4%) dexamethason resistente thymocyten. Deze laatste zijn zwak Thy1+, sterk T200+, T4+, Lyt2- en MEL14+ medullaire thymocyten. Dexamethason resistente thymocyten (d.w.z. thymocyten die resistent zijn tegen 130 mg/kg dexamethason) bleken echter niet representatief te zijn voor medullaire thymocyten, zoals oorspronkelijk werd aangenomen; er ontbreekt n.l. een subpopulatie van zwak Lyt2+ cellen. De perivasculaire ruimten in de medulla bleken een bijzondere subpopulatie thymocyten te bevatten, n.l. MEL14+ cellen met een 'helper' fenotype (T4+, Lyt2-). Waarschijnlijk vertegenwoordigen deze cellen uitgerijpte cellen in de thymus, welke op het punt staan de thymus te verlaten. Het thymus stroma bleek ook gevoelig voor dexamethason en wel bij uitstek het TR4+ corticale stroma. TR5+ medullaire cellen werden niet door dexamethason aangetast, voorzover dit op grond van morfologische criteria kon worden vastgesteld. Ten gevolge van dexamethason behandeling hadden de TR4+ corticale epitheelcellen een 'TNC-achtige' vorm en was expressie van klasse I en II MHC determinanten op het cortexepitheel niet meer membraangebonden.

Hoofdstuk 8 beschrijft de reaktiviteit van de monoclonale antistof ER-TR7 (Hoofdstuk 4), die met fibroblasten reageert. Deze antistof reageert niet alleen met fibroblasten in de thymus, maar ook met fibroblasten in perifere lymfoïde organen en niet-lymfoïde organen. Vriescoupes van milt en lymfeklieren, die met ER-TR7 geïncubeerd werden met behulp van de immunoperoxidase techniek, tonen dat ER-TR7 de verschillende compartimenten in

deze organen op karakteristieke wijze aankleurt. In de milt zijn rode en witte pulpa te onderscheiden. Een onderverdeling van de witte pulpa in marginale zone, follikels (B cel domein) en peri-arteriolaire lymfocytenschede (T cel domein) is mogelijk met ER-TR7. In de lymfklier zijn paracortex (T cel domein), follikels en medulla te onderscheiden. ER-TR7 bleek te reageren met een cytoplasmatische component van fibroblasten en met een extracellulaire component in losmazig bindweefsel. ER-TR7 reageert niet met collageen type I, II, III, IV, V, laminine, fibronectine, heparansulfaat bevattende proteoglycanen, entactine of nidogeen. Deze monoclonale antistof reageert dus met een tot nu toe onbekende verbinding.

In Hoofdstuk 9 wordt beschreven, dat de monoclonale antistof ER-TR9, die opgewekt werd middels de reaktie met een kleine subpopulatie stromale cellen in de thymus, vooral reageert met macrofagen in de marginale zone van de milt. De marginale zone van de milt blijkt een uniek compartiment te zijn, zowel wat betreft de stromale cellen als ook de lymfoïde cellen: het bevat de specifieke TR9+ marginale zone macrofagen en een subpopulatie van niet-recirculerende IgM+, IgD- tot zwak IgD+ B cellen, die wat betreft hun funktie verschillen van IgM+, IgD+ recirculerende B cellen.

CURRICULUM VITAE

De schrijfster van dit proefschrift werd op 7 september 1955 in Den Haag geboren. In 1972 behaalde zij haar HBS-B diploma aan de Wageningse Scholengemeenschap te Wageningen. Vervolgens ging zij aan de Landbouwhogeschool in Wageningen studeren en behaalde daar in 1976 haar kandidaatsdiploma Landbouwwetenschappen. Tijdens de ingenieursstudie deed zij gedurende de praktijkperiode onder leiding van Dr. W.Th.J.M. Hekkens op de afdeling Gastroenterologie van het Academisch Ziekenhuis te Leiden onderzoek naar kwantitatieve bepaling van antistoffen tegen caseïne en β-lactoglobuline. Zij behaalde in juni 1979 haar ingenieursdiploma. In oktober 1979 begon zij op de afdeling Celbiologie en Genetica van de Erasmus Universiteit in Rotterdam onder leiding van Dr. W. van Ewijk aan het in dit proefschrift beschreven onderzoek. Aanvankelijk vond dit plaats in tijdelijke dienst bij de Erasmus Universiteit, later in dienst van de Stichting voor Medisch Wetenschappelijk Onderzoek FUNGO van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek. Sinds 1 oktober 1984 is zij verbonden aan de Rijksuniversiteit te Leiden (afdeling Immunohematologie en Bloedbank, Academisch Ziekenhuis). Zij werkt aan een door de Stichting Research Fonds Diabetes Mellitus gesubsidieerd project betreffende de immunodiagnostiek van diabetes mellitus. Daartoe is zij tot op heden gedetacheerd bij de afdeling Klinische Immunologie van de Stichting Samenwerking Delftse Ziekenhuizen.