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Immunohistochemical characterization of rat thymic non-lymphoid cells

II. MACROPHAGES AND GRANULOCYTES DEFINED BY MONOCLONAL ANTIBODIES

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SUMMARY

A panel of monoclonal antibodies (mAb) raised to antigens of rat thymic non-lymphoid cells (predominantly macrophages and granulocytes) was immunohistochemically characterized. Based on their staining patterns on cryostat thymic sections and double labellings using acid phosphatase activity, anti-cytokeratin mAb to exclude binding to epithelium or ED1 and ED2 mAb, specific for rat macrophages, antibodies were subdivided into four groups: (i) R-MC 39 mAb strongly reactive with macrophages in the cortex and cortico-medullary zone (CMZ) and weakly with some scattered macrophages in the medulla, blood vessels and thymocytes; (ii) R-MC 40, 41 and 42 mAb specific for cortical macrophages and most CMZ macrophages; (iii) R-MC 43 and 44 mAb predominantly recognizing CMZ and medullary macrophages; (iv) R-MC 45 mAb strongly labelling granulocytes and weakly a subset of macrophages throughout the thymus and isolated cells in the medulla. The obtained results show considerable heterogeneity within mobile thymic non-lymphoid cells and the presence of specific or common antigens in macrophages of particular topographic localization in the rat thymus.

INTRODUCTION

Committed haematopoietic T-cell precursors colonize the thymus, undergo a complex development leading to the expression of clonally distributed T-cell receptors and migrate to the periphery as immunocompetent mature T cells (Scollay *et al.*, 1988). Close interactions between developing thymocytes and thymic microenvironment, composed of epithelial cells, macrophages, interdigitating (ID) cells and mesenchymal stroma (Lobach & Haynes, 1987), appear to be essential for thymocyte proliferation and acquisition of major histocompatibility complex (MHC)-restricted functions (Agarwal & Thomson, 1984; Kruisbeek *et al.*, 1985).

In the past few years, attempts were directed toward the delineation of such developmental events and the identification of various surface molecules on thymic cells. Monoclonal antibodies (mAb) raised directly to thymic components, especially to antigens in/on epithelial cells (reviewed by Ritter & Haynes, 1987; Kampinga *et al.*, 1989), enabled better elucidation of inductive microenvironmental signals involved in these processes.

Thymic macrophages and ID cells believed to originate from common precursors in the bone marrow (Veerman, 1974; Johnston, 1988) have been postulated to participate in a stage of thymocyte maturation (Beller & Unanue, 1978), although all their functions are not sufficiently known. They differ markedly in morphology, surface receptors, enzyme contents, tissue distribution and functional properties (Johnston, 1988). In the rat thymus, macrophages in the cortex, corticomedullary zone (CMZ) and medulla are different, as judged by their histochemical reaction, enzyme contents and ultrastructural characteristics (Milićević & Milićević, 1984; Milićević *et al.*, 1987). In contrast, the ED series of mAb (ED1, ED2 and ED3) raised to a subset of rat mononuclear phagocytes (Dijkstra *et al.*, 1985) demonstrated that, although phenotypic heterogeneity within rat thymic macrophages existed, no clear distinction between cortical and CMZ macrophages was observed.

In this paper we demonstrate additional molecular heterogeneity of rat thymic macrophages and granulocytes using a panel of seven mAb raised in our laboratory, and have compared the reactivity of these reagents with the ED series of mAb.

MATERIALS AND METHODS

Animals

AO rats, both sexes, 6–8 weeks old, and female BALB/c mice, 6 weeks old, bred at the Farm for Experimental Animals, MMA, Belgrade, were used in this study.

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Immunization

BALB/c mice were immunized intraperitoneally (i.p.) with 0·4 ml homogenized rat thymus with complete Freund's adjuvant (FA) (Torlak, Belgrade) in physiological saline. Thymic homogenant, which contained enriched non-lymphoid cells, was prepared after excess thymocytes had been removed by teasing. Alternatively, rats were treated with hydrocortisone (Galenika, Belgrade) (150 mg/kg, i.p., 2 days before killing) in order to enrich thymic cells with macrophages. The immunization procedure was repeated with complete FA and without FA, respectively, at 2-week intervals. Fusion was performed 3–4 days after the last challenge.

Fusion

Spleen cells from immunized mice were fused with P3X-63-Ag8.653 myeloma cells (kindly supplied by Dr M. Ritter, Hammersmith Hospital, London, U.K.) using polyethylene glycol 1500 (Serva, Heidelberg, FRG), suspended in RPMI-1640 medium (Serva) with 10% fetal calf serum (FCS), hybridoma grade (Serva), and plated out into 96-well plates (Flow Laboratories, Irvine, Ayrshire, U.K.). Peritoneal macrophages were used as feeder cells. Hybridomas were selected for in hypoxanthine-aminopterin-thymidine (HAT) medium (Serva) according to Köhler & Milstein (1975). Supernatants of growing hybridomas were screened by immunoperoxidase staining on cryostat thymic sections of intact adult rats as described below, so that the possible modulation of macrophage antigen expression caused by hydrocortisone was not relevant for this study. The positive cultures were cloned by limiting dilution and expanded in tissue flasks (Flow Laboratories). Supernatants were collected and stored at $-20°$. Ascites fluid was obtained by i.p. injection of 5×10^6 hybrid cells into BALB/c mice, pretreated with pristane (Serva).

Immunohistochemistry

Cryostate sections of the thymus were fixed in acetone (10 min), before incubation with supernatants for a minimum of 60 min, followed by blocking of endogenous peroxidase activity in 1% H_2O_2 diluted in methanol for 15 min. After that, sections were covered with sheep anti-mouse Ig conjugated with peroxidase (Amersham International, Amersham, Bucks, U.K.) at 1:20 in Tris-buffered saline (TBS) for 30 min. Revelation of the peroxidase activity was performed by 3'3 diaminobenzidine (DAB) (Serva) and 0.01% H_2O_2.

Isotype specificity of mAb as well as their final characterization on lymphatic organs was performed using goat anti-mouse Ig isotype-specific biotynilated antibodies (Amersham) at 1:100 followed by streptavidin-peroxidase (Amersham) and DAB incubation.

The inter-relationship of produced mAb with anti-cytokeratin mAb (K.8.13, IgG2a and CK8, IgG1), purchased from ICN Biomedicals, Farmacos-ICN, Belgrade and Amersham, respectively, as well as ED1 and ED2 mAb, IgG1, specific for rat macrophages (a generous gift of Dr C. Dijkstra, Free University, Amsterdam, The Netherlands), was tested using double immunofluorescence staining. All mAb mutually differed in isotype specificity. Sections were incubated with reagents in the following sequence: first mAb, goat anti-mouse Ig isotype-specific biotinylated antibody, streptavidin-TR (Amersham), second mAb, sheep anti-mouse Ig isotype-specific antibody coupled with FITC (Serotec). After washing following each incubation, sections were mounted in glycerol and observed for green and red fluorescence under a fluorescence microscope (Univar III). Specificity of labelling was checked using appropriate controls, including single staining, omitting the first or second mAb as well as their replacement with an irrelevant mouse mAb of the same isotype (produced in our laboratory). All controls gave negative results. A detailed description of each combination is given in the results.

The suspension of thymic cells was labelled with mAb and subsequent anti-mouse Ig-FITC-coupled antibody using the classic indirect immunofluorescence method. The percentages of positive cells were analysed on an EPICS-CS flow cytometer (Coulter Electronics, FRG).

Enzyme histochemistry

Enzyme histochemistry was used to demonstrate acid phosphatase (AP) activity according to Burnstone (Pearse, 1968) with AS-BI phosphate as substrate and pararosaniline as diazonium salt. This procedure was performed on individual cryostat sections or following streptavidin-biotin immunoperoxidase on the same section as double labelling.

RESULTS

mAb: general characteristics

From four fusions seven mAb predominantly reacting with macrophages in the thymus have been selected for further characterization. Their binding to macrophages has been checked according to cell morphology, localization patterns in the thymus and other lymphatic organs and AP activity. In addition, dual immunolabelling with anti-cytokeratin mAb (to exclude binding to the epithelium) and with ED1 and ED2 mAb (specific for rat cells of the monocyte–macrophage lineage) was performed. Antibodies were subdivided into four groups, whose general staining patterns on the thymus and other lymphatic organs were given in Table 1. The detailed immunohistochemical and flow cytometric characterization of these reagents in peripheral lymphatic and other organs will be published in a separate paper (Popović *et al.*, manuscript in preparation).

Immunohistochemical characterization of mAb reactive with thymic macrophages and granulocytes

R-MC 39 mAb, from the first group, IgG2a isotype, bound to macrophages in the cortex and CMZ as well as to some macrophages in the medulla and capsule (Fig. 1a). In addition, weak staining of blood vessels and thymocytes was seen. This mAb also stained macrophages, lymphocytes and vessels in peripheral lymphatic organs (Table 1).

The second group of three mAb (R-MC 40, 41 and 42), isotyped as IgM, IgG1 and IgG1/G2a/M, respectively, bound to cortical and most CMZ macrophages (Fig. 1b) in similar staining patterns. Positive cells were large, with slender processes extending between surrounding thymocytes. All three mAb also strongly labelled branched macrophages in the capsule and septa, but no labelling was seen in the medulla. Some occasionally R-MC 42^+ cells in the medulla were observed. Subsets of macrophages in the peripheral lymphatic organs (Table 1) were positive. Antibodies of the third group (R-MC 43 and 44), isotyped as IgM, were predominantly reactive

Table 1. Immunoreactivity of R-MC mAb on rat lymphoid organs

Group	I	II	III	IV
mAb	*R-MC 39 (SHC-6D10)	R-MC 40 (R-5G7) R-MC 41 (SHC-5G1) R-MC 42 (SHC-1G2)	R-MC 43 (R-7B5) R-MC 44 (SHC-6E10)	R-MC 45 (R-8C10)
Thymus (M)				
Capsule	++(n)	++++(b)	+(w)	±(w)
Cortex	++++(n)	++++(b)	+(w)	+(w)
CMZ	++++(n)	+++(b)	++++(b)	+(w)
Medulla	+(w)	–	++++(b)	+(w)
Spleen (M)				
Follicle	+++(n)	–	±(w)	–
PALS	+(w)	–	+(w)	+(w)
MZ	++(w)	–	+++(b)†	+(w)
Red pulp	+++(w)	++++(b)	±(w)	++(w)
Lymph node (M)				
Capsule	+(w)	+++(b)	±(n)	–
Follicle	++(w)	–	+(w)	–
Paracortex	++(w)	+(b)	++(n)	+(w)
Medulla	++(n)	+(b)	++(n)	+(w)
Peayer's patches (M)				
Villi	++(n)	++(b)	++++(w)	±(w)
Dome region	++(w)	±(b)	++(n)	±(w)
Follicle	+++(n)	–	++(n)	±(w)
Interfol. area	++(w)	±(b)	+++(b)	+(w)
Other cells	Thymocytes (90%)‡ Lymphocytes and blood vessels in all organs			Granulocytes (in all organs)

* The names of mAb in parentheses are their working names. They are given because some mAb are supplied on request under these names; SHC series of mAb are produced from the fusions in which hydrocortisone was used to enrich thymic macrophages.

† Marginal metalophils.

‡ Flow-cytometric analysis .

M, macrophages; MZ, marginal zone; PALS, periarterial lymphatic sheath; CMZ, cortico-medullary zone; ++++, almost all cells (approximately 90–100%) stained; +++, most cells positive (approximately 75–90%); ++, considerable number of cells positive (approximately 25–75%) stained; +, few cells stained; ±, very few cells stained; b, bright staining; n, normal staining; w, weak staining.

with macrophages in the CMZ and medulla in a similar, granular pattern (Fig. 1c). In positive cells either large or fine granular contents were labelled with mAb. Fine, mostly rare granular staining was also seen in some macrophages in the cortex, capsule and perivascular areas. Both antibodies predominantly stained marginal metalophils in the spleen (Table 1).

R-MC 45 mAb from the fourth group, IgG1, bound strongly to clusters of granulocyte-like cells in the subcapsular region and perivascular area, or to single cells identified as granulocytes by their mophology, scattered throughout the thymus (Fig. 1d). Much weaker positivity of some macrophages in the cortex and isolated cells in the medulla was seen. Granulocytes were positive in the red pulp of the spleen. In addition, weak staining of scattered cells in T-dependent areas (peripheral lymphatic sheath of the spleen, paracortex of the lymph node and interfollicular area in Peyer's patches), resembling ID-like cells, was also demonstrated (Table 1).

Double staining of rat thymic macrophages with R-MC mAb and acid phosphatase

Acid phosphatase (AP) is one of the most characteristic enzymatic markers for macrophages. All macrophages in the cortex, CMZ, medulla and capsule of the rat thymus clearly showed either strong, normal or weak AP activity (Fig. 1e). Spotty staining inside some cells in the medulla (probably ID cells) were also observed. Using dual immunohistochemistry and enzyme-histochemistry on the same thymic section (not shown due to non-recognizable individual stainings on black and white photographs), we observed that our mAb recognized macrophages, but some differences in staining patterns were seen.

Almost all R-MC 39$^+$ macrophages in the cortex and CMZ were AP$^+$. However, more numerous cells in the medulla were AP$^+$ only. Antibodies from the second group (R-MC 40, 41 and

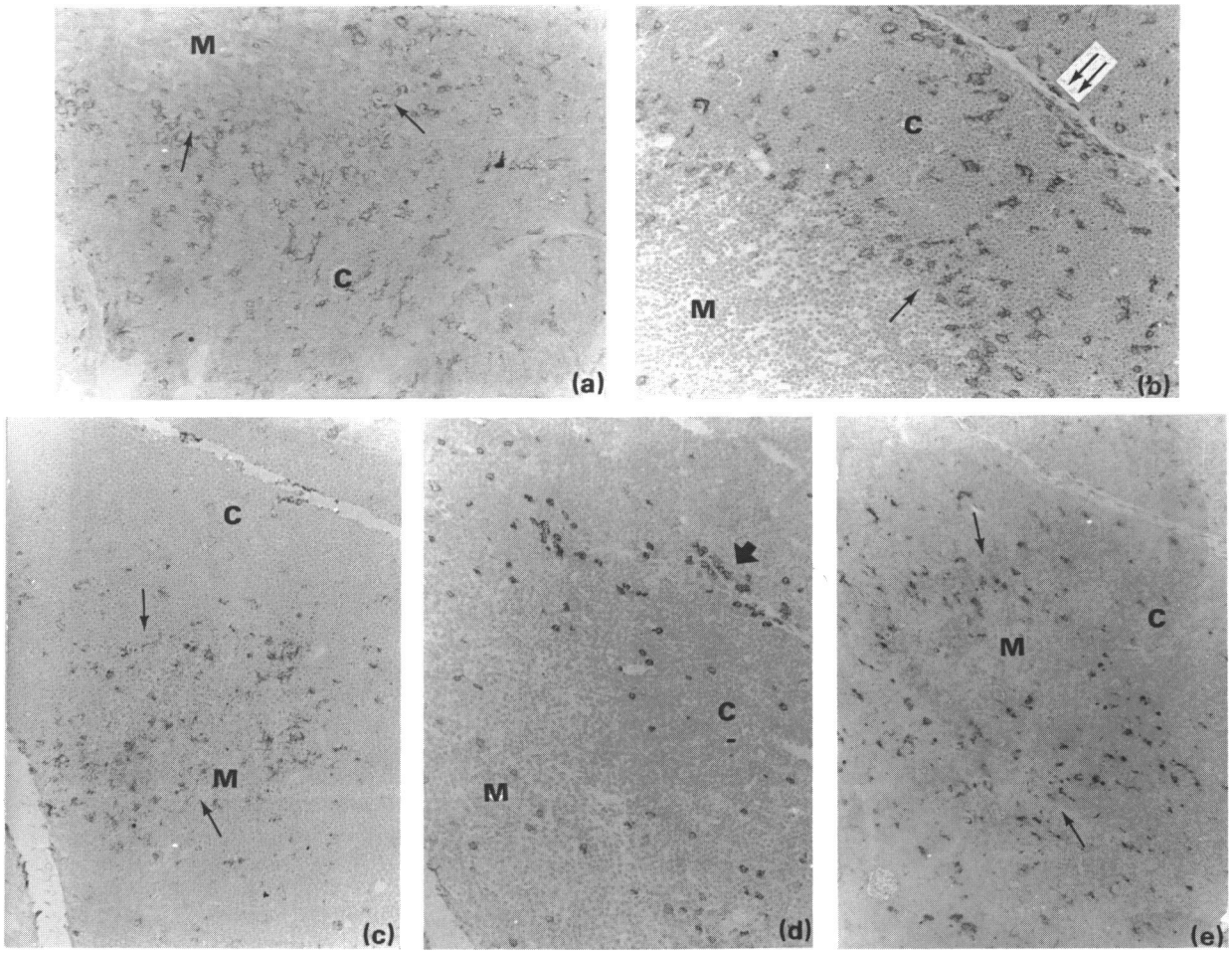

Figure 1. Streptavidin-biotin immunoperoxidase staining of the rat thymus with mAb. (a) R-MC 39. Macrophages in the cortex (C) and CMZ (arrows) are brightly stained. Few cells in the medulla (M) are also weakly positive. (b) R-MC 41. Note labelling of macrophages in the cortex, CMZ (arrow) and capsule (double arrows), while the medulla is negative. (c) R-MC 43. Note predominant labelling of macrophages in CMZ (arrows) and medulla. (d) R-MC 45. Note strong labelling of scattered granulocytes in the cortex and a cluster of the same cells close to the septal area (arrow). Scattered cells in the medulla are stained more weakly. (e) AP staining of the rat thymus. Note labelling in all topographic zones: cortex, medulla and CMZ (arrows). The strongest AP activity is seen in CMZ. Magnifications: × 80.

42) did not bind to all AP^+ cells in CMZ and cortex. R-MC 43 and 44 mAb from the third group bound to almost all AP^+ cells in the CMZ and medulla, but stronger AP activity was observed in the cortex than the reactivity with these mAb. R-MC 45^+ granulocytes were AP^-.

R-MC mAb do not recognize epithelial cells

Double immunostainings of the thymus with R-MC mAb and K 8.13 or CK 8 mAb (both pan epithelial markers in the rat thymus; Čolić *et al.*, 1989) showed that not one of R-MC mAb from this panel bound to keratin-positive cells. An example of such staining is given in Fig. 2a,b.

Inter-relationship of R-MC and ED mAb

ED1 and ED2 mAb have previously been raised as specific markers for rat monocytes and tissue macrophages (Dijkstra *et al.*, 1985). To demonstrate the inter-relationship between our mAb and ED1/ED2 mAb we performed various combinations of dual immunolabelling.

Using a combination of ED1 mAb (pan macrophage marker) and R-MC 39 mAb, we found that in the cortex and CMZ almost all cells were double positive, while in the medulla $ED1^+R^-MC\ 39^-$ cells were much more numerous (not shown). Double staining with ED1 and R-MC 40 showed that almost all cells in the cortex were double positive, while double positive and single $ED1^+$ cells were observed in CMZ (Fig. 2c, d). Similar staining patterns were seen using a combination of R-MC 39 and 40 mAb, except that more single R-MC 40^+ cells in the capsule and septa were found than in the previous combination. Staining with R-MC 44 and ED1 showed double-positive cells in the medulla and CMZ, although more numerous R-MC 44^+ fine granular contents were seen (not shown).

Dual labelling with ED2 mAb (marker for cortical and most CMZ macrophages) and R-MC 40 showed exclusively double positive cells (Fig. 2e, f). Simultaneous staining with other mAb

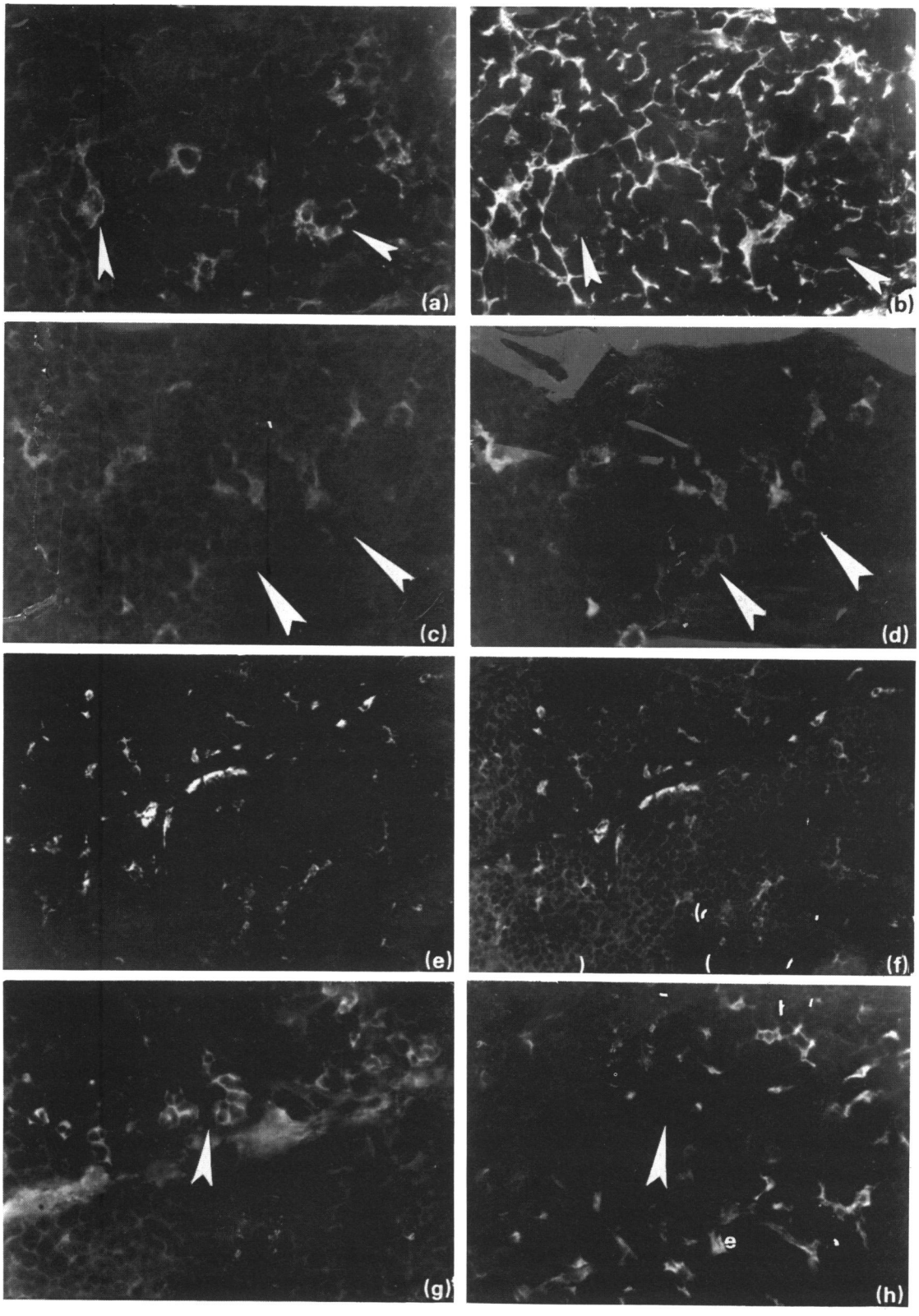

Figure 2. Double immunofluorescence staining of the rat thymus with mAb. (a) R-MC 41 (FITC) and (b) K.8.13. (TR). R-MC 41^+ cells in the cortex are cytokeratin negative. Some of them are arrowed. (c) R-MC 40 (FITC) and (d) ED1 (TR). Note double positive cells in the CMZ and R-MC 40^-ED1^+ cells (arrows). Magnifications: × 240. (e) R-MC 40 (TR) and (f) ED2 (FITC). Note double positive cells in the cortex and capsule. (g) R-MC 45 (FITC) and (h) R-MC 39 (TR). Note single positive cells. Some R-MC 45^+39^- cells in the subcapsular area are arrowed. Magnifications: × 240.

from this group was not performed due to the same isotype of mAb, but dual combination R-MC 40/R-MC 41 again showed double positivity. Using combinations of R-MC 39 or 40 mAb with R-MC 45 mAb we found that R-MC 45$^+$ granulocytes were both R-MC 39 and 40 negative (Fig. 2g, h).

Analysis of cell suspensions by R-MC

Flow cytometric analysis of thymic cells showed that about 90% thymocytes were weakly positive with R-MC 39 mAb (Table 1). No more than 1–2% of thymic cells were stained with R-MC 41, 42 and 45 mAb. The percentage of cells stained with R-MC 40, 43 and 44 mAb was at background levels (not shown).

DISCUSSION

In studies concerned with functional aspects of the thymic microenvironment, it is necessary to define reliable markers which recognize distinct cell subpopulations involved. The results presented here are our first step in phenotypic characterization of mobile non-lymphoid cells (predominantly cells of macrophage lineage and granulocytes) in the rat thymus by a panel of seven mAb raised in our laboratory. The fact that these reagents recognize macrophage/granulocytes is assessed by cell morphology, the localization pattern in lymphatic organs, correlation with AP activity, dual immunoreactivity with ED1 and ED2 mAb specific for rat tissue macrophages (Dijkstra *et al.*, 1985) and non-reactivity with epithelial (cytokeratin-positive) cells. Antibodies were subdivided into four groups according to their distinctive labelling patterns in the thymus.

R-MC 39 mAb, from the first group, detects a membrane antigen as judged by flow cytometric analysis of viable thymic and peritoneal cells (not shown). We did not find data in the literature describing a similar mAb in the rat lymphoid system, but a range of various mAb exist which identify common epitopes on macrophages, vessels and lymphocytes in human tissue. Some of them belong to LFA family (CD11 and CD18) or to the CD14 group (reviewed by Hogg & Horton, 1987). The analysis of our results did not allow more precise grouping of this antibody on the basis of partial immunohistochemistry alone. More detailed immunohistochemical and biochemical characterization of R-MC 39 antigen is now in progress in our laboratory.

The staining patterns of mAb from the second group are similar to that previously reported with ED2 mAb specific for cortical macrophages (Dijkstra *et al.*, 1985; Sminia *et al.*, 1986). Our dual staining (ED2/R-MC 40) shows that all cells are double positive. It is obvious that these mAb are markers for tissue macrophage antigens and probably detect subgroup-specific macrophage molecules. We do not know whether all mAb may be directed against the same or related antigens, even against the same epitopes, or different antigens in the same cell type. Our unpublished results show that R-MC 40 recognizes an intracytoplasmic antigen, while R-MC 41 and 42 detect membrane and maybe intracytoplasmic antigens (flow cytometric and immunocytochemical analysis of rat peritoneal macrophages). In addition, non-competitive binding of ED2 and R-MC 40 mAb to the same cells favours the hypothesis that at least these two reagents recognize different epitopes or antigens. A definitive answer can not be given until the molecules to which they bind have been identified.

mAb from the third group (R-MC 43 and 44) differ from the previous ones by predominant binding to intracytoplasmic, granular content of CMZ and medullary macrophages that could be lyzozomal enzyme(s). There is some similarity in the reactive profile of these mAb and ED1 mAb (Dijkstra *et al.*, 1985), but some difference observed in their distribution pattern in the thymus and peripheral lymphatic organs suggests that ED1 and R-MC 43/44 mAb recognize different antigens. This fact is also demonstrated in our double immunolabelling (ED1/R-MC 44). In the spleen, R-MC 43/44 mAb and ED3 mAb (Dijkstra *et al.*, 1985) stain the strongest marginal metalofils, but they differ in staining patterns in the thymus. Namely, ED3 stains a few cells scattered in the capsule and medulla.

R-MC 45 mAb from the fourth group predominantly reacts with cells of granulocyte lineage localized in the subcapsular area or scattered throughout the whole thymus. Weak staining is also seen in some cortical macrophages and isolated cells in the medulla. Ishii *et al.* (1984) described two mAb, named R2-1A6 and R2-2B1, detecting antigens expressed in rat granulocytes and macrophages, but they differ from our mAb in additional reactivity with medullary cells in the thymus, follicular dendritic cells in lymphoid germinal centre as well as with the specialized endothelium in the marginal sinuses of the spleen and post-capillary venules of the lymph node. Our R-MC 45 mAb probably differ from OX42 mAb in the rat, MAC-1 in the mouse or mAb detecting CD11 epitopes in human (Robinson, White & Mason, 1986) (all specific for C3b) by its non-reactivity with follicular dendritic cells. The possible similarity between R-MC 45 mAb and mAb reacting with CD16 (low affinity FcR, 50,000–70,000 MW) present on human granulocytes and macrophages (Hogg & Horton, 1987) could be determined upon antigen isolation. The presence of common epitopes in granulocytes and cells of the monocyte/macrophage system is in agreement with their common origin (Parwaresch *et al.*, 1985). Foci of myeloid cells extramedullary differentiating in the rat thymus have already been noticed (Olah, Rohlich & Toro, 1975). Our finding of clusters of R-MC 45+ cells localized mainly in the subcapsular area supports the previous morphological observations. It is not clear whether weak R-MC 45$^+$ cells found in T-dependent areas in peripheral lymphatic organs and thymic medulla correspond to ID or ID-like cells. If this is true, it would be in contradiction with findings in the human system. Namely, non of 40 anti-myeloid mAb submitted to the Third International Workshop of Leucocyte Differentiation Antigens (Berti *et al.*, 1987) which detected granulocyte antigens was reactive with ID cells.

Thymic macrohages and ID cells have important roles in differentiation and maturation of thymocytes (Beller & Unanue, 1978), but all their functions are unsufficiently known. Most cortical macrophages possess high phagocytic capacity and are involved in 'cleaning' of the thymus from thymocytes dying in large numbers under physiological conditions (Scollay *et al.*, 1980). Both macrophages and ID cells form multicellular complexes with thymocytes *in vitro* in a rosette form, which probably exist *in vivo* (Kyewski, Rouse & Kaplan, 1982; Brelinska, Houben-Defresne & Boniver, 1986; Wood *et al.*, 1988). This intimate cell–cell contact may play a role in the acquisition of T-cell functions in the thymus. Class II MHC-positive cells (ID cells and some macrophages) can be involved in tolerance induction and self-MHC restriction (Hamblin & Edgeworth, 1988).

Phenotypic analysis of the rat thymus performed in this work and in a previous study (Sminia *et al.*, 1986) shows the presence of different molecules in/on thymic macrophages depending on their topographic localization. The local microenvironments (cortex, CMZ, medulla) seem to determine cell-specific antigenic characteristics. Phenotypic profile of thymic macrophages is not in strict correlation with their morphology. It is especially seen in CMZ in which phenotypically the most heterogenous macrophage population is observed. They share common antigen either with cortical macrophages (ED1, ED2, R-MC 39–43) or with medullary cells (ED1, R-MC 43, 44). No specific mAb for the rat CMZ macrophages has been produced, as can be expected judged by the specific ultrastructural and enzyme features of these cells (Milićević *et al.*, 1987; Milićević & Milićević, 1984).

At present, we do not know the functions of most of these identified molecules, but a panel of mAb which has been raised enables further functional studies of individual cell subsets or molecules both *in vitro* and *in vivo*.

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