Immunology 1990 **70** 66–74

The phenotypic heterogeneity of mouse thymic stromal cells

D. I. GODFREY, D. J. IZON, C. L. TUČEK, T. J. WILSON & R. L. BOYD *Department of Pathology and Immunology, Monash University Medical School, Prahran, Melbourne, Victoria, Australia*

Accepted for publication 1 February 1990

SUMMARY

Sixteen monoclonal antibodies (mAb) were produced against mouse thymic stromal elements. These mAb fell into two groups of reactivity: (i) thymic epithelial markers (screened and presented according to the guidelines proposed in the 1989 Rolduc Thymic Epithelial Workshop); and (ii) non-epithelial thymic markers. Specificities of these mAb included extensive subpopulations of both epithelial and non-epithelial thymic stromal cells, as well as isolated stromal cells, demonstrating some of the complex microspecificities in existence within the thymic microenvironment. Furthermore, six of these mAb demonstrated shared antigenicity between thymocytes and thymic stromal cells, revealing greater similarities than previously recognized between these two components. Three mAb detected antigens illustrating three consecutive layers of the blood–thymus barrier: the vascular endothelium; connective tissue of the capsule and perivascular spaces; and the connective tissue associated with the basal laminae lining these regions. This study illustrates unequivocably that there are indeed complex and varied microenvironments existing within the thymus, and emphasizes the need for reclassification of these cells.

INTRODUCTION

The complex intrathymic processes culminating in the generation of fully functional, self-tolerant, self-major histocompatability complex (MHC)-restricted T lymphocytes have proven difficult to decipher. They appear to involve extensive interactions between T-cell progenitors, thymic stromal cells, and soluble factors from both of these components (Stutman, 1978; Denning *et al.*, 1988; De la Hera *et al.*, 1989). A better understanding of these mechanisms has been hampered by the inability to identify and isolate individual subpopulations from the heterogeneous thymic stroma.

Previous characterization by many investigators (for example Haynes, 1984; Van Vliet, Melis & Van Ewijk, 1984; De Maagd *et al.*, 1985; Lobach, Scearce & Haynes, 1985; Kampinga *et al.*, 1987; Colic *et al.*, 1988) using mAb has revealed phenotypically distinct subpopulations of both epithelial and non-epithelial thymic stromal components. As a consequence, the thymic epithelium can be broadly subdivided into subcapsular, cortical, medullary and Hassall's corpuscles (HC). Interspersed throughout this epithelium are reticular fibroblasts, macrophages (Mϕ) and dendritic/interdigitating cells (DC/IDC) (reviewed by Kendall, 1981, 1988; Lampert & Ritter, 1988). Further heterogeneity of the epithelium has been reported at the ultrastructural level: subcapsular-perivascular (Type 1); pale (Type 2); intermediate (Type 3); dark (Type 4); undifferentiated (Type 5); and large medullary (Type 6) (Van de Wijngaert *et al.*, 1984). Classification of Types 2, 3 and 4 were based upon the electron lucency of the cells. These types were found throughout the organ, whereas type 5 cells were primarily located at the cortico-medullary junction.

In the present study, an extensive panel of 16 mAb has been produced against mouse thymic stromal elements via both spleen and popliteal lymph node fusions. The results presented here confirm and significantly extend those of previous workers, revealing markers specific to subpopulations of both epithelial and non-epithelial thymic stromal cells as well as scattered, isolated stromal cells, reflecting some of the isolated, specific microenvironments within this organ. Additionally, six of these mAb are specific for epitopes common to both thymic stromal cells and thymocytes. Such shared reactivity has been reported previously for the interleukin-4 (IL-4) receptor in the human thymus (Larché *et al.*, 1988). Finally three mAb reacted with, respectively: the vascular endothelium; connective tissue of the capsule and perivascular spaces; and the connective tissue associated with the basal laminae lining these regions. These represent the three consecutive layers of the blood–thymus barrier.

Abbreviations: CTES, clusters of thymic epithelial staining patterns; ECF, epithelial cell free regions; HC, Hassall's corpuscles; IIF, indirect immunofluorescence; KNC, keratin-negative cells; NBS, newborn calf serum; PBS-NBS-NaN_3, phosphate-buffered saline + 5% NBS + 0·02% sodium azide; PVS, perivascular spaces; RPMI-FCS, RPMI-1640 containing 10% fetal calf serum + 2 mM glutamine; RPMI-SF, serum-free RPMI-1640; TEC, thymic epithelial cells.

Correspondence: Dr D. I. Godfrey, Dept. of Pathology and Immunology, Monash University Medical School, Commercial Road, Prahran 3181, Melbourne, Victoria, Australia.

These mAb provide a basis for the characterization and purification of specific thymic stromal cells/molecules for subsequent functional assessment of their contribution to the thymic microenvironment.

MATERIALS AND METHODS

Preparation of thymic stromal cells

Thymic stromal cells from 6–8-week-old female BALB/c or CBA/CaH/WEHI mice (Central Animal House, Monash University) were dispersed by enzymatic digestion of teased thymic fragments, and enriched by sedimentation through newborn calf serum (NBS), essentially as described in Izon & Boyd (1990). The only difference involved the use of trypsin (3 mg/ml; Boehringer-Mannheim, Gmbh, FRG) in the present study, instead of dispase.

Monoclonal antibody production

Two immunization methods were employed for mAb production: (i) splenic priming, the more classical technique resulting in antigen-specific B cells being localized in the spleen; and (ii) popliteal lymph node priming, a novel method devised in our laboratory (Dr D. J. Izon and Dr G. W. Dandie) entailing a significantly shorter immunization schedule and requiring less immunogen.

Rat spleen fusion

LOU/M rats (approximately 1 year old, bred at Monash Central Animal House), were immunized three times intraperitoneally on Day 0, Week 3 and Week 13, followed by intravenous immunization 4 days later with enriched mouse thymic stromal cell suspensions (1 ml of approximately 10^7 cells/immunization). After 4 days the spleen cells were fused with P3-NS-1-Ag 4-1 (NS-1) cells at a ratio of 1 spleen cell to 1 NS-1 cell. The fusion protocol followed that of Galfré *et al.* (1977) with modifications by Goding (1983).

Popliteal lymph node fusion

LOU/M rats (1 year old) were immunized subcutaneously into each hind footpad at Days 0–2 with enriched mouse thymic stromal cell suspensions (0·1 ml containing approximately 10^7 cells). After 4 days, popliteal lymph node cells were fused with NS-1 cells at a ratio of 1:1. Fused cells were resuspended in a mixture of 30 ml of RPMI-FCS with 30 ml of RMPI-1640-Mϕ-conditioned medium (supernatant derived from overnight culture of $1{\cdot}5 \times 10^6$ mouse peritoneal Mϕ in 50 ml of RPMI-FCS). This conditioned medium was used to substitute for the lower feeder cell content of the popliteal lymph node compared to the spleen.

Screening for positive hybrids

After 10 days, the fusion plates were scanned by phase-contrast microscopy for hybrid-containing wells with greater than 70% confluency. Supernatants from these wells were screened by indirect immunofluorescence (IIF) using FITC-conjugated sheep anti-rat Ig (QMF, dilution 1:100, Silenus Laboratories, Hawthorn, Victoria) on 4 μm frozen sections from composite blocks of mouse thymus, small intestine and kidney. NS-1-conditioned medium was used as a conjugate control and 11C3, a rat mAb (IgG) reactive with human but not mouse thymus, served as an antibody control. Hybridomas of interest were cloned twice by limiting dilution. Supernatants were stored at 4° with 0·02% sodium azide (NaN_3; BDH, Poole, Dorset, U.K.).

Tissue and strain specificity

In addition to the initial screening on composite blocks of thymus, small intestine and kidney, the mAb were also screened on frozen sections of uterus, bladder, vagina, colon, duodenum, jejenum, trachea, sciatic nerve, thyroid, adrenal, pancreas, skin, tongue, brain, liver, heart, lung, spleen and lymph node. Strain specificity was determined by screening on frozen sections of BALB/c, CBA/CaH/WEHI and C57BL/6 thymuses. mAb were tested on both sections fixed in acetone (−20°, 20 seconds) as well as unfixed sections for screening acetone-sensitive antigens.

Double labelling with anti-keratin

Reactivity of mAb with thymic epithelial cells was determined via the simultaneous testing of rabbit anti-keratin (broad spectrum; dilution 1:200; Dako, Santa Barbara, CA) with the mAb on frozen mouse thymic sections. The conjugate used for the detection of anti-keratin was rhodamine-conjugated goat anti-rabbit Ig (dilution 1:50; Silenus Laboratories).

Flow cytometry

Thymuses from 6–8-week-old female inbred CBA/CaH/WEHI mice were pushed through a stainless steel mesh (100 μm pore size) and washed (400 g_{max}/4°/5 min) in PBS + 5% NBS + 0·02% NaN_3 (PBS-NBS-NaN_3). Forty microlitres of mAb supernatant were added to 1×10^6 cells in conical test tubes (Johns, Melbourne) and incubated for 30 min at 4°. The cells were then washed once with 1 ml of PBS-NBS-NaN_3, incubated with 40 μl of QMF (tested at dilutions of both 1/100 and 1/1000) as above and washed once as before. Finally, the thymocytes were resuspended in 1 ml of PBS-NBS-NaN_3 for flow cytometric analysis on a FACScan (Becton-Dickinson, Mountain View, CA).

Isotyping of mAb

mAb Ig classes were determined by Ouchterlony double gel immunodiffusion in agar using culture supernatants concentrated 10-fold by negative-pressure dialysis. Antibodies specific for rat IgG and IgM were obtained from Nordic Diagnostics, Tilburg, The Netherlands.

RESULTS

On the basis of their distribution patterns on mouse thymus, 16 hybridomas were selected from over 4000 screened, and cloned twice by limiting dilution. mAb reactive with thymic epithelial cells (TEC) are summarized in Table 1 [some of these mAb also stain keratin-negative cells (KNC) and thymocytes], non-epithelial thymic reactivity is summarized in Table 2, and non-thymic reactivity of these mAb is summarized in Table 3.

Thymic epithelial markers

The epithelial reactivity of these mAb was demonstrated by double labelling with anti-keratin on both thymus and other mouse tissues. This category contains 11 different mAb reactive with thymic epithelium or subpopulations thereof. These mAb have been categorized and presented according to the guidelines proposed at the 1989 Rolduc Thymic Epithelium Workshop.

Table 1. Thymic epithelial markers

MTS	Isotype	CTES	Subcapsule	Cortex	Medulla	Non-epithelial	FACS
39	IgM	I	++	++	++	—	—
10	IgM	II	++	−	++	—	—
44	IgG	III.B	±	++	±	—	—
32	IgG	III.C.2	*	*	−	Thymocytes (cortex)	>95%
20	IgM	IV.A	−	−	±	—	—
29	IgG	IV.A	−	−	±	—	—
7	IgM	XX.c	±	±	±	Capsule; KNC (cortex and medulla)	—
9	IgG	XX.c	±	±	+	Thymocytes (cortex and medulla)	—
37	IgG	XX.c	−	±	±	Thymocytes (cortex and medulla); KNC (medulla); IVC	>95%
33	IgG	XX.d	*	*	±	Thymocytes (cortex)	85–90%
35	IgG	XX.d	*	*	±	Thymocytes (cortex)	75–85%

−, ±, +, ++: 0%, <25%, 25–75%, >75% of TEC are stained.
* Degree of stromal cell staining is masked by thymocytes.
IVC, intravascular cells.

Table 2. Thymic non-epithelial markers

MTS	Isotype	Thymus
12	IgG	Vascular endothelium and thymocytes (FACS: >95%)
14	IgM	Connective tissue: capsule and PVS
16	IgG	Basement membrane-associated connective tissue lining capsule, PVS, and blood vessels
17	IgG	Isolated Mϕ-like cells primarily in medulla, and less in subcapsule, capsule and PVS
28	IgG	Isolated Mϕ-like cells primarily in medulla, and less in cortex

During this workshop, 25 mAb were analysed on their immunohistological staining patterns of reference thymus, thymuses during ontogeny, a variety of other organs and thymuses of other species. Based upon this extensive test protocol, the mAb were subdivided into groups using CTES nomenclature (Clusters of Thymic Epithelial Staining Patterns). A summary of the major findings from this meeting, including four of the mAb presented here (MTS 7, 9, 10 and 33) have been published in a report of the meeting (Kampinga *et al.*, 1989). These four mAb have been presented here in full detail, along with 12 other mAb, seven of which have been assigned CTES classifications.

MTS 39 (CTES I) stained the majority of TEC in subcapsule, cortex and medulla, displaying uniform intensity throughout (Fig. 1A). A consistent finding with both this mAb and anti-keratin was the identification of large epithelial cell-free (ECF) regions within the thymic parenchyma (Fig. 1B). These were primarily observed within the cortex and were clearly distinct from the perivascular spaces (PVS) which are frequently located at the cortico-medullary junction. Extensive expression of both CD4 and CD8 by the majority of cells within these regions (D.I. Godfrey, unpublished observations) suggested a predominance of immature, double positive thymocytes.

MTS 10 (CTES II; Fig. 1C) clearly illustrated the antigenic similarity between Type 1 epithelium lining the thymic capsule and PVS, and the majority of medullary TEC as previously reported in the human (Haynes, 1984; De Maagd *et al.*, 1985) and in the rat thymus (Kampinga *et al.*, 1987; Colic *et al.*, 1988). This mAb also reacted with the stratum basal of the epidemis. Contrasting this MTS 10 thymic staining pattern, cortical reticular epithelium was labelled by MTS 44 (CTES III.B; Fig. 1D); a minor subpopulation of medullary epithelial cells was also positive, whilst the majority of Type 1 epithelium was negative. The extent to which the medulla and subcapsule stained increased with increased concentrations of mAb. This marker was also present on the epidermal stratum lucidium.

MTS 32 (CTES III.C2) stained cortical epithelial cells and thymocytes (Fig. 1E). Shared antigenicity was further illustrated by both flow cytometric analysis and staining of cultured TEC (Tuček, Boyd & Hiai, 1989). This mAb stained greater than 95% of thymocytes, although in tissue section analysis the medulla appeared to be virtually negative. The actual degree of cortical TEC staining was difficult to determine, due to masking by the brightly stained thymocytes. MTS 32 also stained lymphocytes in both the spleen and lymph node, as determined by tissue section analysis. Preliminary flow cytometric results identified these cells as T lymphocytes (C. L. Tuček, D. I. Godfrey, P. Hugo and R. L. Boyd, manuscript in preparation). Limited epithelial reactivity was displayed by MTS 32 on the stratum lucidium of the epidermis. This mAb also reacted extensively with the molecular and granular layers of the brain.

MTS 20 stained isolated cells or small clusters of TEC in the thymus medulla (Fig. 2A). Under high magnification (×320), this staining often appeared granular and extracellular, suggesting the mAb detected a secretory molecule. MTS 29 stained less frequent, isolated, medullary epithelial cells (Fig. 2B). The antigen was also present on epidermal epithelium (stratum granulosum, lucidium and spinosum). (Medullary epithelial staining is categorized CTES IV. Since these mAb detect subpopulations of medullary epithelium, a new category is proposed, CTES IV.A.)

The final group of mAb defined by CTES nomenclature has been classified XX, additional information being required before definite groups can be established. Group XX.c mAb detect markers on a minority of cortical and medullary TEC. MTS 7 labelled isolated epithelial and KNC in the cortex and,

Table 3. Non-thymic reactivity

MTS	39	10	44	32	20	29	7	9	37	33	35	17	28
Spleen	(1)	(5)	4	4	—	—	4	2, 4	2, 4	2	4	4	(2)
Lymph node	(1)	5	4	4	—	—	4	2, 4	2, 4	2	(4)	4	(2)
Skin	1	1	(1)	1, (3)	1	1	—	1	1	1	—	3	5
Tongue	1,5	1	5	5	—	1	1	(5)	1, 5	1	—	3	5
Gut	1	1	5	5	—	—	1, 3, 6	(5), 6	5	5	5	1, 5	5
Uterus	1	—	ND	3	1	—	3	1	—	—	—	—	5
Vagina	(1)	1	(1)	—	1	5	1, 3, 6	6	1, 5	1	(1)	1, 5	5
Bladder	—	—	6	—	1	—	1, 3, 6	6	1, (6)	1	—	—	5
Kidney	(1)	—	(6)	—	1	—	3	1	(1)	—	—	—	—
Lung	—	—	—	—	—	—	5	5	1	—	—	5	4
Thyroid	1	—	—	ND	—	—	3	—	1	—	—	ND	5
Pancreas	(1)	—	—	—	—	—	5	1	1	—	—	ND	5
Salivary gland	—	(1)	—	—	1	—	—	1	1	—	—	3	—
Heart	—	—	—	—	—	—	—	ND	—	—	—	—	—
Brain	—	—	5	7	—	5	7	—	5	—	—	—	5
Sciatic	—	—	—	—	—	—	7	—	(3)	—	—	—	5
Adrenal	5	—	—	—	—	—	(3)	—	—	1	—	—	5
Trachea	1, 3	(1)	—	(3)	1	—	3	6	1, 5	1	—	—	5
Liver	—	—	—	—	—	—	5	1	1, 5	—	—	—	5

—, negative; 1, epithelium; 2, lymphocytes; 3, connective tissue; 4, reticulum; 5, isolated cells; 6, muscle; 7, neuronal cells; ( ), weak staining.

ND, not done.

more frequently, in the medulla (Fig. 2C). This mAb also stained the connective tissue components, these being the capsule and PVS. MTS 9 showed granular reactivity associated with TEC primarily in the medulla, and to a lesser degree in the cortex and subcapsule (Fig. 2D). Small pockets of cortical and most medullary thymocytes were also stained. This antigen was associated with lymphocytes and reticulum in the spleen and non-thymic glandular epithelium (for example: sweat glands, salivary glands, pancreas). Flow cytometric analysis, however, failed to demonstrate the expression of this antigen on the cell surface of lymphocytes.

MTS 37 (CTES XX.c) showed granular staining of isolated epithelial cells in the cortex and to a lesser extent the medulla. KNC were also stained in the medulla (Fig. 2E); these were frequently associated with PVS, often within the actual blood vessel lumen. Weak, granular staining of thymocytes was supported by flow cytometric analysis, demonstrating greater than 95% of these cells to be positive for MTS 37. Tissue section analysis showed most other epithelial tissues to express this antigen, as well as reticulum in the splenic red pulp and lymph node. Lymphocytes were also positive in these organs. Preliminary results have identified these to be primarily B lymphocytes and a minor subpopulation of T lymphocytes (C. L. Tucek *et al.*, manuscript in preparation). As in the thymus, isolated KNC were stained in several of the other organs tested. Peritoneal exudate cells (primarily Mϕ) were also positive.

The final CTES category (XX.d) represents antigens common to a minority of medullary TEC and cortical thymocytes. MTS 33 and MTS 35 fit this pattern although it is possible that cortical and subcapsular stromal cell staining may be masked by the cortical thymocytes. MTS 33 stained medullary epithelial cells that double-labelled strongly with anti-keratin (Fig. 2F). Flow cytometric analysis showed 85–90% of thymocytes to be MTS 33-positive. Non-thymic tissue section reactivity was limited to only a few epithelial tissues, including all epidermal layers, except stratum corneum. MTS 35 stained small, isolated medullary epithelial cells and cortical thymocytes, the brighter thymocyte staining occurring in the outer cortex (Fig. 2G). As shown by flow cytometric analysis, 75–80% of these cells expressed MTS 35.

Non-epithelial thymic markers

In addition to the mAb reactive with TEC, five were produced with specificity for keratin-negative stromal elements within the thymus. Of these, three detected stromal elements in association with the vasculature. Tissue section analysis with MTS 12 detected vascular endothelium throughout the thymus (Fig. 3A). Interestingly, although undetectable on sections, flow cytometric analysis demonstrated this antigen to be present on greater than 95% of thymocytes. MTS 14 stained the mesenchymal connective tissue in direct association with the vasculature, including the entire capsule and PVS (Fig. 3B,C). Directly adjacent to the MTS 14-positive connective tissue, MTS 16 labelled fibrous connective tissue associated with the basal laminae lining the blood vessels, capsule and PVS (Fig. 3D).

MTS 17 detected keratin-negative, Mϕ-like cells in the thymus medulla and, to a lesser degree, the capsule, subcapsule and PVS (Fig. 4A). These cells were often observed in very close association with the epithelial cells and, in some instances, the staining appeared to overlap with isolated TEC. Similar KNC were detected by MTS 28 (Fig. 4B); although analysis of sequential sections showed most of these cells to be distinct from MTS 17-positive cells, they were also more frequent in the cortex. Isolated cells were also detected by these mAb in other tissues, including reticular cells of the splenic red pulp and

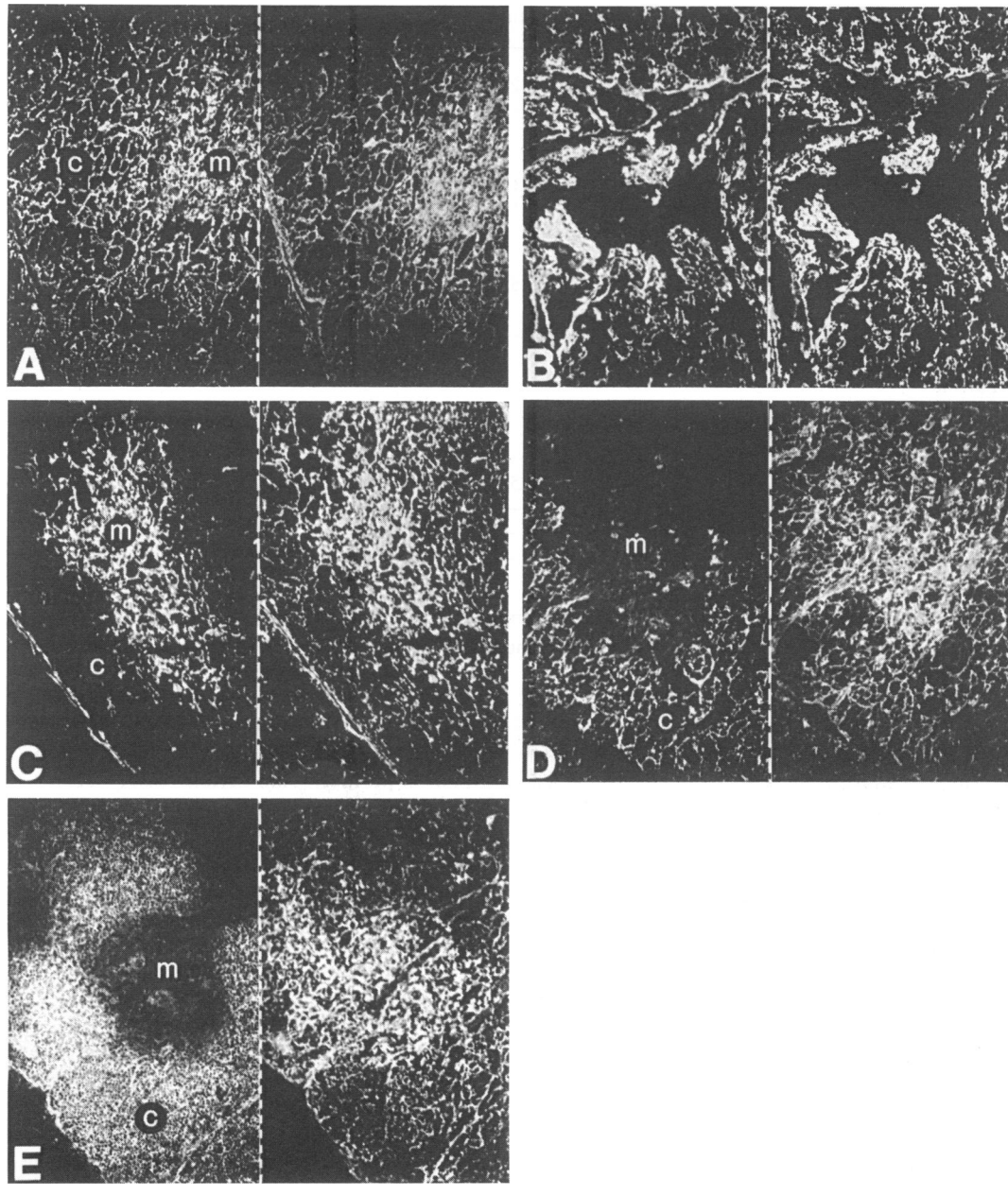

Figure 1. IIF staining. Left panel: mAb; right panel: anti-keratin. c, cortex; m, medulla. MTS 39 stained pan TEC (A; × 120). Large ECF regions were detected by MTS 39 and anti-keratin (B; × 120). MTS 10 labelled subcapsular and medullary epithelium (C; × 120). This pattern was contrasted by MTS 44, which stained cortical epithelium, isolated medullary epithelial cells, but was negative on the subcapsule (D; × 120). Cortical epithelial cells and thymocytes were labelled by MTS 32 (E; × 120).

lymph node cortex, and alveolar Mϕ in the lung. This mAb, and to a lesser degree MTS 28, also stained peritoneal Mϕ.

DISCUSSION

The main purpose of this study has been to phenotypically characterize the heterogeneous mouse thymic stroma, with the ultimate aim of identification, isolation and purification of individual subpopulations, and/or the antigens thereof.

Sixteen mAb were produced and grouped into two broad categories: (i) thymic epithelial markers and (ii) non-epithelial thymic markers. MTS 39 (CTES I) detected the majority of epithelial cells in the thymus. This mAb showed a previously unreported distribution, staining epithelium with uniform intensity throughout the subcapsule, cortex and medulla. The ECF regions detected by MTS 39 and anti-keratin in the thymus reflect MHC antigen-negative/reticular cell-free regions, illustrated by Van Ewijk, Rouse & Weissman (1980) and Van Ewijk (1984). Following the observation by this laboratory that these regions increase in thymuses during ontogeny or reconstitution following castration (Gourlay, unpublished results), it is

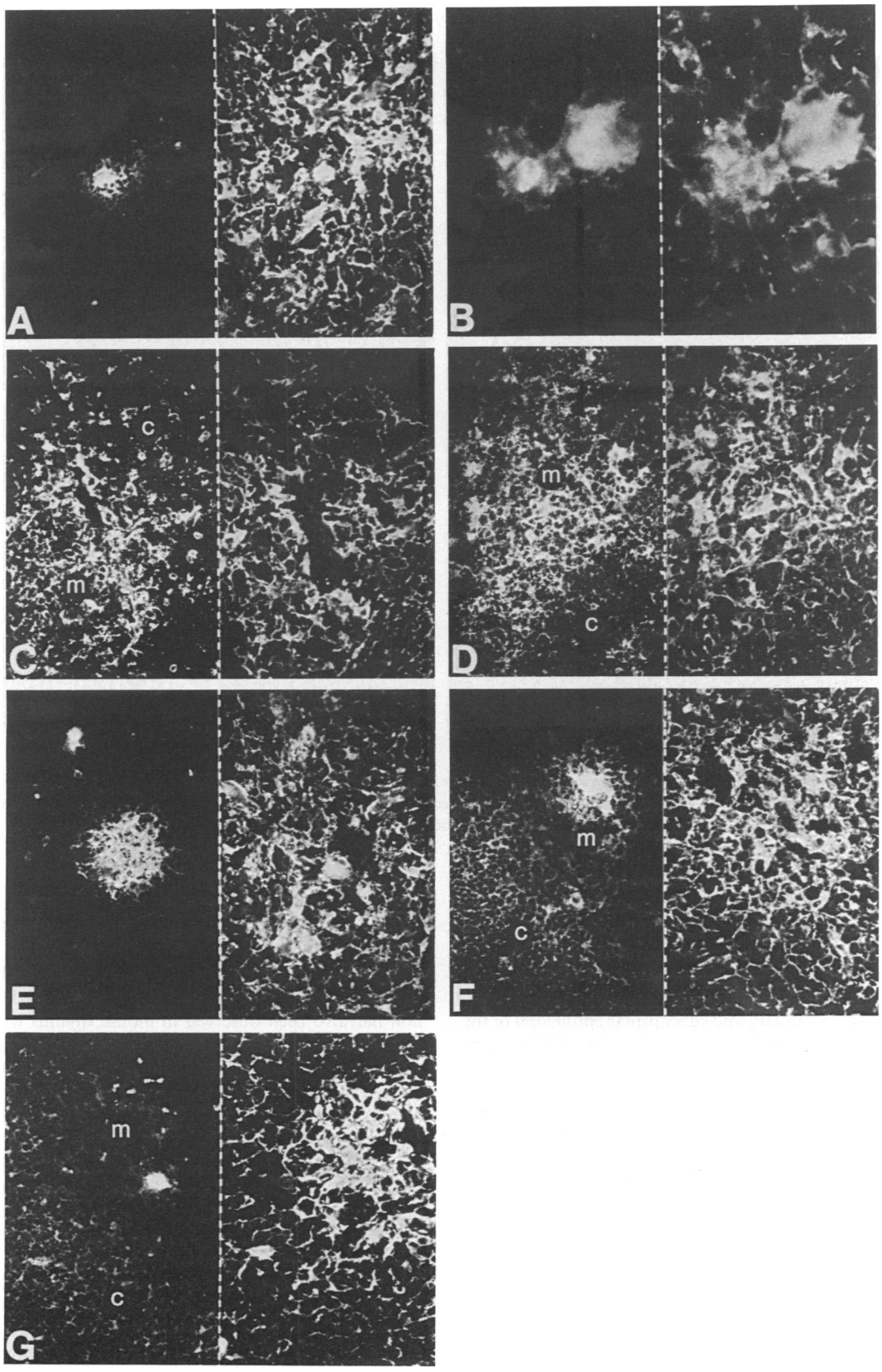

Figure 2. IIF staining. Left panel: mAb; right panel: anti-keratin. c, cortex; m, medulla. Medullary epithelial cell clusters morphologically resembling small HC were detected by MTS 20 (A; × 240; note staining encompasses surrounding cells) and MTS 29 (B; × 600). MTS 7 stained both isolated TEC and KNC in both the cortex and more frequently the medulla, as well as the capsule and PVS (C; × 240). MTS 9 showed granular staining associated with TEC and thymocytes primarily in the medulla, and less frequently in the cortex (D; × 240). MTS 37 showed diffuse granular staining of KNC and TEC (E; × 240). MTS 33 stained cortical thymocytes and medullary epithelial clusters (F; × 240), similarly, MTS 35 stained cortical thymocytes and isolated medullary epithelial cells (G; × 120).

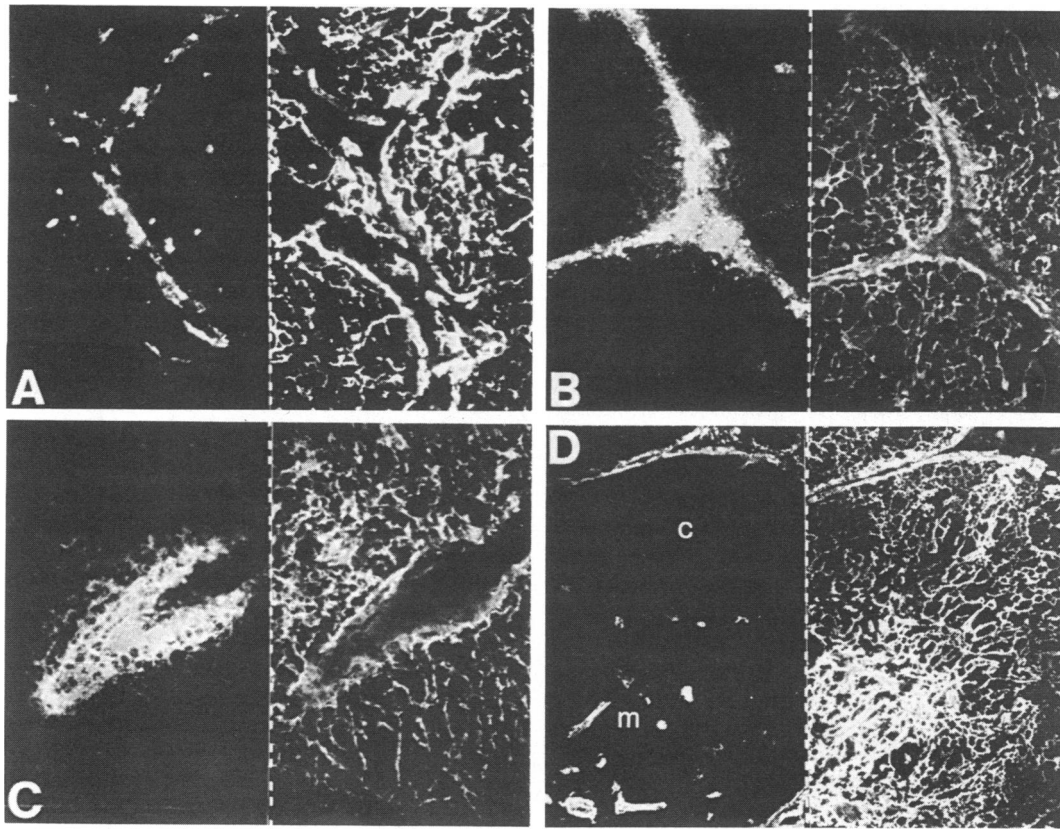

Figure 3. IIF staining. Left panel: mAb; right panel: anti-keratin. c, cortex; m, medulla. MTS 12 stained capillaries in the thymus (A; ×240). MTS 14 showed diffuse granular reactivity with the connective tissue of the capsule (B; ×240) and PVS (C; ×240). MTS 16 labelled connective tissue associated with the basal laminae of blood vessels and epithelial cells lining the capsule and PVS (D; ×120).

hypothesized that they represent isolated pockets of proliferating, immature thymocytes awaiting interaction with the thymic stroma.

MTS 10 (CTES II) and MTS 44 (CTES III.B) illustrated the two major epithelial subpopulations within the thymus. The former stained the medullary and subcapsular epithelium of the thymus, closely resembling mAb produced against the human: anti-p19, A2B5, TE4 (Haynes, 1984), MR10 and 14 (De Maagd *et al.*, 1985); rat: HIS 39 (Kampinga *et al.*, 1987) and RMC 18 and 19 (Colic *et al.*, 1988); and the mouse thymus: ICV4 (presented by M. Ritter at the 1989 Rolduc Thymic Epithelial Workshop and summarized in Kampinga *et al.*, 1989). Contrasting this, MTS 44 was reactive with predominantly cortical and to a much lesser extent medullary and subcapsular epithelium. This pattern has previously been described in the rat (HIS 37; Kampinga *et al.*, 1987) and is similar to ER-TR4 in the mouse (Van Vliet *et al.*, 1984). Interestingly, MTS 10 and MTS 44 stained two distinct layers of epidermal epithelium, the differentiation of which has been well delineated (Doran, Vidrich & Sun, 1980; Watt & Green, 1982). The least differentiated stratum basale was positive for MTS 10, whilst the highly differentiated stratum lucidium was detected by MTS 44. This implies that the subcapsular and the majority of medullary epithelial cells are less differentiated than that of the cortex and a subpopulation of isolated medullary epithelial cells. These subcapsular/medullary epithelial cells may act as a source of epithelial cell progenitors in the adult, since they are the first cells to regenerate in thymic grafts (J. F. A. P. Miller, personal communication).

MTS 32 (CTES III.C2), demonstrated shared antigenicity between thymocytes and cortical epithelial cells. This phenomenon has also been observed in mouse thymus with mAb 6C3 (Adkins, Tidmarsh & Weissman, 1988) and in the human thymus with MR3 (Larché, Ladyman & Ritter, 1987). MTS 32 stained greater than 95% of thymocytes yet, on section, medullary thymocytes appeared to be negative. This may indicate that the antigen on medullary thymocytes is masked *in vivo*, perhaps reflecting a receptor–ligand interaction. Alternatively, the immunohistochemistry technique may be too insensitive to detect the staining of these cells. Detailed flow cytometric studies of all MTS mAb reactive with thymocytes are in progess.

The CTES IV.A markers (MTS 20 and 29) demonstrated some of the complex microspecificities occurring in the thymus microenvironment. The isolated MTS 20-positive epithelial cells were often observed in close association with thymocytes, via their processes and/or secreted products. An intriguing possibility is that these events depict individual steps in T-lymphocyte differentiation. MTS 29 stained a highly restricted and infrequent subpopulation of TEC, usually associated with small, strongly keratin-positive clusters of medullary epithelium (morphologically similar to HC). These cells were apparently related

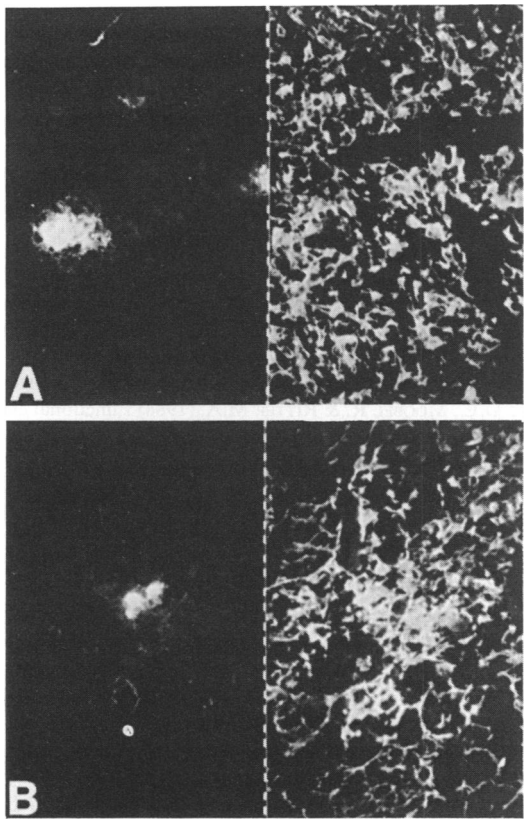

Figure 4. IIF staining. Left panel: mAb; right panel: anti-keratin. Keratin-negative, Mϕ-like cells, primarily in the thymus medulla were stained by MTS 17 (A; × 240), and MTS 28 (B; × 240).

to the more differentiated layers of epidermal epithelium (excluding the terminally differentiated stratum corneum). Considering that the most highly differentiated epithelial cells in the human thymus are associated with HC (Lobach *et al.*, 1985), it follows that these medullary epithelial cells may be associated with the mouse equivalent of HC, or at least a highly differentiated subpopulation of TEC.

The CTES XX.c markers detect a minority of cortical and medullary TEC. Of these, MTS 7 also reacted with KNC in the thymic cortex and medulla, and also the capsule and PVS, showing a resemblance to ER-TR6 (Van Vliet *et al.*, 1984). The major difference, however, was that, unlike MTS 7, ER-TR6 was positive on medullary lymphocytes and negative on cortical epithelial cells. MTS 9 displayed granular reactivity with an epithelial subpopulation in the thymus, as well as thymocytes primarily in the medulla. This antigen was also associated with peripheral lymphocytes and glandular epithelium. The lack of reactivity using flow cytometry suggests that this antigen may be a soluble factor associated with, but not bound to, the lymphocytes or, alternatively, it may be an internal lymphocyte molecule. The third mAb from this category (MTS 37) also stained thymocytes and KNC. The expression of this antigen on Mϕ in the peritoneum and Mϕ-like cells in both spleen and lymph node suggests that the isolated KNC observed in the thymus and other tissues include a population of these cells.

MTS 33 and MTS 35 (CTES XX.d) both detected antigens shared between thymocytes and epithelial cells. For MTS 33, this shared antigenicity was reflected on non-thymic tissues, being expressed on both lymphocytes and epithelium. All epidermal layers, apart from the stratum corneum, were also positive. Similar to MTS 29, the distribution of this antigen in the thymic medulla and on the highly differentiated epidermal layers suggests that the isolated thymic medullary clusters stained by this mAb may be the mouse equivalent of HC. The antigen detected by MTS 35 was largely restricted to the thymus (cortical lymphocytes and isolated medullary epithelial cells), although weak reactivity was also observed with lymphoid reticulum. Further, MTS 35 and MTS 37 are both of particular interest, as recent experiments (Waanders, Godfrey & Boyd, 1989) have shown that masking of the epitopes with these mAb in fetal thymic organ cultures can significantly modulate T-lymphocyte differentiation *in vitro*.

In addition to the thymic epithelial markers, five mAb were specific for non-epithelial cells in the thymus. MTS 12, 14 and 16 collectively stain the three main layers of the blood–thymus barrier. Before cells/protein can cross this barrier, they must pass the vascular endothelium (stained by MTS 12), the connective tissue within PVS (MTS 14) and the basement membrane lining these regions (MTS 16). MTS 14 and MTS 16 display similar reactivity to ER-TR7 in the mouse (Van Vliet *et al.*, 1984) and TE7 in the human (Haynes, 1984), both being reactive with mesodermal-derived thymic stroma. The staining pattern of MTS 14 was less fibrous, more diffuse and granular, suggesting the detection of a secretory product produced by these stromal cells. MTS 16 stained the basal lamina-associated connective tissue, lining blood vessels, capsule and PVS, identical in distribution to type IV collagen or laminin (Berrih, Savino & Cohen, 1985).

MTS 17 and MTS 28 stained isolated Mϕ-like cells in most tissues tested. It is therefore likely that the KNC observed in the thymus represent subpopulations of Mϕ or related cells, although the distribution of these cells in the thymus does not directly follow either anti-Mac-1 or anti-Fc receptor (data not presented). It was difficult to ascertain whether the occasional staining of isolated TEC by these mAb was real, or simply an artefact of two overlapping cells within the tissue section.

Collectively, the mAb described in this paper complement and markedly expand the complex heterogeneity described for thymic stromal cells. While some of these determinants may play a more structural role in the thymus architecture, this study illustrates unequivocably that there are indeed complex and varied microenvironments existing within the thymus. The multiplicity of thymocyte subpopulations is at least matched, if not surpassed, by that of the thymic stromal cell subpopulations and emphasizes the need for reclassification of these cells. It is now apparent that the assignment of function (such as positive and negative selection of thymocytes) to broad thymic stromal populations such as cortical epithelium or Mϕ/IDC may be too simplistic. It is hypothesized that the varied microenvironments comprised by these distinct stromal cell types are reflected by the many different thymocyte subpopulations that exist at some stage in the thymus, and the responsiveness of this organ to exogenous blood-borne stimuli.

ACKNOWLEDGMENTS

The authors wish to thank Mr M. A. Malin for preparation of black and white photographs for this paper; and Dr G. W. Dandie of our

department for assistance in devising the popliteal lymph node immunization method.

This work was supported by grants from the National Health and Medical Research Council (No. 860396) and the Anti-Cancer Council of Victoria (No. 131658).

REFERENCES

ADKINS B., TIDMARSH G.F. & WEISSMAN I.L. (1988) Normal thymic cortical epithelial cells developmentally regulate the expression of a B lineage transformation-associated antigen. *Immunogenetics*, **27**, 180.

BERRIH S., SAVINO W. & COHEN S. (1985) Extracellular matrix of the human thymus: immunofluorescence studies on frozen sections and cultured epithelial cells. *J. Histochem. Cytochem.* **33**, 655.

COLIC M., MATANOVIC D., HEGEDIS L. & DUJIC A. (1988) Immunohistochemical characterization of rat thymic non-lymphoid cells. I. Epithelial and mesenchymal components defined by monoclonal antibodies. *Immunology*, **65**, 277.

DE LA HERA A., MARSTON W., ARANDA C., TORIBIO M.L. & MARTINEZ A.C. (1989) Thymic stroma is required for the development of human T cell lineages *in vitro*. *Int. Immunol.* **1**, 471.

DE MAAGD R.A., MACKENZIE W.A., SCHUURMAN H.-J., RITTER M.A., PRICE K.M., BROEKHUIZEN R. & KATER L. (1985) The human thymus microenvironment: heterogeneity detected by monoclonal anti-epithelial cell antibodies. *Immunology*, **54**, 745.

DENNING S.M., KURTZBERG J., LE P.T., TUCK D.T., SINGER K.H. & HAYNES B.F. (1988) Human thymic epithelial cells directly induce activation of autologous immature thymocytes. *Proc. natl. Acad. Sci. U.S.A.* **85**, 3125.

DORAN T.I., VIDRICH A. & SUN T.-T. (1980) Intrinsic and extrinsic regulation of skin, corneal, and esophageal epithelial cells. *Cell*, **22**, 17.

GALFRE G., HOWE S.C., MILSTEIN C., BURCHER G.W. & HOWARD J.C. (1977) Antibodies to major histocompatibility antigens produced by hybrid cell lines. *Nature (Lond.)*, **266**, 550.

GODING J.W. (1983) *Monoclonal Antibodies: Principles and Practice*. (ed. J. W. Goding) Academic Press, London.

HAYNES B.F. (1984) The human thymic microenvironment. *Adv. Immunol.* **36**, 87.

IZON D.I. & BOYD R.L. (1990) The cytoarchitecture of the human thymus detected by monoclonal antibodies. *Human Immunol.* **27**, 160.

KAMPINGA J., BERGES S., BOYD R., BREKELMANS P., COLIC M. & VAN EWIJK W. (1989) Thymic epithelial antibodies: immunohistological analysis and introduction of CTES nomenclature. *Thymus*, **13**, 165.

KAMPINGA J., KROESE F.G.M., DUIJVESTIJN A.M., MURAWSKA M.B., POL G.H. & NIEUWENHUIS P. (1987) The rat thymus microenvironment: Subsets of thymic epithelial cells defined by monoclonal antibodies. *Trans. Proc.* **19**, 3171.

KENDALL M.D. (1981) The cells of the thymus. In: *The Thymus Gland*. (ed. M. D. Kendall), p. 63. Academic Press Inc., London.

KENDALL M.D. (1988) Anatomical and physiological factors influencing the thymic microenvironment. In: *Thymus Update. 1. The Microenvironment of the Human Thymus* (eds M. D. Kendall and M. A. Ritter), p. 5. Harwood Academic Publishers, London.

LAMPERT I.A. & RITTER M.A. (1988) The origin of the diverse epithelial cells of the thymus: is there a common stem cell? In: *Thymus Update. 1. The Microenvironment of the Human Thymus* (eds M. D. Kendall and M. A. Ritter), p. 5. Harwood Academic Publishers, London.

LARCHÉ M., LADYMAN H. & RITTER M.A. (1987) Human thymic epithelial cells and lymphocytes may share common epitopes. In: *Leukocyte Typing III* (eds A. McMichael, P. Beverley, N. Hogg and M. Horton), p. 257. Oxford University Press, Oxford.

LARCHÉ M., LAMB J.R., O'HEHIR R.E., IMAMI-SHITA N., ZANDERS E.D., QUINT D.E., MOQBEL R. & RITTER M.A. (1988) Functional evidence for a monoclonal antibody that binds to the human IL-4 receptor. *Immunology*, **65**, 617.

LOBACH D.F., SCEARCE R.M. & HAYNES B.F. (1985) The human thymic microenvironment. Phenotypic characterization of Hassall's bodies with the use of monoclonal antibodies. *J. Immunol.* **134**, 250.

STUTMAN O. (1978) Intrathymic and extrathymic T cell maturation. *Immunol. Rev.* **42**, 138.

TUCEK C.L., BOYD R.L. & HIAI H. (1989) Antigens shared by thymic stromal cells and T lymphocytes are abnormally expressed in AKR thymuses. *Thymus*, **14**, 95.

VAN DE WIJNGAERT F.P., KENDALL M.D., SCHUURMAN H.-J., RADEMAKERS L.H.P.M. & KATER L. (1984) Heterogeneity of epithelial cells in the human thymus. An ultrastructural study. *Cell Tissue Res.* **237**, 227.

VAN EWIJK W. (1984) Immunohistology of lymphoid and non-lymphoid cells in the thymus in relation to T lymphocyte differentiation. *Am. J. Anat.* **170**, 311.

VAN EWIJK W., ROUSE R.V. & WEISSMAN I.L. (1980) Distribution of H-2 microenvironments in the mouse thymus. Immunoelectron microscopic identification of I-A and H-2K bearing cells. *J. Histochem. Cytochem.* **28**, 1089.

VAN VLIET E., MELIS M. & VAN EWIJK W. (1984) Monoclonal antibodies to stromal cell types of the mouse thymus. *Eur. J. Immunol.* **14**, 524.

WAANDERS G.A., GODFREY D.I. & BOYD R.L. (1989) Modulation of T-cell differentiation in murine fetal thymus organ cultures. *Thymus*, **13**, 73.

WATT F.M. & GREEN H. (1982) Stratification and terminal differentiation of cultured epidermal cells. *Nature (Lond.)*, **295**, 434.