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The human thymus microenvironment: heterogeneity detected by monoclonal anti-epithelial cell antibodies

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Summary. Monoclonal antibodies were raised against human thymus stromal cells and their specificity for the epithelial component of thymus stroma assessed by double immunofluorescence using anti-keratin antibodies to identify epithelium. Our monoclonal antibodies identify six distinct patterns of epithelial cell antigen expression within the thymus: (1) pan epithelial (antibody IP1); (2) cortex (MR3 and MR6); (3) cortical/medullary junction (IP2); (4) subcapsule and subpopulation of medulla (MR10/MR14); (5) Hassall's corpuscles and adjacent subpopulation of medulla (IP3); (6) Hassall's corpuscles only (MR13/IP4). This heterogeneity of antigen expression suggests that many different epithelial microenvironments exist within the human thymus.

INTRODUCTION

It is well established that the supporting meshwork of the thymus, which is composed mainly of epithelial cells, plays an important part in T lymphocyte maturation (Janossy *et al.*, 1983). Induction of this maturation requires both direct contact between lymphocyte progenitors and thymus stroma as well as the production of soluble thymic hormones by the thymus epithelium (Gelfand *et al.*, 1980). T lymphocyte maturation involves a series of changes in cell surface molecules, with lymphocytes at different stages occupying distinct locations within the thymus (Janossy *et al.*, 1983; Bahn *et al.*, 1980). Such shifts in cell location with differentiation suggest a series of distinct microenvironments within the thymus, each relevant to the induction of a particular stage in T lymphocyte development.

Most studies of thymus lymphocyte development have concentrated on the cell surface molecular characteristics of T-cell subpopulations. However, other work has focussed on the microenvironment, indicating heterogeneity within the epithelial component of the thymus. Morphological differences have been seen by both light and electron microscopy (Arya *et al.*, 1982; Singh, 1981; Van de Wijngaert *et al.*, 1983, 1984) distinguishing between epithelial cells of the subcapsular, cortical and medullary areas, while differences in their secretory capacity have been demonstrated by immunohistochemistry using antisera to thymic hormones (Hirokawa, McClure & Goldstein, 1983; Kater *et al.*, 1979; Savino *et al.*, 1982; Schuurman *et al.*, 1984). In addition, immunohisto-

Abbreviations: FITC, fluorescein isothiocyanate; HAT, hypoxanthine-aminopterin-thymidine; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid; Ig, immunoglobulin; PBS, phosphate-buffered saline; TRITC, tetramethyl rhodamine isothiocyanate; FCA, Freund's complete adjuvant; HRP, horseradish peroxidase.

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chemical staining for known antigens (that is, previously defined in another system) such as Thy-1 (Ritter, Sauvage & Cotmore, 1981), GQ-ganglioside (Haynes, Shimizu & Eisenbarth, 1983b), human T leukaemia virus antigen p19 (Haynes *et al.*, 1983a) and HLA-DR (Bahn *et al.*, 1980) have revealed further heterogeneity.

In this study, we have deliberately raised monoclonal antibodies directly to human thymic stromal cells and have used these to analyse unknown molecular diversity within the thymus microenvironment. Antibodies were produced in both London and Utrecht laboratories, and together these show differential patterns of antigen expression by epithelial cells at different locations within the human thymus.

MATERIALS AND METHODS

Source of tissues

Thymus tissue was obtained from children undergoing cardiac surgery (age range: 2 months–6 years). Other tissues (lymph node, tonsil, spleen, gut, kidney, brain and skin) were from either biopsy or fresh post-mortem material.

Preparation of stromal cells (Utrecht protocol)

Thymus tissue was thoroughly minced. Small explants were washed by repeated sedimentation in HEPES (25 mM) buffered RPMI 1640 (Gibco, Grand Island, NY) and supernatant thymocytes discarded. Explants were then digested in 1·2 mg/ml collagenase in HEPES-buffered RPMI (Worthington, Millipore Corp., 131 U/mg) for 1 hr at 37°, followed by four treatments in 1·75 mg/ml Dispase (protease, neutral, grade 11, Boehringer Mannheim) for 30 min at 37°. After each treatment, the cells in suspension were harvested, pooled and washed three times in Ca/Mg free Hanks' balanced salt solution (Gibco). All media used in enzyme digestion and washing contained 0·02 mg/ml DNAse (deoxyribonuclease 1, grade 11, Boehringer Mannheim). Next the cells were centrifuged over isotonic Percoll (Pharmacia, Uppsala, Sweden) in Hanks' (d = 1·066g/cm^3) for 30 min at 390 g. Interface cells were harvested, and Giemsa-stained cytocentrifuge smears showed the majority of these to be large with abundant cytoplasm—including lymphoblastoid cells, macrophages, interdigitating-like cells and epithelial-like cells.

Preparation of monoclonal antibodies

These were raised independently in London and Utrecht. Immunization protocols differed in the two laboratories, but subsequent fusion, screening and cloning techniques were essentially the same.

London immunization. (BALB/c × CBA)F_1 female mice were injected i.p. with 0·5 ml homogenized human thymus stroma (prepared after excess thymocytes had been removed by teasing) with Freund's complete adjuvant (FCA) in phosphate-buffered saline (PBS). This was repeated twice without FCA, at 3-weekly intervals. Fusion (see below) was done 3 days after the last immunization.

Utrecht immunization. BALB/c female mice were immunized i.p. with 0·3 ml of a suspension containing 2×10^7 stromal cells (see above) and 3×10^9 killed *Bordetella Pertussis* particles (National Institute for Public Health, Bilthoven, The Netherlands) in physiological saline. This was repeated after 2–3 weeks and the fusion performed 3–4 days later.

Fusion. Spleen cells from immunized mice were fused with X63-Ag8.653 non-producer mouse myeloma cells (Kearney *et al.*, 1979) using polyethylene glycol and plated out into 96-well Nunclon microtest plates (Gibco) with mouse peritoneal macrophages as feeders. Hybridomas were selected for in hypoxanthine-aminopterin-thymidine (HAT) medium (Kohler & Milstein, 1975). Supernatants were screened by indirect immunofluorescence or immunoperoxidase on frozen sections of thymus (see below) and positive hybridomas cloned three times by limiting dilution (0·3 cells/well). Positive clones were expanded into 24-well Linbro plates (Flow Laboratories, Irvine, Scotland) and Nunclon flasks (Gibco). Supernatants were collected and stored at either −70° or +4° with 0·02–0·05% sodium azide. The isotype of each monoclonal was determined by immunofluorescence on cytocentrifuge preparations of antibody-secreting hybridoma cells, using subclass-specific antibodies (see below).

Other antibodies

A polyclonal rabbit anti-human keratin antibody (Dakopatts, Copenhagen, Denmark) was used in indirect immunofluorescence with either TRITC-F(ab')$_2$ sheep anti-rabbit Ig (anti-human Ig crossreactivity removed by affinity chromatography) or with TRITC-goat anti-rabbit Ig (GAR/TRITC, Nordic

Immunological Laboratories, Tilburg, The Netherlands). Monoclonal supernates were used in indirect immunofluorescence with FITC-F(ab') rabbit anti-mouse Ig, FITC-goat anti-mouse Ig or anti-mouse Ig (sub)class antibodies (GAM/FITC, GAM/IgM(Fc)/FITC, GAM/IgGl/FITC or GAM/IgG3/FITC, Nordic). For immunoperoxidase staining monoclonal supernates were followed by HRP-rabbit anti-mouse Ig antibody (Dakopatts, Copenhagen, Denmark).

Immunochemistry. Frozen sections (4–6 μm) were air-dried and fixed at room temperature in either acetone (10 min) or methanol (30 sec), followed by rinsing in PBS.

Immunoperoxidase staining. Sections were incubated with supernatant at optimal dilution (neat to 1:40) in PBS with 1% human serum albumin, followed by HRP-rabbit anti-mouse Ig at 1:40 in PBS with 2·5% (v/v) human AB serum, and then developed with 3-amino-9-ethylcarbazol (Aldrich Chemical Co., Milwaukee, WI) and H_2O_2 in 0·1 M acetate buffer, pH 4·6. Sections were mounted in gelatin (18% w/v)/glycerin (50% v/v)/phenol (1% w/v).

Immunofluorescence staining. Sections were incubated with monoclonal supernate for a minimum of 60 min, but usually overnight. This was followed by 20 μl of FITC-F(ab')$_2$ rabbit anti-mouse Ig or FITC-goat anti-mouse Ig (subclass) for 45–60 min. All washes were in PBS. Sections were mounted in PBS/glycerol (9:1) and read using a fluorescence microscope equipped with epi-illumination optics (Leitz Ortholux II, with XB075 illumination and a I2 or K2 (FITC) and N2 (TRITC) filter combination).

Double immunofluorescence staining. Sections were incubated with reagents in the following sequence: monoclonal antibody; FITC-goat anti-mouse Ig(subclass) antibody; rabbit anti-keratin; TRITC-goat anti-rabbit Ig. An alternative sequence was: monoclonal antibody; rabbit anti-keratin; TRITC-F(ab')$_2$ sheep anti-rabbit Ig; normal rabbit serum to block any unsaturated binding sites on the sheep anti-rabbit Ig; FITC-F(ab')$_2$ rabbit anti-mouse Ig. Both procedures gave the same results.

Specificity of labelling. No staining was seen when either primary layer antibodies were omitted (monoclonal antibodies or rabbit anti-keratin), or when the first layer antibody was replaced with an irrelevant mouse monoclonal or normal rabbit Ig.

RESULTS

Monoclonal antibodies: range of specificites raised

Both immunization/fusion protocols led to successful hybridomas secreting antibodies that stained stromal cells in immunohistochemistry on frozen sections of thymus. In addition, many other hybridomas produced antibodies which bound to connective tissue and blood vessels. However, only immunization with enriched thymic stromal cells (Utrecht immunization) yielded appreciable numbers of supernates that were also reactive with lymphoid cells. These gave staining patterns that looked like those of either MHC Class I or II antigens.

Hybridomas secreting antibodies that stained only stromal cells were cloned three times. Those that retained antibody secreting capacity were then tested in double immunofluorescence with anti-keratin antibody (to identify epithelial cells). Six main patterns of binding to epithelial cells were observed (Table 1, Figs 1 and 2). Certain monoclonal antibodies (MR3,MR6,MR13,IP4) also bound to some keratin-negative cells (Table 1, detailed below). Table 2 summarizes the binding of the monoclonals to non-thymic tissue. Antibodies with the prefix 'MR' were raised in London, those with the prefix 'IP' were raised in Utrecht.

Epithelial subpopulations

Although anti-keratin antibodies bound to all thymic epithelial cells, the intensity of immunofluorescent staining showed characteristic differences. Subcapsular and medullary epithelial cells (especially Hassall's corpuscles) showed intense labelling, while epithelial cells in the cortex labelled more weakly (Fig. 2).

Monoclonal antibody IP1 (Fig. 1a,b; Fig. 2a) binds to all epithelial cells of both cortex and medulla, although those in the cortex label more intensely. The pattern of staining is predominantly dendritic, suggesting that the antigen detected is located in the plasma membrane. No labelling of keratin-negative cells was seen. This antibody also binds to epithelium in tonsil, skin, gut and kidney, indicating that the antigen detected may be a general epithelial cell marker.

Monoclonal antibodies MR10 and MR14 (Fig. 1c,d; Fig. 2c) bind to subcapsular epithelium including

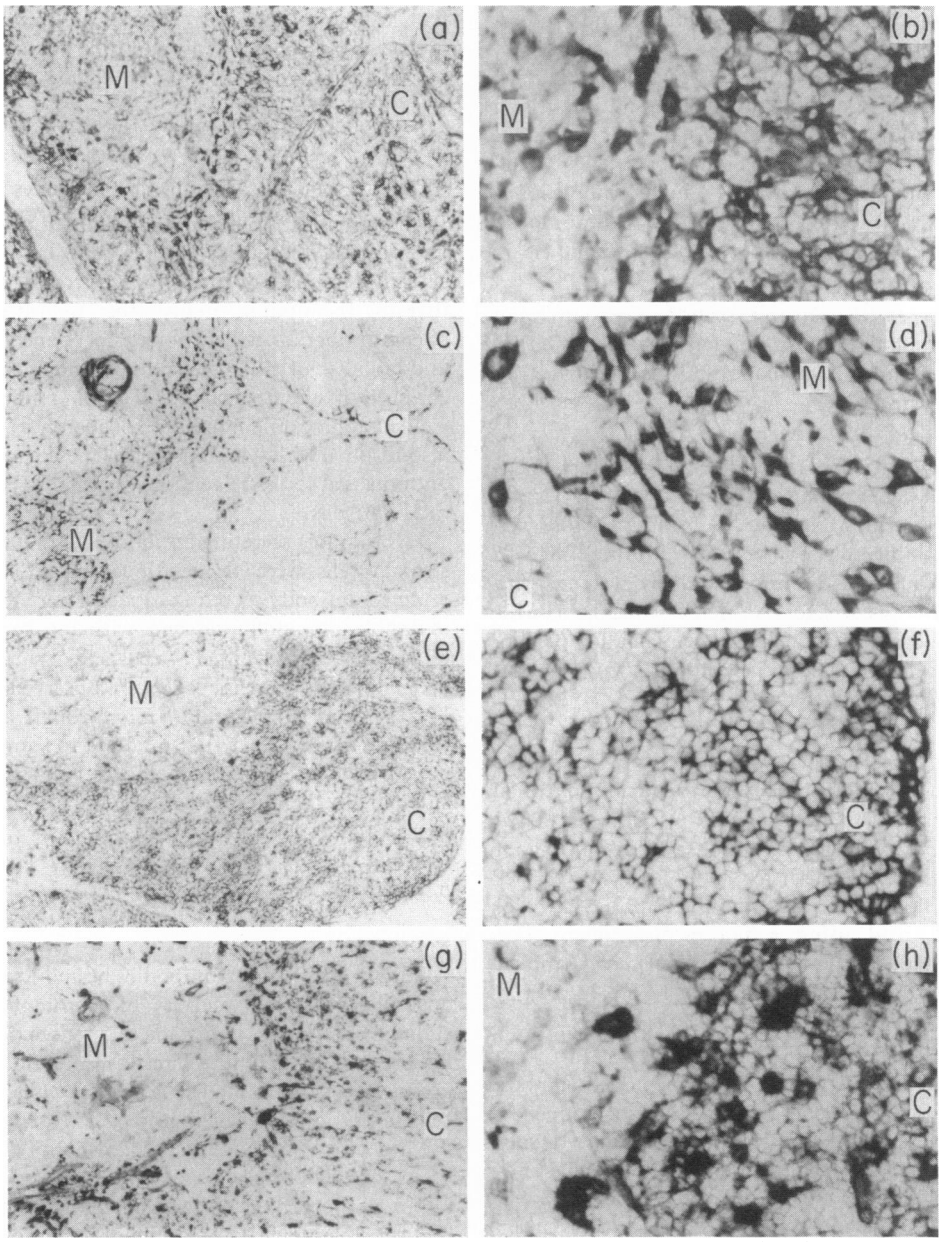

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Figure 1. Immunoperoxidase staining patterns of human thymus tissue sections given by monoclonal anti-epithelial cell antibodies. For each antibody, the staining is shown at × 60 (left) and × 300 (right) magnification. Cortex (C) and medulla (M) are indicated. (a) (b), IP1, [(b) shows cortico-medullary region]; (c) (d), MR10, [(d) shows cortico-medullary region]; (e) (f), MR 3, [(f) shows cortical area]; (g) (h), IP2, [(h) shows cortico-medullary region]; (i) (j), IP3, [(j) shows medullary area]; (k) (l), MR13, [(l) shows subcapsular area, with small cortex and medulla].

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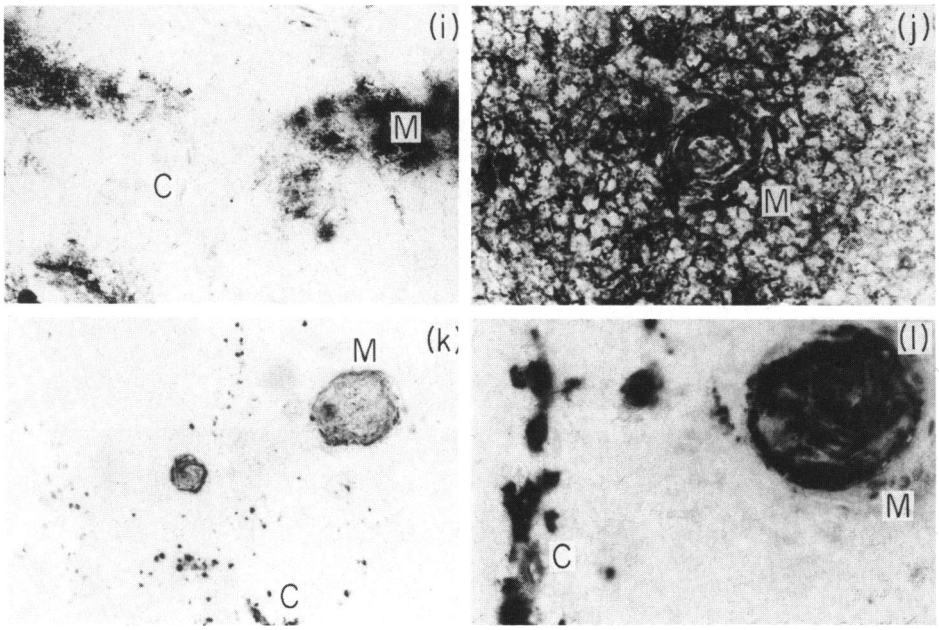

Table 1. Reactivity of monoclonal antibodies to human thymus epithelial cells

Code	Immunoglobulin class	Reactivity pattern†
IPl (U)*	IgM	Epithelial cells in cortex and medulla. Stains only keratin-positive cells.
MR10,MR14 (L)	IgM	Subcapsular epithelial cells and subpopulation of epithelial cells in medulla. Stains only keratin-positive cells.
MR3,MR6 (L)	IgG1	Epithelial cells in cortex (excluding subcapsular area). Stains keratin-negative cells in medulla.
IP2 (U)	IgG1	Some epithelial cells in cortex (predominantly at cortical-medullary junction and excluding subcapsular area). Stains only keratin-positive cells.
IP3 (U)	IgM	Hassall's corpuscles and adjacent epithelial cells. Stains only keratin-positive cells.
MR13 (L)	IgM	Hassall's corpuscles. Stains scattered keratin-negative cells in cortex, medulla and septae.
IP4 (U)	IgG1	

* Within parentheses, laboratory of preparation (Utrecht or London).

† Immunohistochemistry on frozen sections, including double immunofluorescence with anti-keratin antibody.

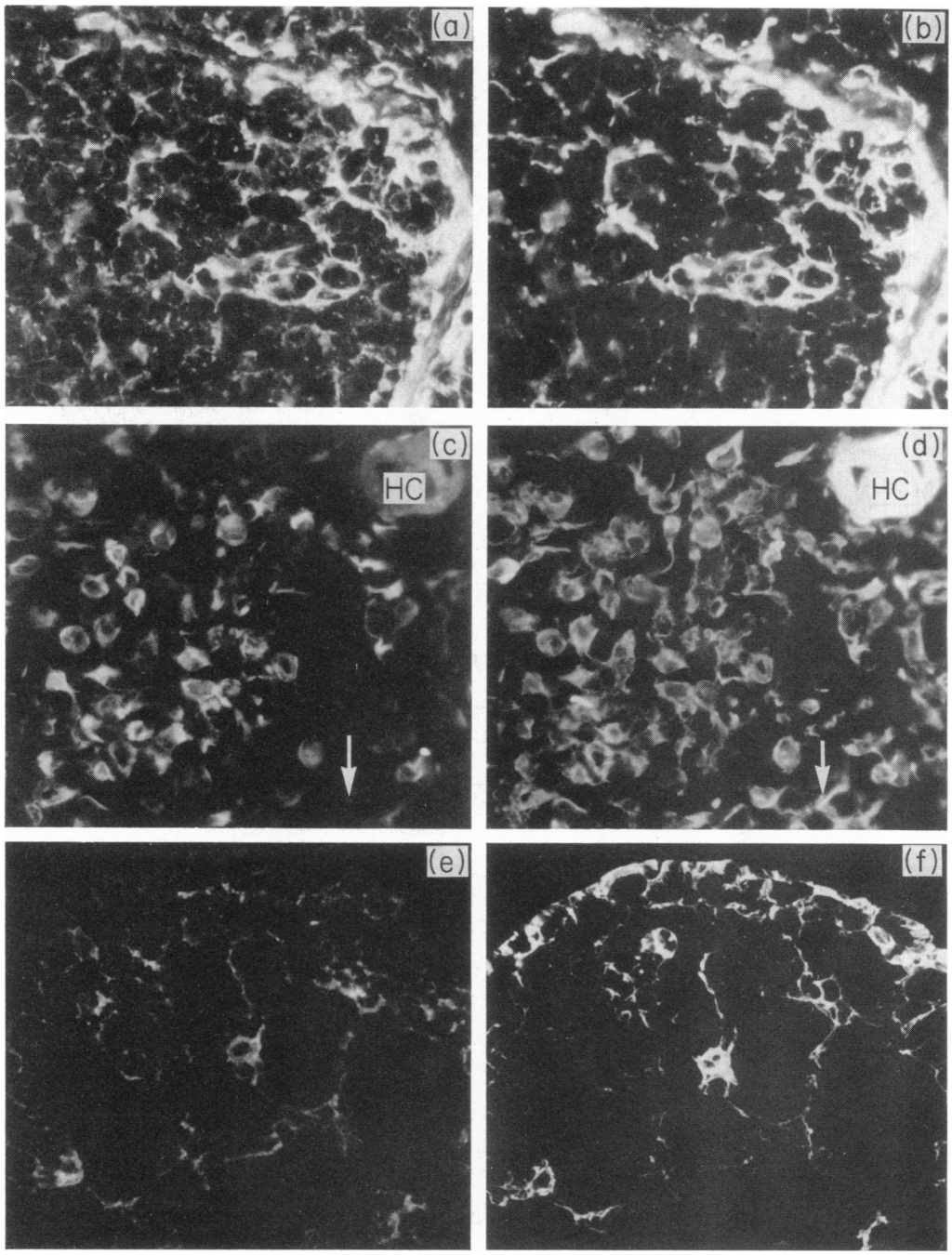

Figure 2. Double immunofluorescence with selected anti-epithelial monoclonal antibodies (left) and rabbit anti-human keratin antibody (right) on thymus tissue sections. Hassall's corpuscles are indicated (HC). (a) (b), IP1, anti-keratin, subcapsular area and cortex; IP1 stains all keratin-positive cells. Anti-keratin staining is strong on subcapsular but weak on cortical epithelial cells. (c) (d), MR14, anti-keratin, medullary area; MR14 stains only keratin-positive cells, giving strong staining of some but not all medullary epithelial cells (an MR10/14$^-$, keratin$^+$ cell is arrowed). Hassall's corpuscle (HC) is only weakly stained. (e) (f) MR3, anti-keratin, subcapsular area and cortex; MR3 stains keratin-positive cortical but not subcapsular epithelial cells. (Magnification × 450.)

Table 2. Reactivity of monoclonal antibodies to non-thymic tissues

Antibody	Lymph node	Tonsil	Spleen	Gut	Kidney	Skin	Brain
MR10	−	−	−	−	−	−	−
MR14							
MR3	+*	+*	+*	−	+	−	−
MR6							
IP2	−	−	−	−	+	−	−
IP3	−	+	−	+	−	+	−
MR13	+*	+*	+*	−	−	−	−
IP4							
IP1	−	+	−	+	+	+	−

+, positive cells which are epithelial.
+*, positive cells which belong to haemopoietic lineages: MR3/6—macrophage; MR13/IP4—neutrophil.

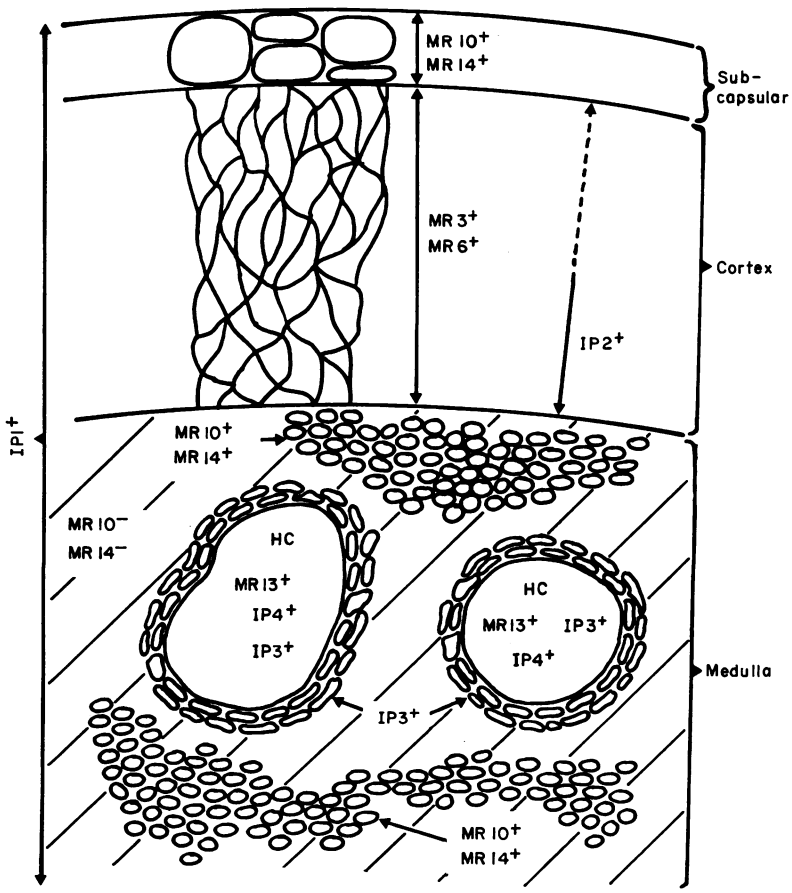

Figure 3. Diagrammatic representation of differential antigen expression by subpopulations of epithelial cells in the human thymus. HC = Hassall's corpuscles.

that lining the trabeculae and to a subpopulation of medullary epithelial cells, with staining of what appears to be plasma membrane and cytoplasm. MR10/14$^+$ medullary epithelium is especially concentrated in areas where the trabeculae extend down into the cortical-medullary junction. In both subcapsular and medullary areas these positive cells are oval-shaped with short cytoplasmic processes. In some parts of the medulla discrete areas of MR10/14$^+$ and MR10/14$^-$ epithelial cells exist (Fig. 1c), whilst in other parts the two populations are interspersed (Fig. 2c). A small proportion of Hassall's corpuscles were also labelled. However, no staining of keratin-negative cells was seen, nor were epithelial cells in other organs labelled by these antibodies. MR10 and MR14 therefore appear to detect a molecule restricted to subcapsular and medullary subsets of thymic epithelium.

Monoclonal antibodies MR3 and MR6 (Fig. 1e, f; Fig. 2e) bind to all cortical epithelial cells, with the exception of those in the subcapsular region. This subcapsular region is more obvious in some thymus sections (Fig. 2e) than in others (Fig. 1e,f) where MR3 staining extends almost to the capsule; in this latter case, almost no subcapsular labelling can be seen with MR10 and MR14 (i.e. very little subcapsular epithelium is present). The staining pattern with MR3 and MR6 is fine and dendritic, characteristic of plasma membrane labelling. These antibodies also show weaker, cytoplasmic staining of keratin-negative dendritic/macrophage-like cells in the medulla, and of similar cells in tonsil, lymph node and splenic white pulp. In addition, tubular epithelium in kidney is MR3/6$^+$. No other epithelium was positive. These antibodies give far less extensive and less intense staining of the thymus medulla than that seen with anti-HLA Class II reagents, and show no binding to B lymphocytes in tonsil, spleen or lymph node. They therefore do not appear to be detecting an HLA Class II product.

Monoclonal antibody IP2 (Fig. 1g, h) binds to a subpopulation of epithelial cells in the thymus cortex which are present predominantly in the area of the cortico-medullary junction. Staining seems to be located in both plasma membrane and cytoplasm. No keratin-negative cells are labelled with this antibody. IP2 binds to tubular epithelium in the kidney, but to no other epithelium tested.

Antibody IP3 (Fig. 1i,j) binds only to Hassall's corpuscles and to a subpopulation of medullary epithelial cells adjacent to them, with a predominantly cytoplasmic pattern of staining. The Hassall's corpuscles were consistently IP3$^+$ in all thymuses, while the extent of adjacent IP3$^+$ staining varied from thymus to thymus. No keratin-negative cells were labelled. This reagent also binds to epithelium in tonsil, skin and gut.

Antibodies MR13 ad IP4 (Fig. 1k,l) bind to Hassall's corpuscles, but to no other epithelial cells in the thymus. The intensity and pattern of this staining was variable between different Hassall's corpuscles, even within the same thymus. Highly keratinized areas (as seen by anti-keratin staining) showed the most intense staining with the monoclonals. Scattered keratin-negative cells in the medulla, cortex and septae are also labelled with these antibodies. Similar cells are found in tonsil, lymph node, spleen, bone marrow and blood, at least some of which are members of the granulocyte lineage. Although MR13 and IP4 stain both cell surface and cytoplasm of what appear to be the same keratin-negative cell subpopulations, their patterns of staining differ, with IP4 giving more prominent labelling of cytoplasm. Neither of these antibodies bind to epithelial cells in any of the other tissues tested.*

DISCUSSION

T lymphocytes at different stages of differentiation occupy distinct locations within the thymus. This differentiation, migration and positioning will depend upon molecular interactions between lymphocyte and local microenvironment, involving cell surface or secreted molecules. The work described here was designed to study such molecular heterogeneity within the thymus microenvironment using monoclonal antibodies raised directly to the epithelial component of the thymus stroma.

Our monoclonals fall into six different groups, each characterized by a distinctive pattern of staining (summarized diagrammatically in Fig. 3). One of these, given by antibodies MR10 and MR14, shows labelling of subcapsular and medullary epithelium and thus has similarities with that of antibodies RFD4 (Janossy *et al.*, 1983), A2B5 (Haynes *et al.*, 1983b) and anti-p19 (Haynes *et al.*, 1983a). However, MR10 and MR14 have a less 'dendritic' staining pattern in the subcapsular area, and bind to only a subpopulation of

* Utrecht monoclonal antibodies have been renamed (previous name given in parentheses): IP1 (18B5); IP2 (7C5); IP3 (8A2); IP4 (4A6).

medullary epithelial cells. These $MR10^+14^+$ cells are typically oval-shaped, in contrast to those that are $MR10^-14^-$ and more irregular in shape with spatulate processes. Thus two distinct subpopulations of epithelial cells reside within the thymus medulla. Subcapsular epithelial cells are also characterized by the presence of Thy-1 antigen (Ritter *et al.*, 1981), but in this case the medullary epithelial cells do not bear the same antigen. Likewise, secretion of thymosin B4 is a property unique to the subcapsular epithelium (Schuurman *et al.*, 1984). Molecular similarities between subcapsular and medullary epithelium may reflect similarities of function, since these are the two regions of the thymus to which bone marrow precursors probably first migrate (Le Douarin & Jotereau, 1981). Molecular differences could reflect subsequent differences in the lymphocytes that develop from these early cells.

A second pattern of staining is given by MR3 and MR6, which bind to all cortical epithelial cells excluding those in the subcapsular area. It is in this region that the postulated enzyme rescue mechanisms would occur (Ma *et al.*, 1983). In most thymuses ring-like staining patterns similar to those seen with anti-Thy-1 (Ritter *et al.*, 1981) and anti-HLA-DR (Bahn *et al.*, 1980) antibodies were observed, which may represent *in situ* Thymic Nurse Cells (Van de Wijngaert *et al.*, 1983). However, heterogeneity exists within this cortical population since, in a third pattern of staining, antibody IP2 binds to only some cortical epithelial cells, particularly those at the cortico-medullary junction. It is interesting that it is from this area that mature lymphocytes are believed to leave the thymus to join the recirculating pool.

Two types of epithelial staining are found only in the medulla. Antibody IP3 binds to Hassall's corpuscles and to a 'halo' of epithelial cells surrounding them, while MR13 and IP4 bind only to the Hassall's corpuscles. Very little is known of the function of these cell aggregates. However, the link between Hassall's corpuscles and neutrophils (both stained by MR13 and IP4) is reminiscent of an early theory (Blau, 1967) that these large epithelial aggregates are 'graveyards' for dead thymocytes and polymorphonuclear leucocytes. Finally, IP1 labels all epithelial cells in the thymus, although staining with this antibody is more intense in the cortex than in the medulla—the reverse pattern to that seen with the anti-keratin reagent.

Polyclonal antisera to different keratin molecules and to polysaccharide antigens have been shown to give differential binding patterns in thymic and non-thymic epithelium (Viac, Schmitt & Thiviolet, 1980; Lyampert *et al.*, 1976), although none give identical staining to that seen with the monoclonal antibodies described in this paper. Whether these monoclonals are detecting similar antigens is not possible to say until the molecules to which they bind have been identified. These studies are currently in progress. Finally, monoclonal antibodies that detect subpopulations of mouse thymic stromal cells (Van Vliet, Melis & Van Ewijk, 1984) and an intracellular antigen in human cortical thymic epithelial cells (McFarland, Scearce & Haynes, 1984) have recently been reported, but again none appear to be the exact counterpart of those to human thymic epithelial cells that we describe here.

In conclusion, we are able to detect antigenic, and hence molecular, heterogeneity within the epithelial component of the thymus microenvironment using a series of monoclonal antibodies raised against thymic epithelium. These reagents should be particularly useful in determining the biochemical nature and ultimately the function of molecules which may be involved in membrane recognition events responsible for the localization and differentiation of lymphocytes within the thymus, and for the release of mature T cells into the periphery.

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