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The human thymus microenvironment: *in vivo* identification of thymic nurse cells and other antigenically-distinct subpopulations of epithelial cells

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Summary. We have studied the human thymus microenvironment in order to identify subsets of cells that may be responsible for the induction of different aspects of T-lymphocyte differentiation, education and MHC restriction. Using immunofluorescence on tissue sections and cell suspensions we have found MHC products (HLA-A, B, C and DR) to be present throughout the thymus epithelium whilst human T-cell antigens are absent from all non-lymphoid cells. In contrast, Thy-1 antigen (expressed on ~1% paediatric human thymocytes) has a differential expression amongst thymic epithelial cells, being confined to those in the subcapsular cortex and to 'thymic nurse cells' (TNC). The former represent the site to which thymocyte precursors first migrate upon entering the thymus. The latter are large epithelial cells, located within the cortex, whose plasma membrane totally encloses a number of thymus lymphocytes; these cells are therefore good candidates for the mediators of direct contact (stromal) induced thymocyte maturation.

INTRODUCTION

Much is known of T-lymphocyte differentiation within the thymus in terms of the acquisition of typical T cell-surface molecules such as Thy-1, TL and Lyt 1, 2 and 3 in mice (Cantor & Boyse, 1977) and antigens defined by the OKT series of monoclonal antibodies in man (Kung, Talle, DeMaria, Butler, Lifter & Goldstein, 1980; Reinherz & Schlossman, 1980). In addition, these T lymphocytes develop functional capabilities governed by MHC restriction, such that foreign antigens on target cell surfaces are recognized in the context of major histocompatibility gene complex products on the target cell (Zinkernagel, Callahan, Althage, Cooper, Klein & Klein, 1978). However, relatively little is known of the thymus microenvironment that is responsible for the induction of at least a major part of these maturation processes.

We were therefore interested to see whether we could identify different subsets of cells within the human thymic microenvironment which might be responsible for the induction of these different phases of T-cell differentiation. To do this we studied the thymus by immunofluorescence on both tissue sections and cell suspensions using antibodies directed against HLA-ABC and DR ('Ia-like') antigens, Thy-1 and a variety of T-cell antigens. Our results demonstrate both antigenic and morphological heterogeneity within the thymus epithelium.

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MATERIALS AND METHODS

Tissues

Foetal thymus was obtained from cases of induced or spontaneous abortion in the second trimester of

pregnancy (kindly supplied by Dr S. Lawler, Royal Marsden Hospital, London). Paediatric thymic tissue was obtained from individuals undergoing open heart surgery (kindly supplied by Dr L. Laywood, Institute of Child Health, London).

Thymus lymphocyte cell suspensions

Thymocyte cell suspensions were prepared at either room temperature or 4° by gently teasing tissues in Dulbecco's modified Eagle's minimal essential medium (DMEM) buffered with 20 mM HEPES and supplemented with 5% heat-inactivated foetal calf serum (FCS).

Preparation of 'thymic nurse cells' and other epithelial cell types in cell suspension

Thymic tissue fragments remaining after the majority of thymocytes had been removed by teasing were treated by sequential trypsinization, based on the method of Werkerle, Ketelsen & Ernst (1980). All cells released from the serial digestions were pooled, layered over FCS and centrifuged for 3 min at 100 *g*. The sedimented cell population was enriched for TNC, Hassall's corpuscles and other epithelial cells. Thymic nurse cells could also be obtained, without enzyme treatment, by thorough mechanical teasing of tissue fragments at room temperature, 4° and at 4° in medium containing 0·2% sodium azide.

Thymus tissue sections

Tissues were fixed for a total of 4–6 hr in three changes of 5% glacial acetic acid/95% absolute ethanol at 4°, washed in 95% ethanol (4°) and embedded in polyester wax (B.D.H.) at 37° (Lebacq & Ritter, 1979). Sections were cut at 2·5 and 5 μm. Both blocks and sections were stored at 4°.

Antibodies and pure Thy-1

The $F(ab')_2$ preparation of a rabbit antiserum to purified rat brain Thy-1 was used at 100 μg/ml to detect the human homologue of Thy-1, and was the gift of Dr R.J. Morris (Morris, Mancini & Pfeiffer, 1980). Quantitative absorption/radioimmune binding assays (Kemshead, Ritter & Greaves, submitted for publication) demonstrate that the component of this antiserum that recognizes human Thy-1 constitutes 60% of that binding to rat–mouse shared determinants which itself constitutes 41% of the total (Morris *et al.*, 1980). Thus, approximately 25% of the total anti-rat Thy-1 antibodies recognize the human Thy-1 molecule. This binding gives strong labelling by indirect immunofluorescence. Monoclonal antibodies that bind to human Thy-1 (gifts of Dr J.W. Fabre and Dr R.M.E. Parkhouse) gave the same pattern of staining as that obtained with the xenoantiserum, but the immunofluorescence was considerably weaker.

Monoclonal antibodies (ascites) against HLA-A, B, C (W6/32) and HLA-DR (DA2) monomorphic determinants were the gift of Dr W. Bodmer-Monoclonal antibodies (ascites) against human T lymphocyte-associated antigens (OKT11a, 10 and 6) were kindly supplied by Drs P. Kung and G. Goldstein (Ortho Pharmaceutical Corporation, Raritan, New Jersey, U.S.A.)—Kung *et al.*, 1980; Greaves, Delia, Sutherland, Rao, Verbi, Kemshead, Robinson, Hariri, Goldstein & Kung, 1981). NA134 was the gift of Dr A. McMichael (McMichael, Pilch, Galfre, Mason, Fabre & Milstein, 1979).

These antisera were followed in immunofluorescence by fluorescein isothiocyanate (FITC) conjugated $F(ab')_2$ sheep anti-rabbit $F(ab')_2$ (F/P ratio = 3·28) at 60 μg/ml and FITC or tetra-methyl rhodamine isothiocyanate (TMRITC)-conjugated $F(ab')_2$ goat anti-mouse Ig as appropriate.

Purified rat Thy-1 was the generous gift of Dr R.J. Morris. Human Thy-1 was purified by deoxycholate extraction, lentil lectin affinity chromatography and repeated gel filtration (Cotmore, Crowhurst & Waterfield, 1981).

Immunofluorescence and histology

Cell suspensions were stained by indirect immunofluorescence as in Ritter, Gordon & Goldschneider (1978) using the above reagents.

Tissue sections were treated for immunofluorescence, photographed and counterstained with ammoniacal silver nitrate/gold chloride, haematoxalin-eosin or Giemsa (Ritter & Morris, 1980).

Specificity of immunofluorescence

Specificity of staining for Thy-1 was demonstrated by abrogation of FITC fluorescence on both cell suspensions and tissue sections by prior incubation (overnight at 4°) of the anti-Thy-1 antibody with: (i) a three-fold molar excess of pure human Thy-1 or (ii) a three-fold molar excess of pure rat Thy-1 (Fig. 1c). No immunofluorescence was seen when either the FITC-sheep anti-rabbit $F(ab')_2$ or the TMRITC-goat anti-mouse Ig (to follow monoclonals) antibodies were used in the absence of primary layer antibodies, or when the mouse monoclonal reagents were replaced by a non-immune ascites preparation.

RESULTS

Tissue sections

In section, the human thymus can be seen by phase contrast or after histological staining to be composed of many lobules each separated by a connective tissue septum containing blood vessels and migratory cells. Each lobule consists of an outer, cortical, area where epithelial cells with long processes form a framework into which many lymphocytes are packed in such a way that many, though not all, are in direct contact with the epithelial cells. The cells of the medulla are more loosely packed, consisting of epithelial cells with shorter processes, Hassall's corpuscles (whorl-like structures of epithelial cells) and lymphocytes. Macrophages are also present within the thymus.

Thy-1 immunofluorescence in paediatric thymus was confined to the thymic cortex, the medulla being consistently negative (Fig. 1d). Within the cortex three distinct Thy-1$^+$ patterns of immunofluorescent staining were identified. Firstly, most of the Thy-1$^+$ staining was situated in the peripheral area of the cortex, characteristically showing a layer of staining one to three cells deep immediately under the thymic capsule and along the septae (Fig. 1a). The immunofluorescence showed a dendritic pattern of staining and by phase contrast could be seen to be associated with the processes of epithelial cells (Fig. 1b). These processes surround one or, more usually, two or more lymphocytes. A small proportion of outer cortical lymphocytes were also Thy-1$^+$.

Secondly, 'rings' of Thy-1 immunofluorescence were seen in the mid/outer cortex (Fig. 1e,g). By phase contrast and after Giemsa post-staining the immunofluorescence was seen to be associated with the membrane of a single very large cell containing its own nucleus and many small lymphocytes (Fig. 1f, h, i). These 'rings' are therefore probably 'thymic nurse cells' in cross section. The TNC were most clearly seen in 2·5 μ sections where there is very little superimposition of cells to obscure the detail. Although most TNC contained Thy-1$^-$ lymphocytes, an occasional TNC contained Thy-1$^+$ cells. Thirdly, groups of epithelial cells in the mid/outer cortex were also Thy-1$^+$. By phase contrast these cells contained no lymphocytes.

All immunofluorescent structures were checked for their appearance by phase contrast. In addition, some structures of each type (peripheral epithelial, TNC and epithelial groups) were post-stained with either silver nitrate/gold chloride or May–Grunwald Giemsa. All Thy-1$^+$ immunofluorescence described above was associated with cell membranes and none with collagen. The only two situations in which Thy-1 staining correlated with that for collagen were the basement membrane of capillaries in the cortex and the thymic capsule, which stained weakly with anti-Thy-1 antibodies (see also Ritter & Morris, 1980).

Thy-1 immunofluorescence on sections of foetal thymus showed a similar pattern to that of paediatric thymus, although the intensity of staining was considerably weaker.

Cell suspensions

Cell suspensions prepared conventionally by teasing of thymic tissue were composed almost entirely of lymphocytes, although an occasional thymic nurse cell or Hassall's corpuscle was seen. Sequential trypsinization or prolonged physical teasing followed by centrifugation through FCS increased the yield of epithelial cells considerably. In these latter suspensions four types of non-lymphoid cells could be identified in both foetal and adult thymus: TNC, characterized as large cells with an intact plasma membrane completely enclosing a nucleus together with a variable number of lymphocytes, ranging from two up to at least twenty; Hassall's corpuscles, identified as whorls of epithelial cells; large epithelial cells with no lymphocyte inclusions, present as aggregates or as single cells; and large single cells, probably macrophages, surrounded by a rosette of lymphocytes (Schulte-Wissermann, Borzy, Albrecht & Hong, 1979). A summary of the immunofluorescence data is given in Table 1. All TNC in paediatric thymus were HLA-ABC$^+$, DR$^+$ and Thy-1$^+$ (Fig. 2a–e). However, in the foetal thymus TNC exhibited a variety of phenotypes (by dual immunofluorescence): HLA--ABC$^+$ Thy-1$^-$, HLA-ABC$^+$ Thy-1$^+$, HLA-DR$^-$ Thy-1$^-$, HLA-DR$^+$ Thy-1$^-$ and HLA-DR$^+$ Thy-1$^+$. All TNC exhibited clear ring immunofluorescence typical of cells within a viable intact cell membrane. Simple epithelial cells (no lymphocyte inclusions) occurring both singly and in clumps were all HLA-ABC$^+$ DR$^+$; some of these were also Thy-1$^+$, whilst others lacked the Thy-1 antigen (Fig. 3a,b). A small proportion of single HLA-ABC$^+$ DR$^+$ non-lymphoid cells formed rosettes with the thymic lymphocytes and were probably thymic macrophages (Fig. 3c,d). Hassall's corpuscles were all HLA-ABC$^+$ DR$^+$ but Thy-1$^-$ (Fig. 3e,f). No anti-T cell-monoclonal antibodies (NA134, OKT6, 10, 11a) bound to any epithelial cells or macrophages. In addition, no lymphocytes within TNC bound any of these reagents unless the TNC-plasma membrane was

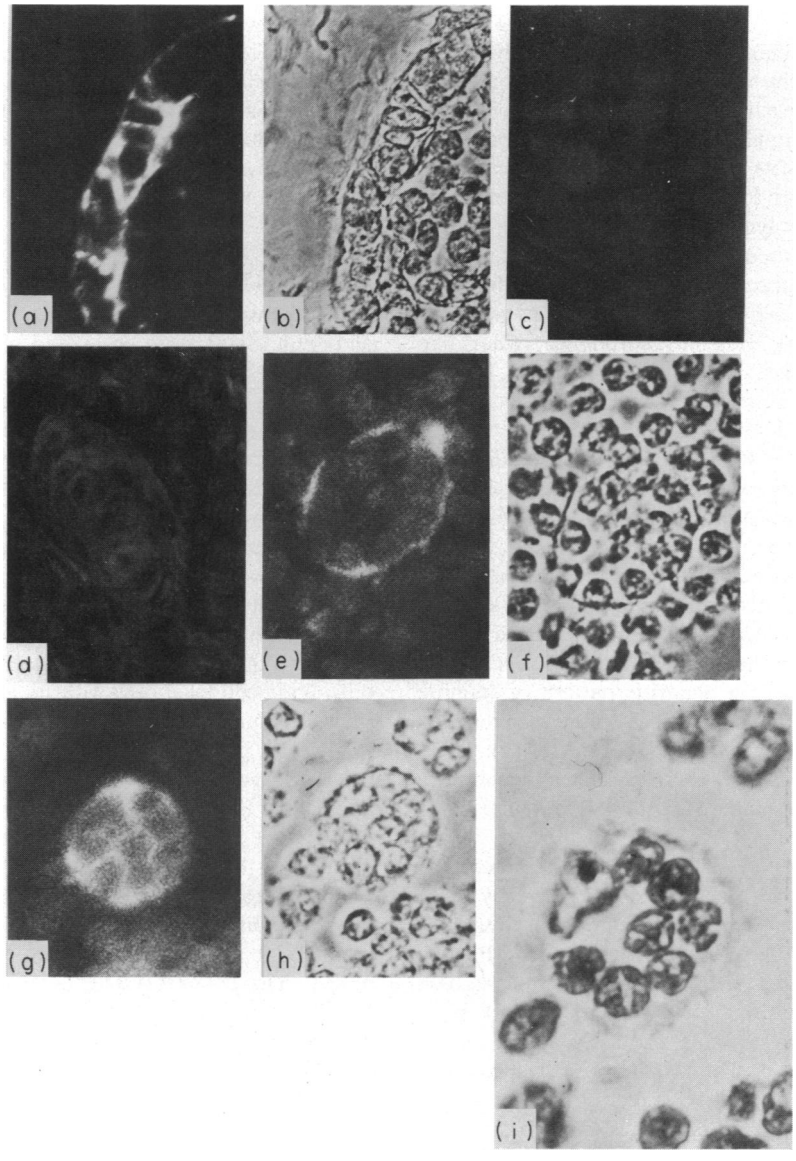

Figure 1. Thy-1 immunofluorescence in tissue sections of human thymus. (a) Thy-1$^+$ epithelial cells in the peripheral cortex; (b) the same field by phase contrast; (c) immunofluorescence control for peripheral cortex (anti-Thy-1 antibodies preabsorbed with pure human Thy-1); (d) Thy-1$^-$ cells (including epithelial cells and Hassall's corpuscle) in the medulla; (e) Thy-1$^+$ 'thymic nurse cell' containing Thy-1$^-$ lymphocytes; (f) the same cells by phase contrast; (g) Thy-1$^+$ 'thymic nurse cell' containing Thy-1$^+$ lymphocytes; (h) the same cell by phase contrast; (i) the same cell after Giemsa staining, showing the 'thymic nurse cell' membrane enclosing its epithelial nucleus and seven lymphocytes. Magnification: $\times$ 538 (d); $\times$ 806 (a–c, e–h); $\times$ 1290 (i).

Table 1. Antigenic phenotype of human thymus epithelial subpopulations

	Thy-1	HLA-ABC	HLA-DR	T-cell antigens*
Thymic nurse cells	+	+	+	−
Simple epithelial cells				
(either)	+	+	+	−
(or)	−	+	+	−
Hassall's corpuscles	−	+	+	−

* Antibodies used to detect T-cell antigens: OKT6, 10, 11a, NA134. Cells were stained in suspension by indirect immunofluorescence.

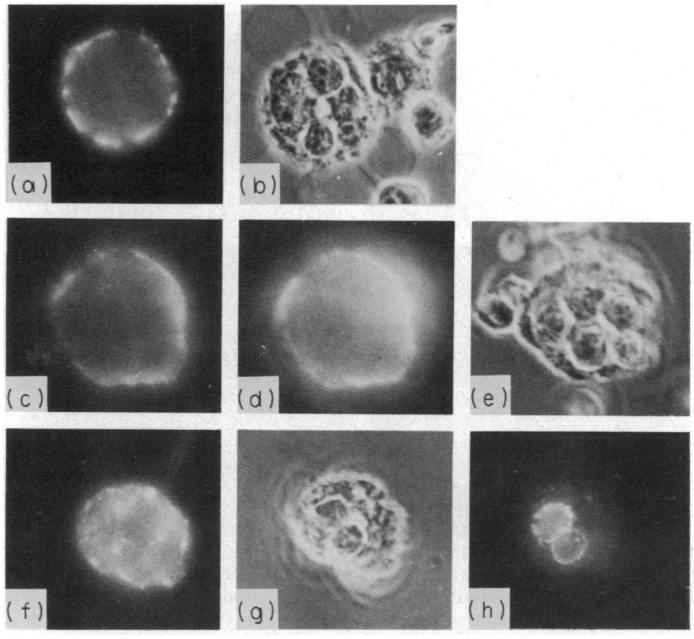

Figure 2. Thy-1 immunofluorescence on human 'thymic nurse cells' in suspension. (a) Thy-1$^+$ 'thymic nurse cell'; (b) the same cell by phase contrast showing the epithelial nucleus and three lymphocytes within the 'thymic nurse cell' membrane; (c) Thy-1$^+$ 'thymic nurse cell'; (d) the same cell showing HLA-DR$^+$ immunofluorescence; (e) the same cell by phase contrast; (f) Thy-1$^+$ 'thymic nurse cell' with permeabilised cell membrane; (g) the same cell by phase contrast; (h) the same cell after immunofluorescence staining with NA134 monoclonal antibody, showing two NA134$^+$ lymphocytes within the 'thymic nurse cell'. Magnification: ×804.

first permeabilized (e.g. by fixation (Fig. 2f,g,h) thus demonstrating that the lymphocytes within the TNC are totally enclosed within a viable TNC-plasma membrane. The antigenic phenotype of TNC and other thymic epithelial cells was the same whether the cell suspension was prepared by trypsinization or by extensive physical teasing. Approximately 1% or fewer paediatric thymocytes and up to 10% foetal thymocytes were Thy-1$^+$. Double-staining experiments showed these cells were also OKT10$^+$ and some (approximately 50%) were also OKT 6$^+$.

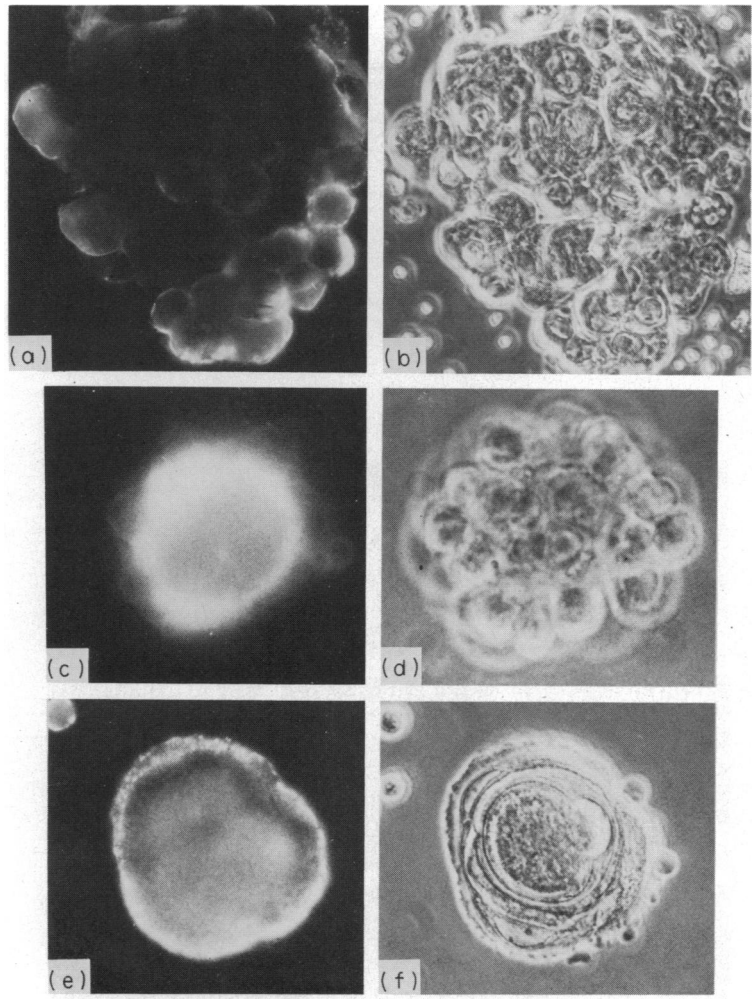

Figure 3. HLA-DR immunofluorescence on human thymus cells in suspension. (a) HLA-DR$^+$ group of simple (no lymphocyte inclusions) epithelial cells (all cells in the clump were positive by immunofluorescence; however, only those in the plane of focus appear strongly stained in the photograph); (b) the same cells by phase contrast; (c) HLA-DR$^+$ macrophage; (d) the same cell by phase contrast showing its rosette of lymphocytes; (e) HLA-DR$^+$ Hassall's corpuscle; (f) the same cell by phase contrast. Magnification: ×538 (a and b); ×804 (c–f).

DISCUSSION

The work presented in this paper was aimed at dissecting the human thymic microenvironment into different compartments that might be responsible for the induction of different stages of T-lymphocyte differentiation. Two major findings emerge: firstly, we have identified human 'thymic nurse cells' both in tissue sections of *in vivo* material and in cell suspensions *in vitro*; secondly, we have found the Thy-1 antigen to be predominantly associated with human thymic epithelial cells rather than with thymocytes.

Our immunofluorescence studies show that whilst MHC products are present essentially throughout the thymic epithelium, T-cell antigens are totally absent, being confined to cells of the lymphoid lineage. In contrast, Thy-1 antigen showed a differential expression: TNC in the mid/outer cortex, subcapsular

epithelial cells and small groups of cells in the mid/outer cortex being Thy-1$^+$ whilst other cortical epithelial cells and all those of the medulla were negative.

The MHC phenotypes fit well with those seen in frozen sections of both human and mouse thymus Janossy, Tidman, Selby, Thomas, Granger, Kung & Goldstein, 1980; Bhan, Reinherz, Poppema, McCluskey & Schlossman, 1980; Rouse, van Ewijk, Jones & Weisman, 1979). However, the predominant association of Thy-1 with human thymus epithelial cells, with only a minority of thymocytes (outer cortical, immature) being Thy-1$^+$, is in sharp contrast to the situation in rodents where essentially all thymic lymphocytes bear Thy-1 antigen (Reif & Allen, 1964; Douglas, 1973). In addition, it has been generally assumed that Thy-1 is absent from rodent thymus epithelium, although Thy-1$^+$ 'epithelium-like' cells have been identified in long term cultures of both rodent and human thymus (Raedler, Arndt, Raedler, Jablonski & Thiele, 1978; Raedler, Arndt & Thiele, 1979).

The Thy-1$^+$ phenotype of human TNC contrasts clearly with the situation in rodents where TNC are Thy-1$^-$ (Werkerle *et al.*, 1980). It is interesting that in the human foetus, although all TNC are HLA-ABC$^+$, some lack HLA-DR and/or Thy-1. Since all adult TNC are HLA-ABC$^+$ DR$^+$ Thy-1$^+$ it seems likely that the various foetal TNC phenotypes represent the sequential acquisition of first HLA-DR and then Thy-1 by maturing TNC during human thymus ontogeny, rather than separate subsets of cells. That these surface antigen differences are not an artefact resulting from the trypsinization process is demonstrated by the fact that epithelial cells derived by mechanical teasing show the same characteristic combinations of surface molecules as those derived by enzyme treatment.

Another problem to be considered is that of the TNC themselves since their existence in mice has only been demonstrated in cell suspensions (Werkerle *et al.*, 1980), and might therefore be an artefact resulting either from cell damage during trypsinization or from endocytosis of lymphocytes during cell suspension preparation. However, we can routinely obtain TNC by thorough teasing in the absence of enzymes, at either 37° or 4°, and in the presence of sodium azide, thus excluding the involvement of either active uptake or enzyme damage. Nevertheless, the crucial evidence in support of the physiological nature of the TNC is our identification of these cells in tissue sections of *in vivo* material. The failure of others to find TNC in sections may be due to the use of either frozen sections with relatively poor preservation and resolution, or electron microscopy in which it is difficult to find relatively rare structures at high magnification. We have used fixed tissues embedded in low melting point (37°) wax to give both good antigenic and histologic preservation at the light microscope level.

The thymus plays a vital role in T-cell maturation (Cantor & Weissman, 1976; Cantor & Boyse, 1977; Reinherz & Schlossman, 1980) and the development of MHC restriction (Zinkernagel *et al.*, 1978). Its influence on T-cell maturation is mediated in two ways: firstly, by direct contact of thymocyte precursors with the thymus stroma and subsequently via humoral factors (Stutman, Yunis & Good, 1969; Goldstein, 1977; Marshall, Low, Thurman & Goldstein, 1978; Bach, Pleau, Dardenne & Bach, 1978); thymic epithelial cells are believed to be responsible for both effects. It is interesting that the Thy-1$^+$ epithelial cells at the periphery of the human thymic cortex are in the area to which thymocyte precursors first migrate and proliferate after their arrival in the thymus, and thus may play an important part in the early stages of thymocyte differentiation via a 'stromal' type of induction. The Thy-1$^+$ TNC are also good candidates for mediating 'stromal' induction since the thymocytes are contained totally within this specialized epithelial cell. The phenotype and location of the small percentage of Thy-1$^+$ lymphocytes in the thymus suggests that they may represent thymocyte precursors/early thymocytes (McKenzie & Fabre, 1981) which lose Thy-1 as an early maturational step. Since a small proportion of TNC in sections contain Thy-1$^+$ lymphocytes, it is possible that this loss occurs in or around the phase of migration through the TNC; hence the Thy-1 on TNC might have been acquired from that shed by differentiating lymphocytes rather than synthesized by the epithelial cells themselves (Vitetta, Uhr & Boyse, 1974). What role Thy-1 is playing in this process is not known, although previous studies in the rat (Ritter, 1980; Morris & Ritter, 1980; Ritter & Morris, 1980) suggest that the molecule may be involved in cell–cell/cell-connective tissue adhesion. Since essentially all thymic epithelial cells and macrophages bear MHC products, any or all of these cells could be involved in MHC restriction.

Thus we have demonstrated the presence of human TNC both *in vivo* and *in vitro*. These specialized cells together with the subcapsular epithelial cells, are Thy-1$^+$ and hence are antigenically distinct from the

remainder of the cortical and all medullary epithelial cells. It is proposed that, by virtue of their location and special characteristics, these Thy-1$^+$ epithelial cells may be important in the induction of early lymphocyte differentiation. *In vitro* experiments are therefore in progress to study the possible role of these cells in both T-lymphocyte maturation and MHC restriction.

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