PHENOTYPIC CHARACTERIZATION AND ONTOGENY OF MESODERMAL-DERIVED AND ENDOCRINE EPITHELIAL COMPONENTS OF THE HUMAN THYMIC MICROENVIRONMENT

By BARTON F. HAYNES, RICHARD M. SCEARCE, DAVID F. LOBACH, and LUCINDA L. HENSLEY

From the Department of Medicine, Division of Rheumatic and Genetic Disease, and the Department of Microbiology and Immunology, Duke University School of Medicine, Durham, North Carolina 27710

The thymic microenvironment is a complex specialized tissue derived from at least three sources: endoderm of the third pharyngeal pouch, ectoderm derived from the third brachial cleft, and mesenchymal stromal cells derived from embryonic mesoderm (1–6). Pharyngeal pouch endoderm and brachial cleft ectoderm give rise to epithelial components, while mesodermal-derived mesenchymal cells form the thymic capsule, vessels, and interlobular septae (1–6). Early in thymic ontogeny, mesodermal-derived connective tissue induces epithelial cell maturation and fetal thymic lobulation (3, 4). Ultrastructurally, the nonlymphoid postnatal thymic microenvironment is composed of macrophages (7), fibroblasts, and dark and pale epithelial reticular cells (7, 8). In postnatal human thymic medulla, Hassall's bodies, or keratinized epithelial swirls, are frequently present (7, 8).

Microscopists have long appreciated the dendritic nature of thymic epithelial cells (7, 9). Cortical and medullary epithelial cells have long, thin processes that connect with other epithelial cells via desmosomes, and form a complex interdigitating meshwork filled with thymocytes (7, 9). Some thymic epithelial cells have been shown to express class I and II major histocompatibility complex (MHC)[1] antigens (10–12), and functional data have demonstrated that the thymic microenvironment is responsible for conferring to developing thymocytes the ability to recognize self-class I and II MHC antigens (13–17). Kruisbeek et al. (18) have recently suggested that during early T cell development, education of class I MHC-restricted T cells is conferred by different thymic elements than those involved in the education of class II MHC-restricted T cells. Moreover, a subset of epithelial cells within the thymic microenvironment contains a variety

This work was supported by grants CA28936, CA11265, K0400695, and AI1-19368 from the National Institutes of Health, and by a Basic Research Grant from the March of Dimes Foundation. Address reprint requests to B. F. Haynes, Box 3258, Duke University School of Medicine, Durham, NC 27710.

[1] *Abbreviations used in this paper:* F/P, fluorescein to protein ratio; HB, Hassall's bodies; HTLV, human T cell leukemia/lymphoma virus; MHC, major histocompatibility complex; RITC, rhodamine isothiocyanate.

of thymic hormones that are postulated to induce certain stages of T cell maturation (19–22). However, in spite of the important role the thymic microenvironment plays in the maturation of T cells, little is known regarding antigenically definable subsets of cells within the human thymic microenvironment.

Recently (21, 23) we have described complex ganglioside antigens on human and rodent endocrine thymic epithelium, and demonstrated that these thymopoietin- and thymosin α_1-containing epithelial cells are present in two distinct regions of the thymus: the subcapsular cortex and medulla. In the present study, we use novel monoclonal antibodies raised against human thymic stroma, TE-4 and TE-7, to define two mutually exclusive thymic microenvironment components. The TE-4$^+$ epithelial component contains thymosin α_1 and keratin, and strongly expresses class I HLA-A, -B, and -C (or HLA) and class II Ia-like (or Ia) MHC antigens. In contrast, antibody TE-7 defines the mesodermal component of the thymic microenvironment that does not contain thymosin α_1 or keratin, weakly expresses HLA antigens, and does not express Ia antigens. Thus, TE-4$^+$ thymic epithelium constitutes an HLA$^+$, Ia$^+$ endocrine subset of thymic epithelial cells and is a prime candidate for a cell type of the human thymic microenvironment that might participate in conferring MHC restriction to maturing T lymphocytes. TE-7$^+$ stroma most likely represents the mesodermal-derived thymic component that early in development induces thymic epithelial differentiation.

Materials and Methods

Production and Characterization of Monoclonal Antibodies TE-4 and TE-7. Antibodies TE-4 and TE-7 were the products of a lymphocyte hybrid fusion between P3X63/Ag8 murine myeloma cells (TE-4) or 653 murine myeloma cells (TE-7) and BALB/c spleen cells from an animal immunized with fresh human thymic stroma. Murine hybridomas were fused, selected, and cloned as previously described (24, 25). Human thymus was obtained from either three normal subjects (all less than 16 yr old) at the time of corrective cardiovascular surgery for congenital heart disease or from three patients with myasthenia gravis at the time of therapeutic thymectomy (age range, 17–31 yr). Thymocytes were gently teased from 10-mm^3 pieces of stroma by mechanical dissociation, the stroma cut into <1-mm pieces with scissors and drawn into a 1-cc syringe in RPMI 1640 media (Gibco Laboratories, Grand Island, NY) through a 19 gauge needle. Female BALB/c mice were immunized intraperitoneally with 0.5 cc of stroma on days 0, 7, 14, and 21. On day 24, the spleen was removed and a single-cell suspension was obtained. Fusion was performed in 0.8 ml of 50% polyethylene glycol 1000 (J. T. Baker Chemical Co., Phillipsburg, NJ) (24, 25). Normal nonimmunized BALB/c spleen cells were added to the fused cell suspension (5,000 cells/well) as feeder cells. To each well of a Linbro flat-bottomed microtiter plate (Flow Laboratories, McLean, VA), 0.25 ml of the fused cell suspension was added. After 8–10 d, the supernatant of visible colonies was removed and assayed for reactivity with thymic microenvironment components by indirect immunofluorescence on 4-μm acetone-fixed frozen human thymus tissue sections as previously described (26).

In the TE-4 fusion, 418 wells were seeded, from which 180 colonies were assayed (43% fusion efficiency). 20 of 180 screened colonies were positive in indirect immunofluorescence for reactivity with human thymic tissue sections, from which hybridoma TE-4 was selected and serially passed. In the TE-7 fusion, 418 wells were seeded from which 121 colonies were assayed (29% fusion efficiency). 69 of 121 screened colonies were positive for reactivity with human thymus sections, from which hybridoma TE-7 was selected and serially passed. TE-4 and TE-7 hybridoma cell lines were cloned by limiting dilution until all clones assayed were positive; select positive clones were sequentially passed as mouse ascites tumors. Immunoglobulin isotyping was performed by Ouchterlony gel analysis.

After all clones were positive, they were subcloned three additional times; antibody TE-7 was shown to be IgG_1 and antibody TE-4 was shown to be IgM.

Other Monoclonal and Polyclonal Antisera. Monoclonal antibody A2B5 was raised against chick retinal cells (27) and binds to complex GQ gangliosides on a variety of human and rodent neuroendocrine tissues (28, 29). In human thymus, A2B5 defines the endocrine component of thymic epithelium (21, 23). Monoclonal antibody 12/1-2 (anti-p19) reacts with the 19,000 dalton internal core protein (p19) of human T cell leukemia-lymphoma virus (HTLV) and was the generous gift of Dr. M. Robert-Guroff, National Institutes of Health (30). Moreover, anti-p19 also defines an antigen acquired during ontogeny on normal, $A2B5^+$, non–HTLV-infected human thymic epithelium (31). Antibody 3F10 defines a nonpolymorphic antigen on the HLA-A and -B backbone molecule (32) and antibody L243 (American Type Tissue Collection, Rockville, MD) defines a nonpolymorphic antigen on the human Ia-like backbone molecule (33). Monoclonal antibody AE-1 binds to human keratin and was the generous gift of Dr. Tung Tien Sun, New York University School of Medicine, New York (34). Monospecific rabbit polyclonal anti-thymosin α_1 antiserum was the generous gift of Dr. Allan Goldstein, George Washington University, Washington, DC, and was absorbed for specificity as previously described (23).

Indirect Immunofluorescence Assays. Human thymus tissue was obtained from eight normal subjects (age 3 mo to 16 yr) at the time of corrective cardiovascular surgery for congenital heart disease. Staining of 4-μm acetone-fixed tissue sections with monoclonal antibodies in indirect and direct immunofluorescence was performed as previously described in detail (26). Each single or double immunofluorescence assay was performed on at least three thymuses.

Monoclonal antibodies TE-4, TE-7, 3F10, and L243 were directly fluoresceinated as previously described (35). Briefly, fluorescein isothiocyanate (Research Organics, Inc., Cleveland, OH) (50 μg/mg of antibody) was added to ammonium sulfate-purified monoclonal antibody (1 mg/ml in 0.1 M borate buffer in 0.85% saline, pH 9.2), incubated 2 h at 37°C, and dialyzed against phosphate-buffered saline. Fluorescence to protein (F/P) ratios of the monoclonal antibodies and their saturating titers in direct immunofluorescence were as follows: P3 (control IgG, myeloma protein), 7.7; 3F10, 3.6 (1:50); L-243, 4.6 (1:20); TE-4, 9.0 (1:50); and TE-7, 3.3 (1:1,000). Double indirect immunofluorescence simultaneously comparing the reactivity of TE-4 or TE-7 with A2B5, L243, 3F10, AE-1 (antikeratin), TE-4, or TE-7 on thymus tissue was performed as follows: Unconjugated antibody A was incubated with thymus sections and the sections washed, followed by incubation with goat anti-mouse IgG conjugated with rhodamine isothiocyanate (RITC) (1:50; TAGO, Inc., Burlingame, CA). After a second washing, the slides were then incubated with directly fluoresceinated antibody B, washed, and then viewed for green fluorescence using filters for fluorescein excitation (515W, B Nikon) or for red fluorescence using filters for rhodamine excitation (580W, G Nikon). Controls for double immunofluorescence with two murine monoclonal antibodies included thymus sections sequentially incubated with (*a*) P3X63/Ag8 IgG1 parent myeloma protein, RITC-conjugated goat anti-mouse IgG, directly fluoresceinated antibody B, or directly fluoresceinated P3X63/Ag8 parent myeloma protein, and (*b*) antibody A, RITC-conjugated goat anti-mouse IgG, followed by directly fluoresceinated P3X63/Ag8 parent myeloma protein. Double indirect immunofluorescence assays with rabbit antithymosin α_1 antisera and monoclonal antibodies TE-4 and TE-7 were performed as previously described in detail (21, 23).

Results

Reactivity Patterns of Antibodies TE-4 and TE-7 on Normal Thymic Tissue. TE-4 reacted with normal thymus in a pattern similar to that described for monoclonal antibody A2B5 (21) and, regarding thymus >36 mo postnatal, anti-p19 (31). Epithelial reticular cells in the subcapsular cortex and medullary areas of the thymus were $TE\text{-}4^+$ (Fig. 1 *A* and *B*). $TE\text{-}4^+$ cells in the medulla went up to and surrounded Hassall's bodies (HB), while HB themselves were nonreactive with

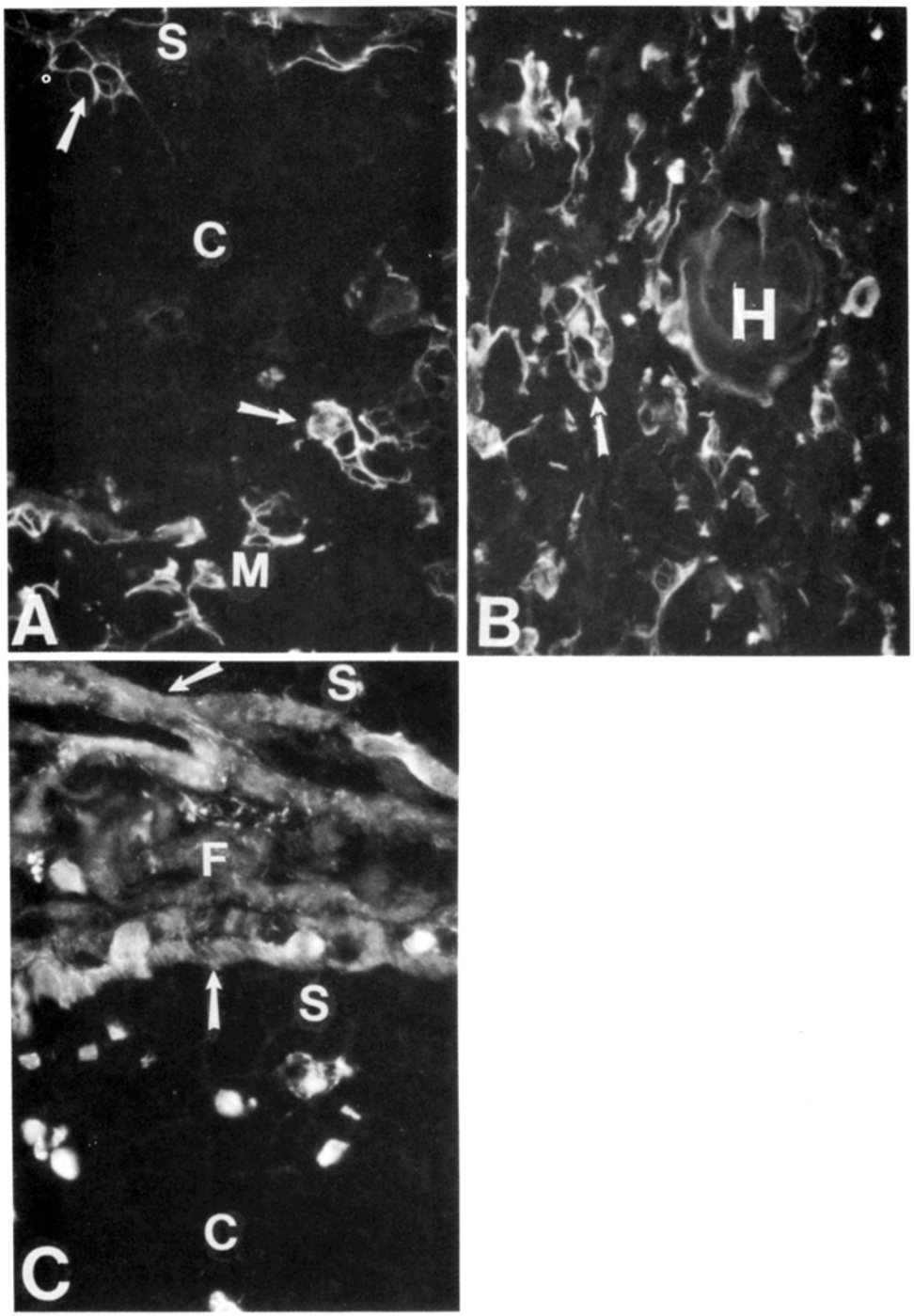

FIGURE 1. Reactivity pattern of antibodies TE-4 and TE-7 with normal human thymus. Using indirect immunofluorescence, TE-4 reacted (*A*) with normal thymus from a 3-mo-old subject in a dendritic pattern (arrow) in the subcapsular cortex (S) and medulla (M). Epithelial cells in the cortex were predominantly TE-4$^-$ (C). The fibrous capsule (above the subcapsular cortex) and the interlobular septae were TE-4$^-$. TE-4$^+$ epithelial cells (arrow) in the thymic medulla (*B*) went up to and surrounded HB (H). The center of HB themselves were TE-4$^-$. (*C*) TE-7$^+$ fibrous interlobular septum (F) between two thymic lobules. Antibody TE-7 did not react with epithelium in the subcapsular cortex (S), cortex (C), or medulla (not shown), but rather reacted with the thymic capsule (arrows) and the fibrous tissue and vessels in interlobular septae (F). × 400.

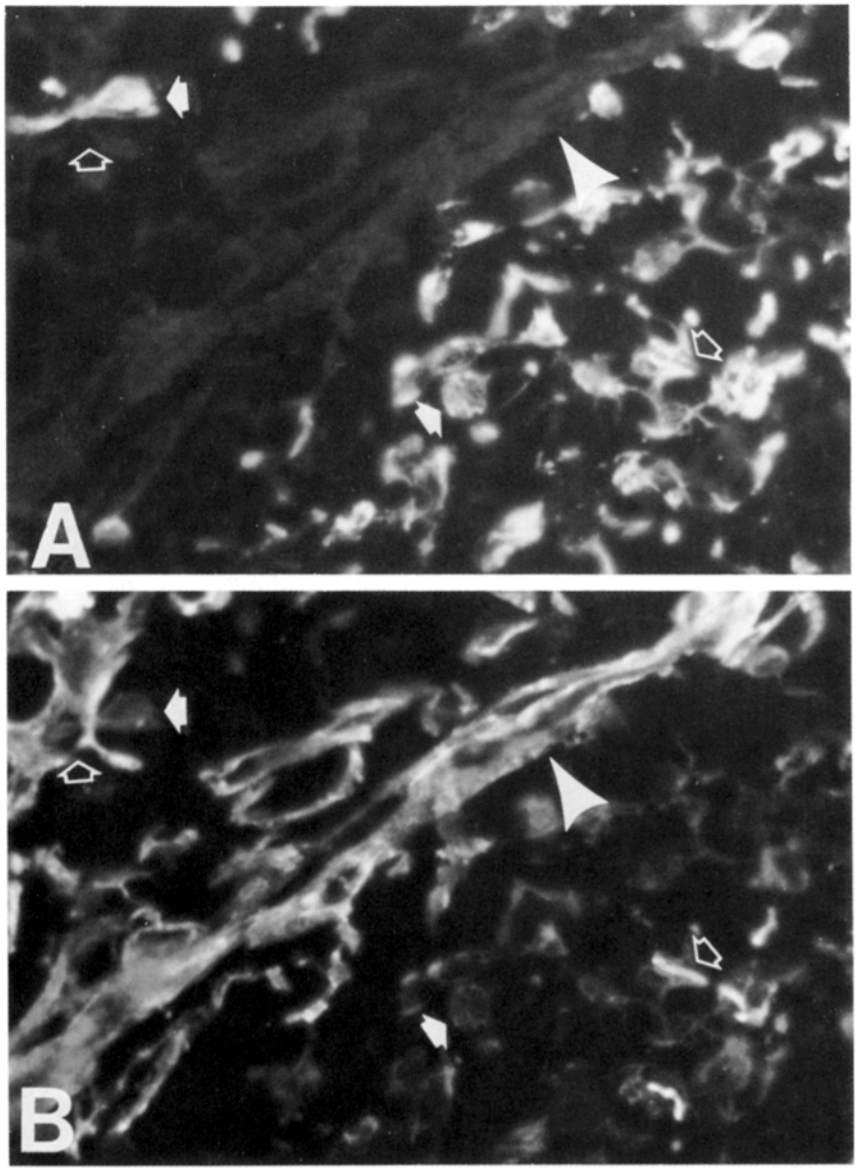

FIGURE 2. Direct comparison of reactivity of directly fluoresceinated TE-7 and indirectly rhodaminated TE-4. (*A*) TE-4$^+$ dendritic cells in normal 3-mo-old thymic medulla (solid small arrows) and TE-4$^-$ adjacent stroma (large arrow). (*B*) TE-7$^+$ adjacent stroma (large arrow) with TE-4$^+$, TE-7$^-$ cells (solid small arrows). Although TE-4$^+$ and TE-7$^+$ cells were mutually exclusive, it was seen that TE-4$^+$ epithelial cells and TE-7$^+$ stromal cells were frequently in close physical contact (small open arrows, *A* and *B*). × 400.

TE-4 (Fig. 1*B*). Fibrous stroma, thymic capsule, and interlobular septae did not react with TE-4. In contrast, antibody TE-7 reacted with all structures within the interlobular septae, with the thymic capsule, and with coarse bands of stroma penetrating into the cortex and medulla (Fig. 1*C*). To determine the surface reactivity of TE-4 and TE-7 with thymocytes in suspension and also with viable

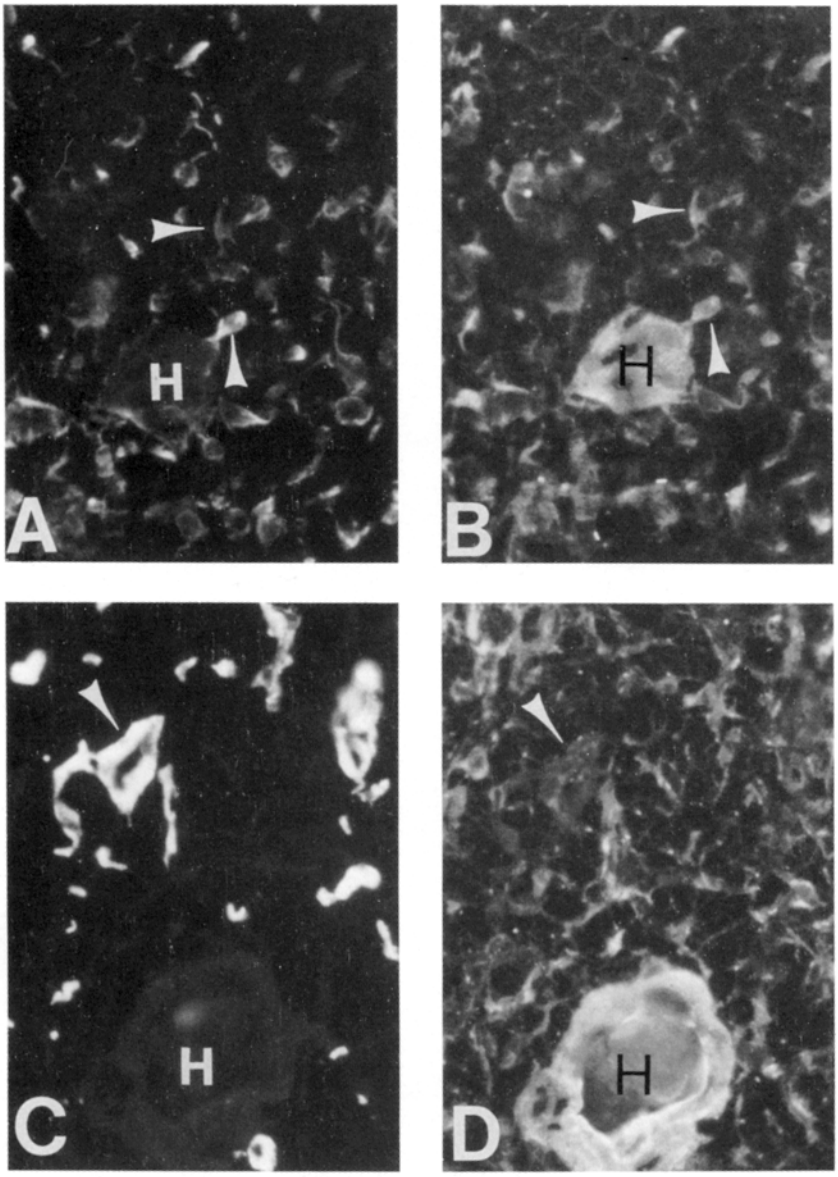

FIGURE 3. TE-4$^+$ epithelial cells contain thymosin α_1 while TE-7 stromal cells do not. TE-4 or TE-7 was sequentially incubated with rabbit anti-thymosin α_1 on normal 3-mo-old thymus sections in double immunofluorescence assay (see Materials and Methods). While TE-4$^+$ thymic epithelial cells were uniformly anti-thymosin α_1^+ (*A* and *B*, arrows), TE-7$^+$ cells were thymosin α_1^- (*C* and *D*). HB (H) stained strongly with anti-thymosin α_1 (*B*) while only the outermost layer of epithelial cells surrounding HB were TE-4$^+$ (*A*). × 400.

thymic stromal cells, thymocytes were teased from stroma and assayed for reactivity with TE-4 and TE-7 in indirect immunofluorescence and analysis on an Ortho 50H cytofluorograph (Ortho Diagnostic Systems, Inc., Westwood, MA). In each of three normal thymocyte suspensions tested, neither TE-4 nor

TE-7 reacted with viable thymocytes. Similarly, viable thymic stromal tissue was minced and stained in suspension with TE-4 or TE-7 and assayed for surface fluorescence using indirect immunofluorescence by placing stained (1 mm^3) pieces of stroma in glycerol under coverslips and viewing with fluorescent microscopy. We found in three separate experiments that both TE-4 and TE-7 antibodies reacted with viable thymic stroma cells, indicating surface reactivity of antibodies TE-4 and TE-7.

Next, in double fluorescence studies using directly fluoresceinated TE-7 with TE-4, we found that the TE-4^+ and TE-7^+ stromal elements in thymus were mutually exclusive components of the thymic microenvironment (Fig. 2*A* and *B*). It was observed, however, that TE-4^+ and TE-7^+ stromal cells were frequently in close contact (small open arrows, Fig. 2*A* and *B*).

Relationship of Expression of TE-4 and TE-7 Antigens to Reactivity with Anti-Thymosin α_1, A2B5, Anti-p19 and AE-1 (Antikeratin). TE-4 or TE-7 antibody was sequentially incubated with rabbit anti-thymosin α_1 on normal thymus sections in a double immunofluorescence assay. While TE-4^+ thymic epithelial cells were uniformly thymosin $\alpha_1{}^+$ (Fig. 3*A* and *B*), TE-7^+ cells were uniformly thymosin $\alpha_1{}^-$ (Fig. 3*C* and *D*). Interestingly, HB stained strongly with anti-thymosin α_1 (Fig. 3*B*) while only the outermost layer of epithelial cells surrounding HB were TE-4^+ (Fig. 1*B*, 3*A* and *B*).

We have previously shown (21, 31) antibodies A2B5 and anti-HTLV p19 to be markers of endocrine (thymosin α_1-containing) thymic epithelium. Therefore, we investigated the reactivity of A2B5 or anti-p19 with either TE-4 or TE-7 antibodies in double immunofluorescence experiments. As expected from the pattern of anti-thymosin α_1 reactivity with TE-4^+ and TE-7^+ stroma, we found that TE-4^+ cells were A2B5$^+$, p19$^+$, while TE-7^+ cells were A2B5$^-$, p19$^-$ (not shown). In double immunofluorescence assays comparing AE-1 (antikeratin) reactivity and TE-4 or TE-7, we found that TE-4^+ cells were AE-1^+, whereas TE-7^+ cells were uniformly AE-1^- (not shown). Whereas AE-1 reacted strongly with HB, TE-4 did not.

Expression of MHC Antigens by Endocrine Thymic Epithelium (TE-4^+) and Nonendocrine Thymic Stroma (TE-7^+). To determine the MHC antigens expressed by TE-4^+ or TE-7^+ thymic stroma cells, either directly fluoresceinated anti-HLA-A, -B, and -C monoclonal antibody (3F10) or anti-Ia monoclonal antibody (L-243) was sequentially incubated on thymus sections that had been previously labeled with either TE-4 or TE-7 antibodies and rhodaminated goat anti-mouse Ig. In these double-labeling experiments, we found TE-4^+ thymic epithelium strongly expressed HLA and Ia antigen (Fig. 4*A–D*). While more cells were HLA$^+$ and Ia$^+$ than were TE-4^+, all TE-4^+ cells were clearly HLA$^+$ and Ia$^+$. In contrast, TE-7^+ thymic stroma was uniformly Ia$^-$. Areas of TE-7^+ stroma were also HLA unreactive, while foci of TE-7^+ thymic stroma reacted weakly with anti-HLA antibody (Figs. 4*E–H*). Table I summarizes the phenotype of TE-4^+ and TE-7^+ human thymic microenvironment components.

Tissue Distribution of TE-4 and TE-7 Reactivity. Reactivity of TE-4 and TE-7 antibodies with a variety of normal tissues and cell types was determined using indirect immunofluorescence (Table II). TE-4 reacted only with the endocrine thymic epithelium and, interestingly, with the basal layer of squamous epithelium

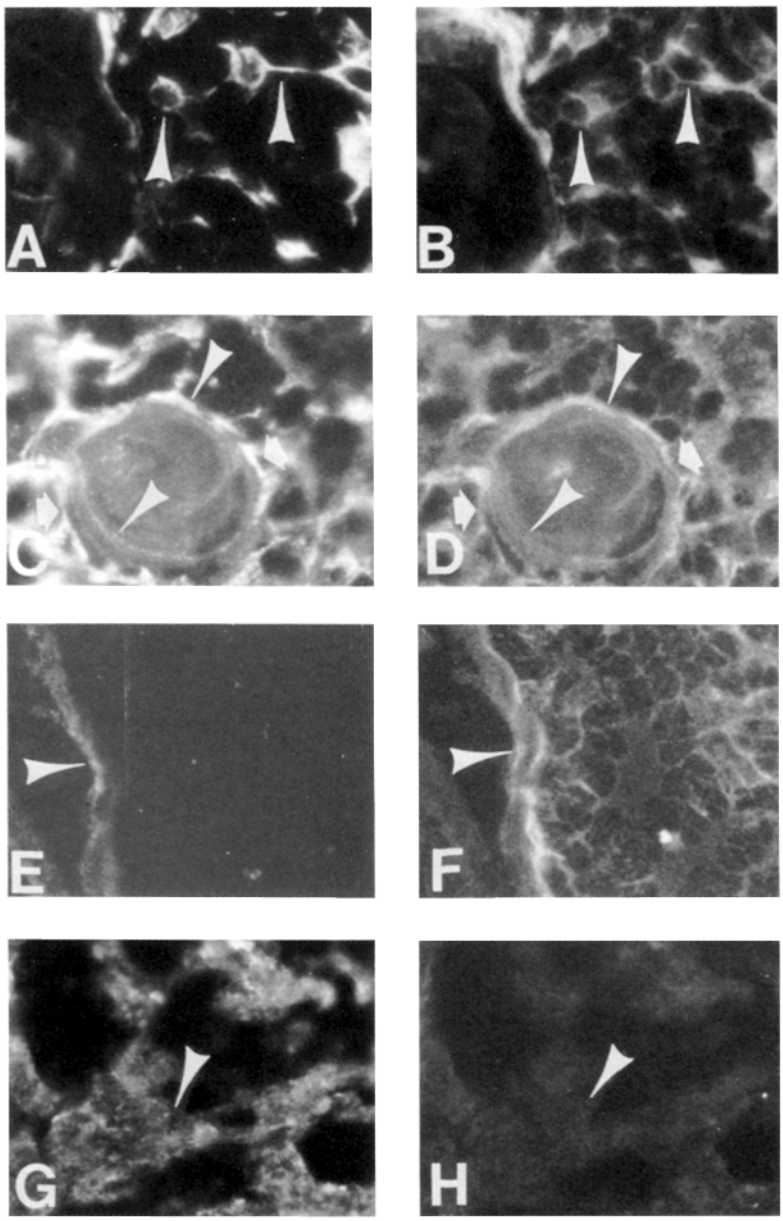

FIGURE 4. Expression of MHC antigens by endocrine thymic epithelium (TE-4$^+$) and thymic stroma (TE-7$^+$). Either directly fluoresceinated anti-HLA (3F10) or anti-Ia (L-243) was sequentially incubated on normal 3-mo-old thymus sections previously labeled with either TE-4 or TE-7 and rhodaminated goat antimouse Ig. TE-4$^+$ epithelium (*A* and *C*, arrows) strongly expressed HLA antigens (*B*, arrows) and Ia antigens (*D*, arrows). In *C* and *D*, it is easiest to compare reactivity of TE-4 and anti-Ia antibody around HB. As TE-4$^+$ epithelial cells flatten to form the concentric layer of cells within HB, the simultaneous expression of TE-4 and Ia can be seen (large and small arrows). The TE-7$^+$ stroma (*E* and *G*, arrows) was either weakly HLA$^+$ or HLA$^-$ (*F*, arrow) while all TE-7$^+$ stroma was Ia$^-$ (*H*, arrow). × 400.

TABLE I

Characteristics of TE-4$^+$ and TE-7$^+$ Components of Human Thymic Microenvironment

	TE-4	TE-7
Localized in subcapsular cortex and medulla	Yes	No
Localized in thymic capsule, interlobular septae	No	Yes
Contained thymosin α_1	Yes	No
Reactivity with:		
Anti-HLA (3F10)	Positive	Weakly positive to negative
Anti-Ia (L-243)	Positive	Negative
A2B5	Positive	Negative
Anti-p19	Positive	Negative
Antikeratin (AE-1)	Positive	Negative

TABLE II

*Reactivity Patterns of TE-4 and TE-7 Monoclonal Antibodies with Normal Tissues**

Tissue	Antibodies	
	TE-4	TE-7
Thymus‡	Endocrine thymic Epithelium	Non-endocrine thymic stroma and vessels
Spleen	Negative	Fibrous stroma and vessels
Tonsil	Basal layer of squamous epithelium	Fibrous stroma and vessels
Lymph node	Negative	Fibrous stroma and vessels
Skin	Basal layer of squamous epithelium	Fibrous stroma and vessels
Esophagus	Basal layer of squamous epithelium	Fibrous stroma and vessels
Liver	Negative	Fibrous stroma and vessels
Kidney	Negative	Fibrous stroma and vessels
Brain (cortex)	Negative	Fibrous stroma and vessels
Brain (medulla oblongata)	Negative	Fibrous stroma and vessels
Pancreas	Negative	Fibrous stroma and vessels
Adrenal	Negative	Fibrous stroma and vessels
Thyroid	Negative	Fibrous stroma and vessels
Pituitary	Negative	Fibrous stroma and vessels
Conjunctiva	Basal layer of squamous epithelium	Fibrous stroma and vessels
Appendix	Negative	Fibrous stroma and vessels

* Reactivity of monoclonal antibodies was determined by indirect immunofluorescence on acetone-fixed frozen tissue sections.

‡ In addition to assay on frozen tissue sections, reactivity of TE-4 and TE-7 with peripheral blood mononuclear cells (three subjects), thymocytes (three subjects), and tonsillar lymphocytes in suspension (two subjects) were assayed by indirect fluorescence, analyzed on an Ortho 50H cytofluorograph and found to be TE-4$^-$ and TE-7$^-$ in all cases. Reactivity of TE-4 and TE-7 was also determined on human foreskin fibroblast lines. Fibroblasts growing in culture were strongly TE-7$^+$ with both surface and intracytoplasmic reactivity; human foreskin fibroblasts did not react with antibody TE-4.

in skin (Fig. 5*A*), esophagus, tonsil (Fig. 5*B*), and conjunctiva. In tonsil, the TE-4$^+$ epithelial layer at the base of tonsillar crypts splayed out into a dendritic pattern (not shown). Unlike antibody A2B5, which reacted with most endocrine cell types (28, 29), antibody TE-4 did not react with islet cells of pancreas, anterior pituitary, or adrenal medulla (Table II). In contrast to TE-4, antibody

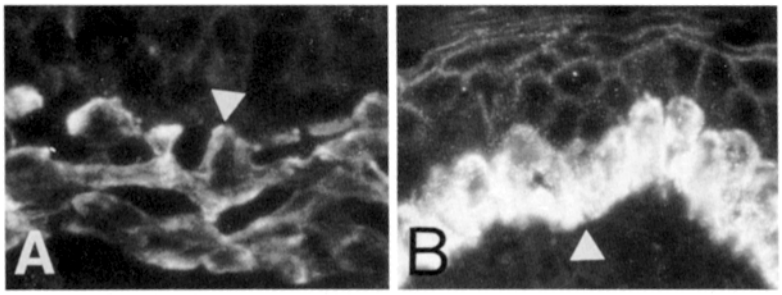

FIGURE 5. Reactivity of antibody TE-4 with extrathymic epithelial cells. Antibody TE-4 reacted with the basal layer of tonsillar (*A*, arrow) and skin (*B*, arrow) epithelium. Suprabasilar epithelial cells were faintly TE-4$^+$ while stratum granulosum and stratum corneum were TE-4$^-$ (not shown). × 400.

TABLE III

*Reactivity of TE-4 and TE-7 Monoclonal Antibodies with Fresh Malignant Tumor Cells and Malignant Cell Lines**

Tumor (No. of samples tested)	Antibody	
	TE-4	TE-7‡
Fresh tissue		
Melanoma in skin (1)	Negative	Negative
Cutaneous T cell lymphoma in skin (3)	Negative	Negative
T cell lymphoma of ileum (1)	Negative	Negative
Peripheral blood T cell acute lymphoblastic leukemia cells (1)	Negative	Negative
Breast carcinoma (3)	Negative	Negative
Lung squamous cell carcinoma (1)	Negative	Negative
Fibrosarcoma in skin (1)	Negative	Positive
Ovary adenocarcinoma (1)	Negative	Negative
Prostate adenocarcinoma (1)	Positive	Negative
Prostate adenocarcinoma (3)	Negative	Negative
Thymoma (4)	Positive	See Text
Cell lines		
4475-Breast adenocarcinoma	Negative	Negative
5577-Lung carcinoma	Negative	Negative
HUT 102 HTLV$^+$ cutaneous T cell lymphoma cell line	Negative	Negative

* Reactivity of monoclonal antibodies was determined by indirect immunofluorescence on acetone-fixed frozen tissue sections or on cytocentrifuge preparations of cell suspensions.

‡ While TE-7 reactivity was uniformly negative on tumor cells in all tissues except fibrosarcoma, TE-7 reacted with the nonmalignant supporting stroma in all tissues tested.

TE-7 was not tissue specific, since TE-7 reacted with fibrous stroma in every tissue tested (Table II).

Next, reactivity of TE-4 and TE-7 monoclonal antibodies with malignant cells was tested (Table III). TE-4 was nonreactive with carcinomas of lung and breast, but was reactive with 1 of 4 adenocarcinomas of prostate tested. All other tumor

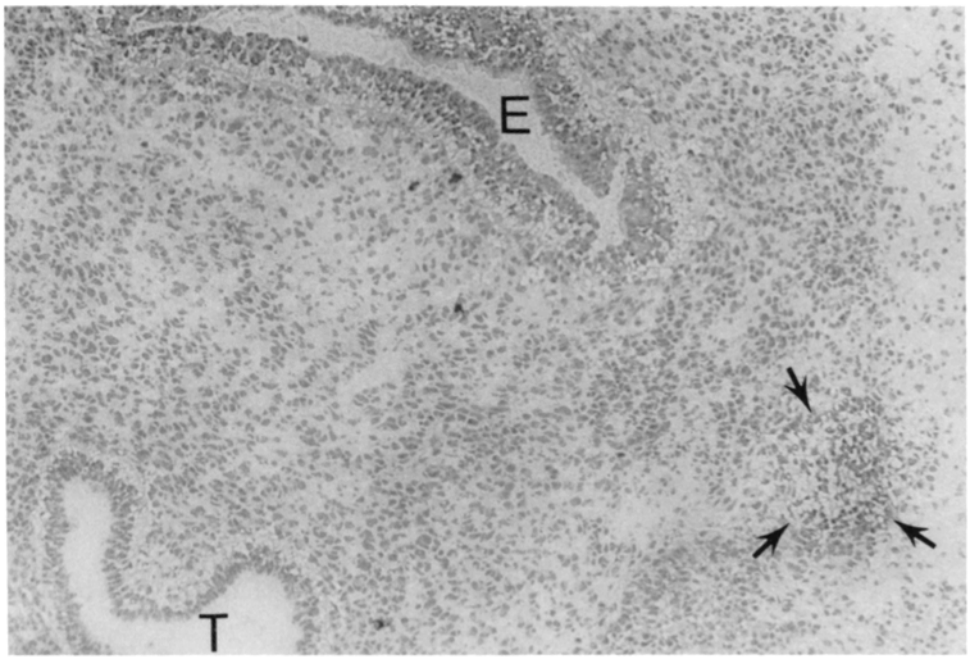

FIGURE 6. Human 7-wk fetal thymus. This transverse section through the upper thorax of a 17-mm fetus shows the esophagus (E), trachea (T), and the left lateral thymic rudiment (arrows) (hematoxylin and eosin stain). × 100.

types tested were TE-4 nonreactive except thymomas, which contained strands of TE-4$^+$ epithelial cells uniformly distributed throughout lymphoid areas.

The only malignant cells that were TE-7$^+$ were in skin fibrosarcoma (Table III). TE-7$^+$ stroma was present throughout thymoma tissue. Given that the malignant cell type in thymomas is not clearly understood, we could not assess whether the thymoma TE-7$^+$ stroma was benign or malignant. Phylogenetically, TE-4 and TE-7 were not reactive with rat, rabbit, and mouse thymus.

Thus, the antigen defined by antibody TE-7 appears to be a marker for mesodermally derived (mesenchymal) human connective tissue and tumors derived from such tissue (e.g., fibrosarcomas). In contrast, antibody TE-4 defines A2B5$^+$, p19$^+$, thymic epithelial cells that contain thymic hormones and keratin and are physically located in the thymic subcapsular cortex and medulla. Unlike antibody A2B5, antibody TE-4 is specific for thymic epithelial cells, with the exception of TE-4 expression on the basal cell layer of squamous epithelium and on rare tumors of epithelial origin (prostate). Unlike antikeratin (AE-1), TE-4 did not bind to HB.

Ontogeny Study of TE-4 and TE-7 Components of Human Thymus Microenvironment. During morphogenesis of the human thymus gland, the fibrous capsule, stroma, and vessels are derived from mesodermal or mesectodermal (mesenchymal) tissue (3, 4, 36). The cortical epithelial component of the thymic microenvironment is thought to be derived from the ectodermal brachial cleft, while the medullary epithelium is thought to be derived from the third pharyngeal pouch endoderm (1, 2, 5, 6). Auerbach (3, 4) has demonstrated that the mesodermal

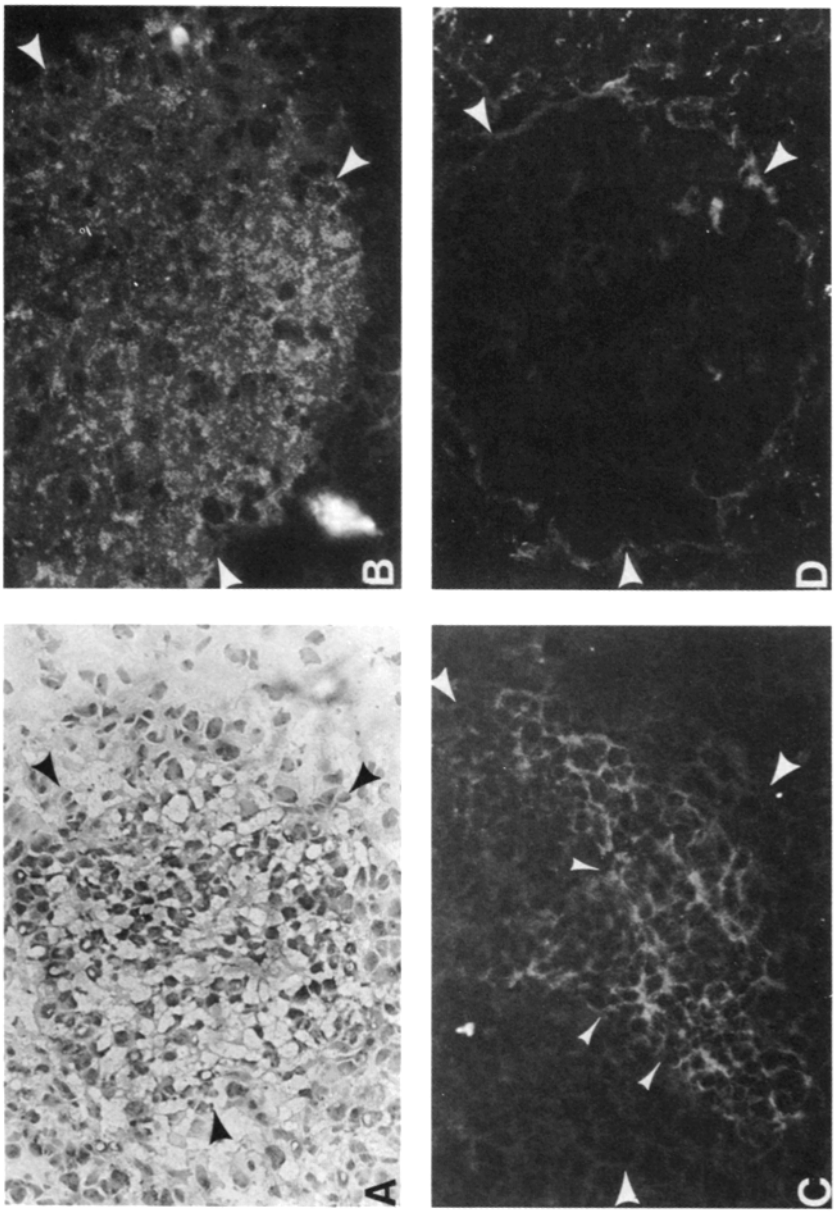

FIGURE 7. Phenotypic characterization of human 7-wk fetal thymus. (*A*) High power view (× 200) of 7-wk thymus (hematoxylin and eosin); (*B*) the entire rudiment stained with A2B5 in a granular pattern; (*C*) a central portion of the thymic rudiment was TE-4$^+$; and (*D*) while the entire epithelial component of the thymic rudiment was TE-7$^-$, TE-7$^+$ thymic stroma surrounded the thymus. (*A–D*) Arrows demarcate the thymic rudiment. × 400.

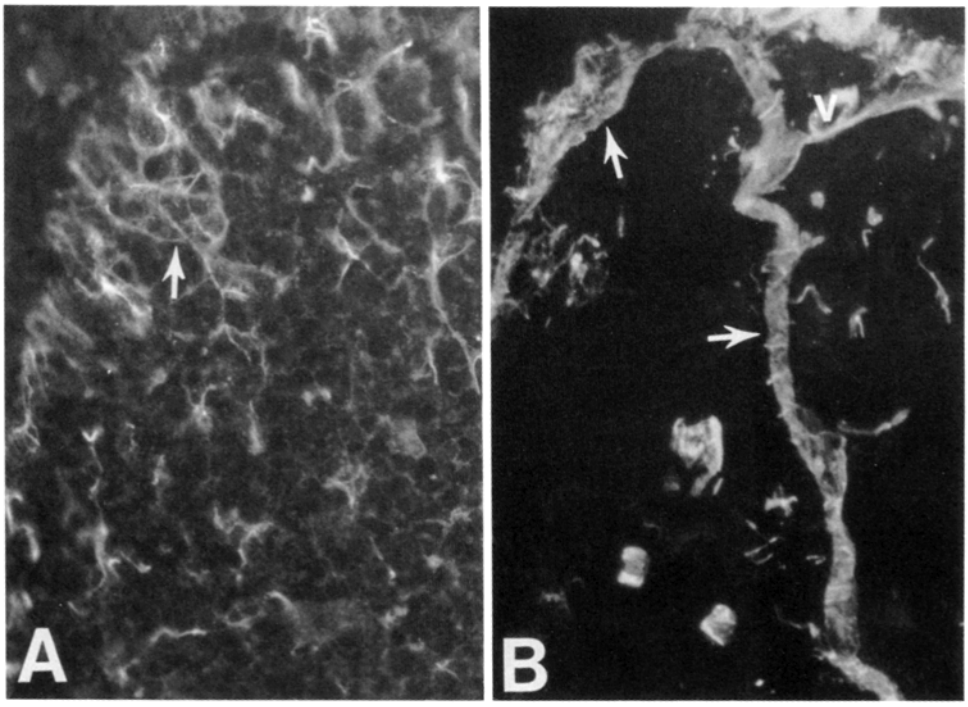

FIGURE 8. Human 12-wk fetal thymus. (*A*) TE-4$^+$ epithelium in a dendritic pattern (arrows) in the 12-wk fetal thymus, which is now populated with E-rosette$^+$, 3A1$^+$ thymocytes (T cell markers not shown). (*B*) TE-7$^+$ fibrous capsule, vessels (V), and interlobular septum (arrows) in the lobulated 12 wk fetal thymus. × 400.

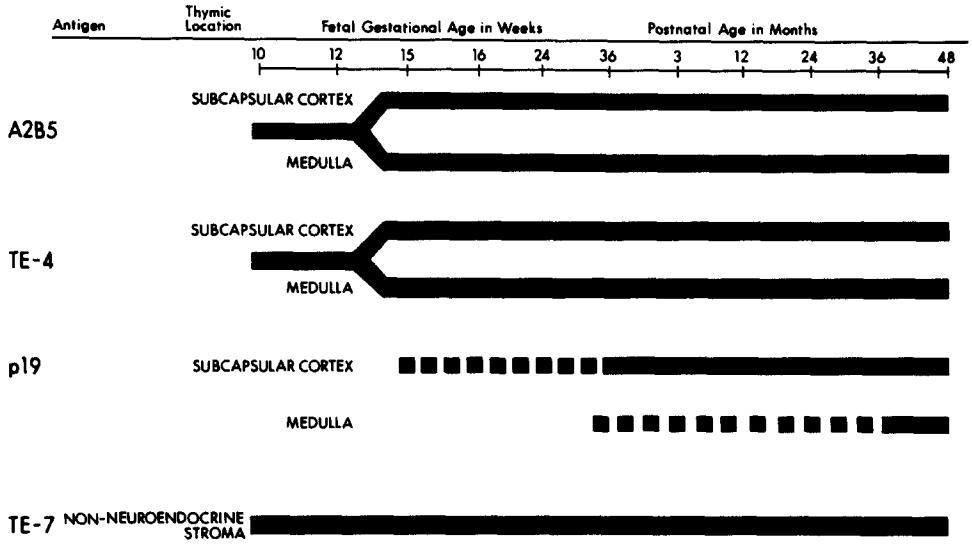

FIGURE 9. Acquisition of human thymic epithelial antigens during ontogeny. Whereas A2B5, TE-4, and TE-7 antigens were preserved throughout ontogeny, the p19 antigen was selectively acquired during ontogeny (31).

component of the thymus microenvironment (fibroblasts and vessels) surrounds the thymic primordia in the human at 6–7 wk gestation (3, 4). Thus, from the characterization of the reactivity of TE-4, TE-7, A2B5, and p19 monoclonal antibodies, we expected TE-7 to identify the outer mesenchymal connective tissue of the preseptated thymic primordia (7–9 wk gestation), and to identify interlobular septal tissue, vessels, and thymic capsule of lobulated fetal thymus (10–12 wk gestation). In contrast, TE-4 and A2B5, if conserved during ontogeny, should be present on epithelial components of thymic epithelium.

An ontogeny study of the reactivity of antibodies A2B5, p19, TE-4, and TE-7 was performed on seven fetal and five infant thymuses of the following ages: Fetal– 7, 9, 10, 13, 15, 16, and 24 wk gestation; postnatal– 3, 6, 12, 36, and 48½ mo. The thorax of the earliest fetus available for study (7 wk gestational age with a 17-mm crown-rump length) was serially sectioned (4 μm) from the base of the heart through the oral cavity. Hematoxylin and eosin (H and E) stains of sections were performed at 80-μm intervals. At 7 wk, the thymic rudiments were still paired tubular structures ~160 μm in length ventral to the carotid artery, which had not yet fully migrated caudally and fused at the midline (1, 2, 6). Thymic epithelial rudiments until the 9th to 11th wk were devoid of lymphoid cells entirely (6, 37). Fig. 6 demonstrates a low power view (× 100) of the 7 wk thymic rudiment, Fig. 7*A*, a high power (× 200) view. In Fig. 7*B* the A2B5$^+$ thymic epithelium is depicted (arrows), while 7*C* shows TE-4$^+$ thymic epithelium. Fig. 7*D* shows the outer rim of TE-7$^+$ mesodermally derived connective tissue (arrows) surrounding TE-7$^-$ epithelial thymus. The entire epithelial thymus at 7 wk was p19$^-$ (not shown). From 9 to 13 wk, TE-4$^+$, A2B5$^+$ epithelium was arranged in lobulated zones surrounded by TE-7$^+$ areas with no discernable cortical-medullary junction (Fig. 8*A* and *B*); no HB were seen. After 9 wk gestational age, numerous thymocytes were present that expressed the T cell antigens 35.1 (E-rosette receptor) and 3A1 (24, 35). By 15 wk, a cortical-medullary junction was present and the TE-4$^+$ component had compartmentalized into the subcapsular cortical and medullary areas. Furthermore, at 15 wk HB were present as seen in normal infant thymuses (not shown). As previously described, anti-p19 defined an antigen on endocrine (TE-4$^+$) epithelium that was acquired during ontogeny, first appearing in the subcapsular cortex region at between 12 and 15 wk fetal gestation (31).

Fig. 9 summarizes the results of the ontogeny study of reactivity of A2B5, TE-4, anti-p19, and TE-7 antibodies with human thymic microenvironment components beginning at 10 wk of fetal gestation.

Discussion

Using two novel monoclonal antibodies, TE-4 and TE-7, our study characterizes two components of the human thymic microenvironment: the TE-4$^+$ endocrine epithelial cells and TE-7$^+$ nonendocrine fibrous stroma.

The thymic microenvironment is a complex epithelial-stromal compartment within which thymocytes mature and undergo a variety of phenotypic and functional changes. Embryonically, data suggest that at least three separate tissues come together during fetal development to form the thymic microenviroment. Mesodermal cells give rise to fibroblasts and other connective tissue cells. Thymic

epithelial cells have been postulated (1, 2) to be derived from brachial cleft ectoderm and the third pharyngeal pouch endoderm. Moreover, neural crest ectoderm has also been suggested to contribute to the thymic microenvironment (36).

In both mouse (5) and man (1, 2), it has been shown that the thymus develops primarily from the third endodermal pharyngeal pouch and the third ectodermal cleft. In man (4–5 wk) and mouse (day 11), proliferation of the ectoderm of the third brachial cleft covers the endodermally derived epithelium (1–6). The endodermal thymic epithelial component appears to chemically attract immigrant blood-borne stem cells (3, 36) that in man first appear in the thymus at weeks 8–9 (6, 37). Thus, the epithelial component of mature (neonatal) normal thymic microenvironment is thought to be composed of a central endodermal region and a peripheral ectodermal region (2); cortical epithelial cells are thought to be ectodermal in origin while medullary epithelial cells are endodermal in origin (2, 5). Whether the TE-4$^+$ population of subcapsular cortical and medullary epithelial cells constitute the endodermal or the ectodermal component of thymic epithelium at present remains speculative. Our ontogeny study did not include embryos before 7 wk gestation, necessary to resolve this question. Clearly there are cortical epithelial cells that are TE-4$^-$, HLA$^+$, Ia$^+$, and contain keratin (10, 11). Moreover, we have recently developed a monoclonal antibody (TE-3) that selectively defines the cortical but not medullary component of thymic epithelium (E. McFarland, and B. F. Haynes, manuscript submitted).

The most likely origin for TE-4$^+$ epithelium, based on its physical location in thymus (medulla and subcapsular cortex) is pharyngeal pouch endoderm (2). However, the shared expression of TE-4 antigen by thymic epithelial cells with cells of known ectodermal origin (basal layer of squamous epithelium of conjunctiva and skin) suggests possible ectodermal origin of TE-4$^+$ thymic epithelium.

Regarding the shared expression of TE-4 antigen of thymic epithelial cells with basal epithelial cells, it should be noted that each of the markers of endocrine thymic epithelium (TE-4, p19, and A2B5) are expressed on the basal layer of squamous epithelium (21, 23, 31). Whereas A2B5 is also expressed on a variety of neuroendocrine tissues, the basal layer of squamous epithelium is the only known normal human tissue other than thymic epithelium that expresses p19 and TE-4. Interestingly, skin and tonsil epithelium have both been implicated in promoting extrathymic T cell maturation or activation (26, 38–40). Further, the basal layer of keratinocytes in skin have recently been shown to contain both thymopoietin-like and thymulin-like thymotrophic hormones (41, 42). Thus, regardless of the endodermal vs. ectodermal origin of TE-4$^+$ thymic epithelium, the presence of TE-4, A2B5, and p19 antigens on thymic epithelium and basal keratinocytes of squamous epithelium might signify common T cell trophic functions, and identify extrathymic as well as intrathymic T cell-inductive environments.

Using the tonofilament stain, tannic acid-phosphomolybdic acid-amido black (TPA), Pereira and Clermont (43) identified two components of the thymic microenvironment: TPA$^+$ epithelial reticular cells localized to the subcapsular cortex, cortex, and medulla, and TPA$^-$ fibrous capsule, interlobular septae, and

vessels. Most likely, a subset of TPA^+ epithelial reticular cells corresponds to $TE\text{-}4^+$ epithelium, and the TPA^- component to $TE\text{-}7^+$ stroma.

Several lines of evidence suggest that TE-4 does not bind to known keratin-like molecules. While all $TE\text{-}4^+$ cells contain keratin as defined by antibody AE-1, not all keratinized cortical epithelial cells were $TE\text{-}4^+$ nor were $AE\text{-}1^+$ HB reactive with TE-4. Moreover, TE-4 had surface reactivity with thymic epithelial cells, and skin and thymic epithelial cells grown in culture frequently are $TE\text{-}4^-$ and $AE\text{-}1^+$ ($antikeratin^+$) (K. Singer, E. A. Harden and B. F. Haynes, unpublished observations). TE-4, though having a similar reactivity pattern to anti-p19, differed from anti-p19 in that TE-4 was conserved in ontogeny beginning at 7 wk, and did not react with HTLV-infected T cells (HUT 102 cells).

At 5–7 wk gestation, mesenchymal cells derived from the fetal mesodermal germ layer form a network of fibroblasts that surround the human thymic rudiment (2–4). From 7 to 9 wk gestation, the surrounding connective tissue invaginates the thymus and sends fibrous septae throughout the epithelial rudiment, effecting thymic lobulation (2–4, 36). Mesoderm-derived mesenchymal tissue also gives rise to vessel endothelium, cartilage, and a variety of other tissues (6). While antibody TE-7 in the adult was specific for fibroblasts and vessels in all organs tested, it reacted with cartilage, vessels, and mesenchymal interstitial cells in early fetal tissues. Notocord, neural crest ectoderm, spinal cord, and all endodermal tissues were $TE\text{-}7^-$. Thus, TE-7 identified primarily cells of mesodermal origin in fetal and adult tissues. Regarding the thymus, TE-7 identified the mesodermal-derived fibrous septae and vessels. The results of our ontogeny study agreed with light microscopic descriptions of the relationship of fibrous ($TE\text{-}7^+$) stroma to thymic epithelial rudiments from 7 wk gestation through birth (2–4). We further demonstrated the TE-7 antigen to be conserved in ontogeny as early as 7 wk gestation.

In the quail-chick hybrid system, LeDouarin and Jotereau (36) demonstrated that mesenchyme from neural crest mesectoderm gave rise to thymic connective tissue and vessels. On the other hand, it has been suggested (44) that the neural crest contribution to the thymus forms the endocrine epithelial component, based on the known common function of neuroendocrine secretory cells and on the common expression of the complex ganglioside antigen defined by monoclonal antibody A2B5 on thymic epithelium and neural and neural crest-derived human and rodent tissues (21, 27–29). In the present study, we showed that all $TE\text{-}4^+$ cells that contained thymosin α_1 and keratin were $A2B5^+$. Conversely, all $TE\text{-}7^+$ mesenchymal-derived connective tissue cells were $A2B5^-$. Thus, the neural crest mesectodermal component of the thymic microenvironment proposed by LeDourain and Jotereau (36) most likely corresponds conceptually to the $TE\text{-}7^+$, $TE\text{-}4^-$ component of human thymic stroma. The presence of neural crest-derived mesenchyme in human thymus tissue, however, remains to be demonstrated.

In the postnatal thymus, the $TE\text{-}7^+$ component of thymic stroma was located in the interlobular septae and throughout the cortex and medulla as fibrous bands of Ia-negative and HLA weakly positive connective tissue. The importance of TE-7 as an antigenic marker of the mesodermal-derived component of thymic stroma is indicated by the observations of Auerbach (3) and others (36) that

mesodermal stroma may play an important role in inducing murine thymic epithelial differentiation and fetal thymic lobulation at the stage of fetal development just before lymphoid cell population of the thymic rudiment.

Thus, the availability of antibodies TE-4 and TE-7 should allow for the physical separation and in vitro study of the physiology of the mesodermal and endocrine epithelial components of fetal and postnatal thymic microenvironments. This approach should facilitate the study of these human thymic microenvironment components in normal thymus development and in the generation of T cell diversity and MHC restriction. Limited quantities of TE-4 and TE-7 antibodies are available to interested investigators upon request.

Summary

Using murine monoclonal antibodies TE-4 and TE-7 raised against human thymic stroma, we identified two distinct and mutually exclusive thymic microenvironment components: the thymic endocrine epithelium (TE-4^+) and mesodermal-derived fibrous stroma (TE-7^+). TE-4-reactive epithelium did not react with antibody TE-7, contained thymosin α_1 and keratin, and expressed other known markers of thymic endocrine epithelium (A2B5 and p19). Moreover, TE-4^+ thymic epithelial cells strongly expressed class I (HLA-A, -B and -C) and class II (Ia-like) major histocompatibility complex (MHC) antigens. In contrast, TE-7^+ thymic fibrous stroma did not react with antibody TE-4, did not contain thymosin α_1 nor keratin, and did not express the thymic endocrine epithelium markers A2B5 and p19. TE-7^+ thymic stromal cells weakly expressed class I and did not express class II MHC antigens. Both TE-4^+ and TE-7^+ thymic microenvironment compartments were identifiable in thymus from 7 wk gestation through adult life. At 7 wk fetal gestation, TE-7^+ stroma surrounded a cylindrical TE-4^+, A2B5$^+$ thymic epithelial rudiment. Between 10 and 15 wk fetal gestation, TE-7^+ thymic stroma surrounded early thymic lobules. By 15 wk fetal gestation, antibody TE-4 defined subcapsular cortical and medullary zones of endocrine thymic epithelium, while antibody TE-7 bound to interlobular fibrous septae, vessels, and thymic fibrous capsule. While otherwise specific for endocrine thymic epithelium, antibody TE-4 reacted with the basal layer of squamous epithelium in skin, tonsil, conjunctiva, and upper esophagus.

We thank Ms. Winifred Ho for expert technical assistance and Ms. Joyce Lowery for expert secretarial assistance.

Received for publication 31 October 1983 and in revised form 28 December 1983.

References

1. Weller, G. L. 1933. Development of the thyroid, parathyroid and thymus glands in man. *Contrib. Embryol. Carnegie Inst.* 22:95.
2. Norris, E. H. 1938. The morphogenesis and histogenesis of the thymus gland in man: in which the origin of the Hassall's corpuscles of the human thymus is discovered. *Contrib. Embryol. Carnegie Inst.* 27:193.
3. Auerbach, R. 1960. Morphogenetic interactions in the development of mouse thymus gland. *Dev. Biol.* 2:271.
4. Auerbach, R. 1961. Experimental analysis of the origin of cell types in the development of mouse thymus. *Dev. Biol.* 3:336.
5. Cordier, A. C., and S. M. Haumont. 1980. Development of thymus, parathyroids,

and ultimo-branchial bodies in NMRI and nude mice. *Am. J. Anat.* 157:227.
6. Patten, B. M. 1968. Human Embryology. McGraw-Hill Book Co., New York.
7. Kendall, M. D. 1981. The cells of the thymus. *In* The Thymus Gland. M. D. Kendall, editor. Academic Press, Inc., London. 63–83.
8. Rouse, R. V., and I. L. Weissman. 1981. Microanatomy of the thymus: its relationship to T cell differentiation. *In* Microenvironments in Haemopoietic and Lymphoid Differentiation. Ciba Foundation Symposium 84. Pitman, London. 161–177.
9. Kessel, R. G., R. H. Kardon. 1979. Tissues and Organs, A Text-Atlas of Scanning Electron Microscopy. W. H. Freeman & Co., San Francisco. p. 72.
10. Janossy, G., J. A. Thomas, F. J. Bollum, S. Granger, G. Pizzolo, K. F. Bradstock, L. Wong, A. McMichael, K. Ganeshaguru, A. V. Hoffbrand. 1980. The human thymic microenvironment: an immunohistological study. *J. Immunol.* 125:202.
11. Bhan, A. K., E. L. Reinherz, S. Poppema, R. T. McCluskey, and S. F. Schlossman. 1980. Location of T cell and major histocompatibility complex antigens in the human thymus. *J. Exp. Med.* 152:771.
12. Jenkinson, E. J., W. van Ewijk, and J. J. T. Owen. 1981. Major histocompatibility complex antigen expression on the epithelium of developing thymus in normal and nude mice. *J. Exp. Med.* 153:280.
13. Zinkernagel, R. M., and P. C. Dougherty. 1979. MHC-restricted T-cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction specificity, functionality and responsiveness. *Adv. Immunol.* 27:51.
14. Fink, P. J., and M. J. Bevan. 1978. H-2 antigens of the thymus determine lymphocyte specificity. *J. Exp. Med.* 148:766.
15. Kruisbeek, A. M., R. J. Hodes, and A. Singer. 1981. Cytotoxic lymphocyte responses by chimeric thymocytes. Self-recognition is determined early in T cell development. *J. Exp. Med.* 153:13.
16. Kruisbeek, A. M., S. O. Sharrow, B. J. Mathison, and A. Singer. 1981. The H-2 phenotype of the thymus dictates the self-specificity expressed by thymic but not splenic cytotoxic T lymphocyte precursors in thymus-engrafted nude mice. *J. Immunol.* 127:2168.
17. Singer, A., K. S. Hathcock, and R. J. Hodes. 1982. Self-recognition in allogeneic thymic chimeras. Self-recognition by helper T cells from thymus-engrafted nude mice is restricted to the thymic H-2 haplotype. *J. Exp. Med.* 155:339.
18. Kruisbeek, A. M., M. J. Fultz, S. O. Sharrow, A. Singer, and J. J. Mond. 1983. Early development of the T cell repertoire. In vivo treatment of neonatal mice with anti-Ia antibodies interferes with differentiation of I-restricted T cells but not K/D-restricted T cells. *J. Exp. Med.* 157:1932.
19. Schmidt, D., J. C. Monier, M. Dardenne, J. M. Pleau, P. Deschaux, and J. F. Bach. 1980. Cytoplasmic localization of FTS (facteur thymique serique) in thymic epithelial cells. An immunoelectron microscopical study. *Thymus.* 2:177.
20. Goldstein, A. S., T. L. K. Low, G. B. Thurman, M. M. Zatz, N. Hall, J. Chen, S. K. Hu, P. B. Naylor, and J. E. McClure. 1981. Current status of thymosin and other hormones of the thymus gland. *Recent Prog. Horm. Res.* 37:369.
21. Haynes, B. F., K. Shimizu, and G. S. Eisenbarth. 1983. Identification of human and rodent thymic epithelium using tetanus toxin and monoclonal antibody A2B5. *J. Clin. Invest.* 71:9.
22. Beardsley, T. R., M. Piersebacher, G. D. Wetzel, and E. F. Hays. 1983. Induction of T-cell maturation by a cloned line of thymic epithelium (TEPI). *Proc. Natl. Acad. Sci. USA.* 80:6005.
23. Haynes, B. F., R. W. Warren, R. H. Buckley, J. E. McClure, A. L. Goldstein, F. W.

Henderson, L. L. Hensley, and G. S. Eisenbarth. 1983. Demonstration of abnormalities of expression of thymic epithelial surface antigens in severe cellular immunodeficiency diseases. *J. Immunol.* 130:1182.

24. Haynes, B. F., G. S. Eisenbarth, and A. S. Fauci. 1979. Production of a monoclonal antibody that defines functional thymus-derived subsets. *Proc. Natl. Acad. Sci. USA* 76:5829.
25. Scearce, R. M., and G. S. Eisenbarth. 1984. Production of monoclonal antibodies reacting with the cytoplasm and surface of differentiated cells. *Methods Enzymol.* In press.
26. Haynes, B. F., L. L. Hensley, and B. V. Jegasothy. 1982. Differentiation of human T lymphocytes. II. Phenotypic difference in skin and blood malignant T cells in cutaneous T cell lymphomas. *J. Invest. Dermatol.* 78:323.
27. Eisenbarth, G. S., F. S. Walsh, and M. Nirenberg. 1979. Monoclonal antibody to a plasma membrane antigen of neurons. *Proc. Natl. Acad. Sci. USA.* 76:4913.
28. Eisenbarth, G. S., K. Shimizu, M. Conn, B. Mittler, and S. Wells. 1981. Monoclonal antibody F12 A2B5: expression on neuronal and endocrine cells. In Monoclonal Antibodies to Neural Antigens. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 209–218.
29. Eisenbarth, G. S., K. Shimizu, M. A. Bowring, and S. Wells. 1982. Expression of receptors for tetanus toxin and monoclonal antibody A2B5 by pancreatic islet cells. *Proc. Natl. Acad. Sci. USA.* 79:5066.
30. Robert-Guroff, M., F. W. Ruscetti, L. E. Posner, B. J. Poiesz, and R. C. Gallo. 1981. Detection of the human T cell lymphoma virus p19 in cells of some patients with cutaneous T cell lymphoma and leukemia using a monoclonal antibody. *J. Exp. Med.* 154:1957.
31. Haynes, B. F., M. Robert-Guroff, R. S. Metzgar, G. Franchini, V. Kalyanaraman, T. J. Palker, and R. C. Gallo. 1983. Monoclonal antibody against human T-cell leukemia virus p19 defines a human thymic epithelial antigen acquired during ontogeny. *J. Exp. Med.* 157:907.
32. Haynes, B. F., E. G. Reisner, M. E. Hemler, J. L. Strominger, and G. S. Eisenbarth. 1982. Description of a monoclonal antibody defining an HLA allotypic determinant that includes specificities within the B5 cross reacting group. *Hum. Immunol.* 4:273.
33. Lampson, L., and R. Levy. 1980. Two populations of Ia-like molecules on a human B cell line. *J. Immunol.* 125:293.
34. Woodcock-Mitchell, J., R. Eichner, W. G. Nelson, and T. T. Sun. 1982. Immunolocalization of keratin polypeptides in human epidermis using monoclonal antibodies. *J. Cell. Biol.* 95:580.
35. Haynes, B. F., D. L. Mann, M. E. Hemler, J. A. Schroer, J. A. Shelhamer, G. S. Eisenbarth, C. A. Thomas, H. S. Mostowski, J. L. Strominger, and A. S. Fauci. 1980. Characterization of a monoclonal antibody which defines an immunoregulatory T cell subset for immunoglobulin synthesis in man. *Proc. Natl. Acad. Sci. USA.* 77:2914.
36. LeDouarin, N. M., and F. V. Jotereau. 1975. Tracing of cells of the Avian thymus through embryonic life in interspecific chimeras. *J. Exp. Med.* 142:17.
37. Papiernik, M. 1970. Correlation of lymphocyte transformation and morphology in the human fetal thymus. *Blood.* 36:470.
38. Rubenfeld, M. R., A. E. Silverstone, D. M. Knowles, J. P. Halper, A. Desota, C. M. Fenoglio, and R. L. Edelson. 1981. Induction of lymphocyte differentiation by epidermal cultures. *J. Invest. Derm.* 77:221.
39. Oosterom, R., L. Kater, and L. H. P. M. Rademakers. 1981. How unique is the thymus? Conditioned media from thymus and tonsil epithelial cultures share biological

activities in T-cell maturation. *Clin. Immunol. Immunopathol.* 19:428.
40. Sauder, D. N., C. S. Carter, S. I. Katz, and J. J. Oppenheim. 1982. Epidermal cell production of thymocyte-activating factor (ETAF). *J. Invest. Dermatol.* 79:34.
41. Chu, A. C., J. A. K. Patterson, G. Goldstein, C. L. Berger, S. Takezaki, and R. L. Edelson. 1983. Thymopoietin-like substance in human skin. *J. Invest. Dermatol.* 81:194.
42. Kato, K., S. Ikeyama, M. Takouki, A. Shino, M. Takeuchi, and A. Kakinuma. 1981. Epithelial cell components, immunoreactant with antiserum thymic factor (FTS): possible association with intermediate sized filaments. *Cell.* 24:885.
43. Pereira, G., and Y. Clermont. 1971. Distribution of cell web-containing epithelial reticular cells in the rat thymus. *Anat. Rec.* 169:613.
44. Kissel, P., J. M. Andre, and A. Jacquier. 1980. The endocrine cells of the thymus. *In* The Neurocrestopathies. Manon Publishing, New York. 131–134.