# LOCATION OF T CELL AND MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGENS IN THE HUMAN THYMUS*

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The essential role of the thymus in the differentiation and functional maturation of bone marrow-derived precursors into immunocompetent T lymphocytes is well established (1, 2). It is clear that most of the maturation occurs within the thymus itself, although there is evidence that some of the peripheral T lymphocytes are not completely mature and require the presence of the thymus or thymic products for full development (1). The process of intrathymic differentiation of T cells has been most thoroughly studied in the mouse, where it has been possible to trace cells with distinctive chromosomal markers in irradiated recipients (2) and to identify T cell-restricted surface antigens that become expressed on cells undergoing thymus-dependent differentiation (3). The early stages in differentiation from stem cell to thymocyte are associated with the acquisition of TL, Thy-1, and Ly-123 antigens (3). The great majority of lymphocytes in the thymus are Ly-123$^+$, TL$^+$, and Thy-1$^+$. With further maturation, the lymphocytes become TL$^-$, show a diminution of Thy-1 antigen, and show an increase in H-2 antigens, thus resulting in features that are characteristic of virtually all peripheral T cells. In addition, the Ly-1$^+$ and Ly-23$^+$ subsets develop, at least in part, within the thymus and are programmed for their respective roles as inducer (helper) and cytotoxic/suppressor cells. Moreover, the receptors capable of recognizing self major histocompatibility complex (MHC)[1] products are acquired within the thymus (4).

Until recently, studies of differentiation in the human thymus have been limited because of the paucity of antibodies capable of recognizing specific T cell surface antigens on human lymphocytes that correlate with functional properties. Lately, however, a series of such monoclonal antibodies has been developed (5, 6). Studies performed with these antibodies on suspensions of thymocytes and peripheral T cells have provided evidence for stages of differentiation of human thymocytes that are analogous to those of the murine system (5, 6).

The earliest cells found within the human thymus bear antigens shared by some bone marrow cells, but lack mature T cell antigens. These immature cells, which account for ~10% of the thymic lymphocytes, are reactive with two monoclonal antibodies, anti-T9 and anti-T10 (stage I). With maturation, thymocytes lose T9,

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[1] *Abbreviations used in this paper:* MHC, major histocompatibility complex; PAP, peroxidase anti-peroxidase; PB, peripheral blood; PBS, phosphate-buffered saline, pH 7.3.

retain T10, and acquire a thymocyte-distinct antigen defined by anti-T6. Concurrently, these cells express antigens defined by anti-T4, anti-T5, and anti-T8 (stage II). The $T4^+$, $T5^+$, $T6^+$, $T8^+$, and $T10^+$ thymocytes account for ~70% of the total thymic population. With further maturation, thymocytes lose T6 antigen, acquire the T1 and T3 antigens, and segregate into $T4^+$ and $T5^+/T8^+$ subsets (stage III). Immunologic competence is acquired at this stage, but it is not fully developed until thymic lymphocytes are exported.

The studies cited above did not provide evidence concerning the sites within the thymus where various stages of differentiation occur. In our study we have used a series of monoclonal antibodies against T cell antigens, as well as antibodies directed against $\beta_2$-microglobulin and Ia antigen, with immunofluorescence and immunoperoxidase techniques applied to tissue sections to determine the location within the thymus of various T cell and histocompatibility antigens.

## Materials and Methods

*Processing of Tissue.* Normal human thymic tissue was obtained from four patients, ranging from 2 mo to 10 yr of age, during cardiac surgical procedures. The blocks were frozen in OCT Compound (Ames Co., Div. of Miles Laboratory, Inc., Elkhart, Ind.).

*Monoclonal Antibodies.* A series of monoclonal antibodies that are reactive with thymocyte and/or peripheral T lymphocyte surface antigens was employed in this study (7–11). The antibodies were obtained in the form of ascitic fluid from mice injected with hybridoma cells. Control ascitic fluid was obtained from animals injected with nonsecreting hybridoma cells (7–11). The methods of production and characterization of the antibodies have been described elsewhere (7–11). The reactivity of the T cell-specific antibodies with thymocyte suspensions and peripheral T lymphocytes, as determined in previous studies by indirect immunofluorescence using a Cytofluorograf (Fc 200/4800A; Ortho Instruments, Westwood, Mass.), is shown in Table I.

In addition, anti-Ia monoclonal antibody (anti-I1) reactive with a nonpolymorphic region of the human Ia-like antigens (10) and a monoclonal antibody reactive with $\beta_2$-microglobulin (88) (gift of Dr. Lee Nadler, Sidney Farber Cancer Institute, Harvard Medical School, Boston, Mass.) were used.

*Immunofluorescence Studies.* 4-$\mu$-thick frozen sections were air dried and fixed in acetone for 5 min before staining. (In preliminary studies it was found that the use of unfixed frozen sections gave less satisfactory results.) Sections were incubated with a 1:20–1:500 dilution of ascitic fluid

TABLE I

*Reactivity of Monoclonal Antibodies with Human Thymocytes and Peripheral Blood (PB) T Cells and PB Non-T Cells (E Rosette Negative)**

| Monoclonal antibody | Suspensions of thymus | PB T cells | PB non-T cells |
|---|---|---|---|
| | | % | |
| Anti-T1 | 10‡ | 100 | 0 |
| Anti-T3 | 10 | 100 | 0 |
| Anti-T4 (inducer) | 75 | 60 | 0 |
| Anti-T5 (cytotoxic/suppressor) | 80 | 20 | 0 |
| Anti-T8 (cytotoxic/suppressor) | 80 | 30 | 0 |
| Anti-T6 | 70 | 0 | 0 |
| Anti-T10 | 95 | <5 | 10 |

* Data from reference 6.

‡ Percentage of reactivity (approximate).

containing one of the monoclonal antibodies at room temperature for 30 min; they were then washed in phosphate-buffered saline, pH 7.3 (PBS) and stained with fluorescein-labeled or unlabeled $F(ab')_2$ fragments of goat anti-mouse IgG (N. L. Cappel Laboratories, Inc., Cochranville, Pa.). Sections that were washed and coverslipped at this stage generally showed only very faint specific staining; accordingly, most sections were processed with an additional step, which consisted of incubation with fluorescein-labeled rabbit anti-goat IgG (N. L. Cappel Laboratories, Inc.) for 30 min; they were then washed and coverslipped. Some sections were incubated with control ascitic fluid in the first step and then were treated as described above for use as controls. In other controls, the first step was omitted entirely, but the fluorescein conjugates were applied as above.

*Immunoperoxidase Staining.* After being fixed in acetone for 5–10 min, 4-$\mu$-thick frozen sections were stained by a four-step peroxidase anti-peroxidase (PAP) method. The sections were incubated with a 1:500 dilution of ascitic fluid that contained monoclonal antibodies for 60 min at room temperature, followed by 30-min incubations with rabbit anti-mouse IgG (N. L. Cappel Laboratories, Inc.), swine anti-rabbit IgG, and finally with peroxidase-rabbit-anti-peroxidase reagent (Dakopatts A/S, Copenhagen, Denmark). Each incubation was followed by repeated washing with PBS. Staining was achieved by incubation of sections in an acetate buffer solution (pH 5.0) that contained 3-amino-9-ethyl carbazol (Aldrich Chemical Co., Inc., Milwaukee, Wis), dimethylformamide, and hydrogen peroxide (12). The sections were washed in acetate buffer and mounted in Elvanol (E. I. Du Pont de Nemours & Co., Wilmington, Del.).

## Results

In control sections of thymus, which were stained by the immunofluorescence or immunoperoxidase technique without the use of monoclonal antibodies, nonspecific staining was generally of low intensity, except for Hassalls' corpuscles, which stained moderately or intensely with all of the conjugates. However, very faint smooth or granular staining was sometimes seen around cells in both cortex and medulla in sections treated with control ascitic fluid. In addition, there was moderate to bright granular or diffuse nonspecific intracytoplasmic staining of large cells, presumably mast cells, that were most often seen in the capsules or septa, and, less often, scattered throughout the thymus.

The staining patterns and estimates of the percentages of positive cells in the cortex and medulla seen after incubation of sections with the monoclonal antibodies are recorded in Table II; for purposes of comparison, the reactivity of thymocyte suspensions with these antibodies is also given.

In sections treated with antibodies to T4, T5, T6, T8, or T10, the great majority of the cells in the cortex were stained, indicating coexpression of multiple antigens. The staining with all these reagents was seen at the periphery of the cells, which probably resulted from cell membrane staining (Figs. 1 and 2). The staining was very intense in sections treated with T6 and T8 antibodies. In studies performed by the immunofluorescence technique, where various dilutions of antibodies were used, the intensity of fluorescence was not related to the dilution of antibody. Although the pattern of staining in the cortex with all the monoclonal antibodies was similar by both immunofluorescence or immunoperoxidase techniques, the latter method seemed to define a larger number of reactive cells in the medulla. This was particularly apparent with anti-T4 antibody; $T4^+$ cells were not seen in the medulla when indirect immunofluorescence was used, whereas with the PAP method, approximately one-half of the cells stained (Fig. 3). However, with either technique, the staining of medullary cells with antibodies to T4, T5, T6, T8, or T10 was much less intense than

TABLE II

*Location of Cell Surface Antigens in the Human Thymus by the Use of Monoclonal Antibodies*

| Monoclonal antibody | Cell suspension* | Tissue sections‡ | |
|---|---|---|---|
| | | Cortex | Medulla |
| | % | | |
| Anti-T1 | 10 | Intense peripheral staining of a small number of cells; faint peripheral staining of many cells | Intense peripheral staining of numerous cells |
| Anti-T3 | 10 | Same as anti-T1 | Same as anti-T1 |
| Anti-T4 | 75 | Peripheral staining of most of the cells | Peripheral staining of numerous cells§ |
| Anti-T5 | 80 | Peripheral staining of most of the cells | Peripheral staining of a minority of cells |
| Anti-T6 | 70 | Peripheral staining of most of the cells | A minority of cells show peripheral staining |
| Anti-T8 | 80 | Peripheral staining of most of the cells | Peripheral staining of a minority of cells |
| Anti-T10 | 95 | Peripheral staining of most of the cells | A minority of cells show peripheral staining |
| Anti-I1 (anti-Ia) | 0 | Ring of staining surrounding groups of unstained cells or individual cells in clusters | Diffuse‖ staining of an appreciable minority of cells (probably epithelial) |
| Anti-$\beta_2$-microglobulin (88) | 100 | Peripheral staining of all the cells; intense diffuse staining of scattered cells (probably epithelial) | Intense diffuse or peripheral staining of almost all the cells |

* Cytofluorographic analysis of thymus cell suspension performed by indirect immunofluorescence with fluorescein-conjugated goat anti-mouse IgG on a Cytofluorograf Fc200/4800A as described previously (6).

‡ Detection of antigens in 4-$\mu$m-thick acetone-fixed frozen sections, performed by the immunofluorescence and immmunoperoxidase (PAP) techniques (Materials and Methods).

§ Staining detected only with use of the PAP method (Materials and Methods).

‖ The term diffuse is used to describe staining throughout the cytoplasm; with this pattern, it cannot be determined whether membrane staining is also present.

that shown by the majority of cortical cells. Moreover, even with the more-sensitive PAP technique, only a minority of medullary cells stained with anti-T5, anti-T6, anti-T8, and anti-10 antibodies. An example of this type of staining is shown in Fig. 4.

Treatment of sections with antibodies to T3 and T1 resulted in distinctive staining patterns, which are similar to one another. About one-third to one-half of all cells in the medulla were stained by the immunofluorescence technique. The fluorescence was at the periphery of the cells and generally surrounded the entire cell in a pattern that was consistent with membrane staining. With the immunoperoxidase technique, relatively larger numbers of medullary cells (about two-thirds) were strongly stained (Fig. 5). In contrast, by both techniques, only a small percentage of cortical cells showed membrane staining (Figs. 5 and 6). The latter cells were present as either isolated cells or as small groups of cells scattered at random throughout the cortex.

Taken together, the above findings indicated that most cortical cells were T4, T5, T6, T8, and T10 reactive, whereas most medullary cells were reactive with anti-T1 and T3 and, to a lesser extent, with anti-T4.

To define the distribution of HLA-related antigens, we utilized monoclonal antibodies to both $\beta_2$-microglobulin and Ia antigens. With anti-$\beta_2$-microglobulin anti-

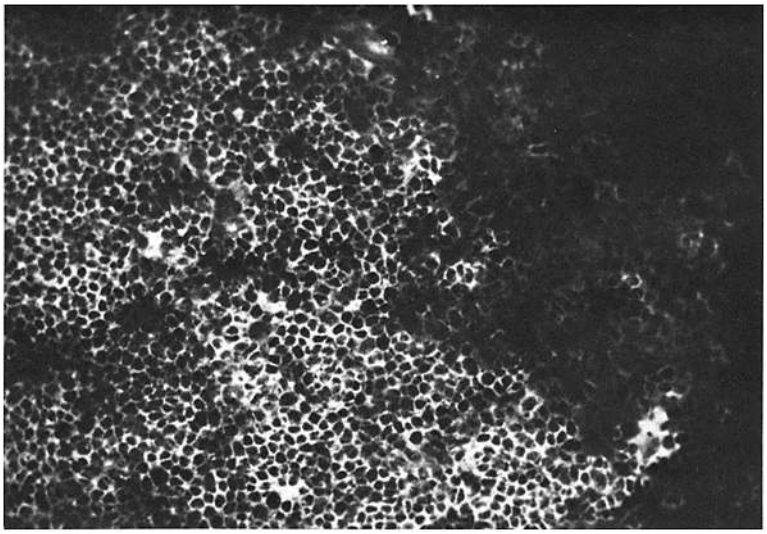

FIG. 1. Indirect immunofluorescence staining of thymus with anti-T6 antibody. A majority of cells in the cortex (on the left) show bright membrane staining. A few cells in the medulla are also stained. × 300.

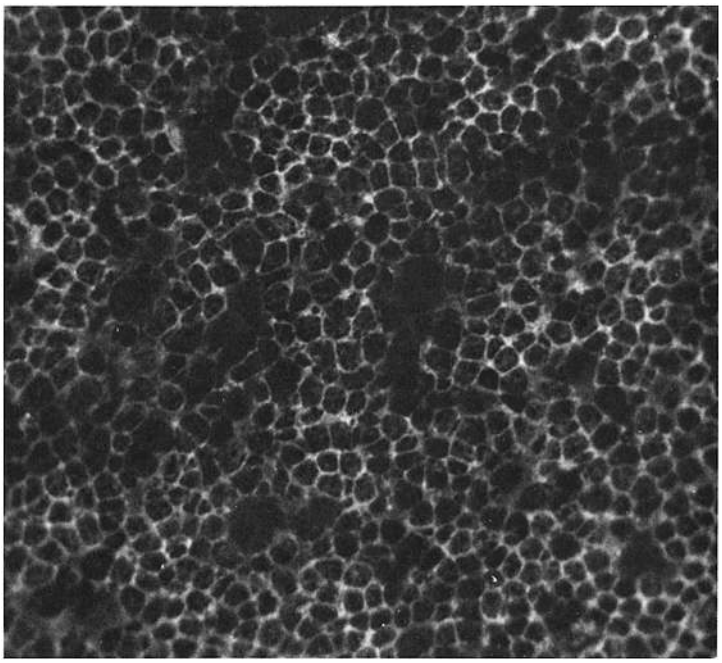

FIG. 2. Higher magnification of the section of thymus shown in Fig. 1. In between the majority of brightly stained thymocytes are unstained cells, which probably represent epithelial cells and some thymocytes. × 500.

body, the cells in the medulla were very brightly stained and showed both membrane staining and/or intracytoplasmic staining. Although most cells in the cortex showed membrane staining, the overall intensity of staining was considerably less than in the

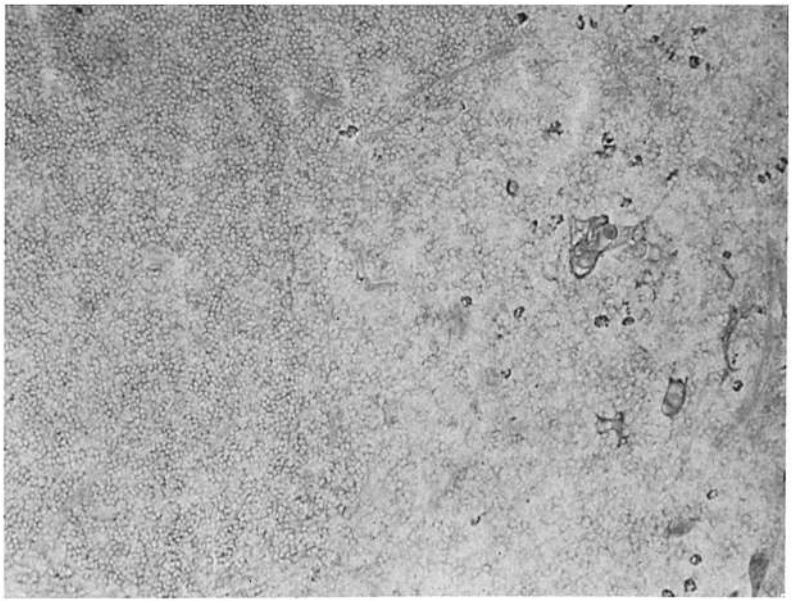

FIG. 3. Low-power view of thymus stained with anti-T4 antibody by the PAP method. A majority of cortical cells (on left side) show membrane staining, whereas smaller numbers of cells in the medulla show less-intense staining. Hassall's corpuscles and mast cells in the medulla are nonspecifically stained. × 160.

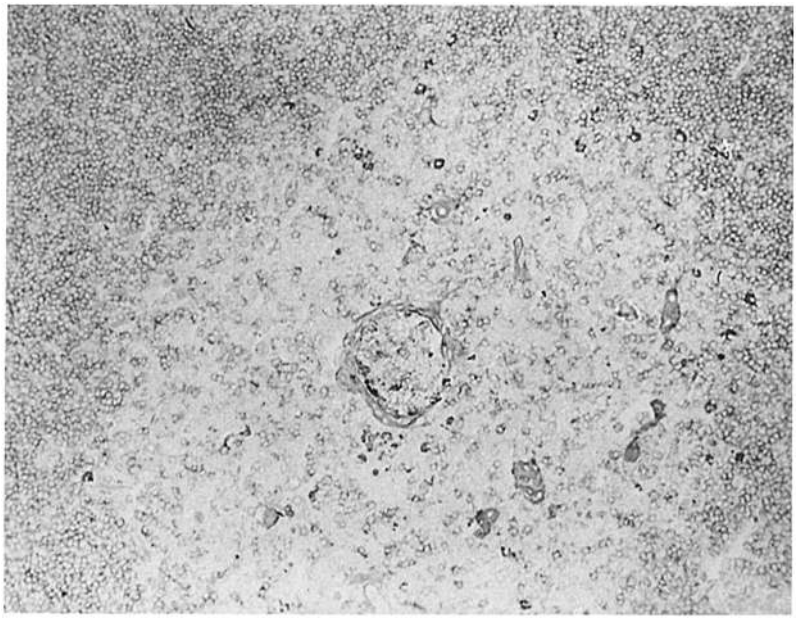

FIG. 4. Section of thymus stained with anti-T8 antibody by the PAP method. Central area, representing medulla, contains a Hassall's corpuscle, and shows a minority of cells with membrane staining, whereas the majority of cells in the surrounding cortex show intense membrane staining. × 160.

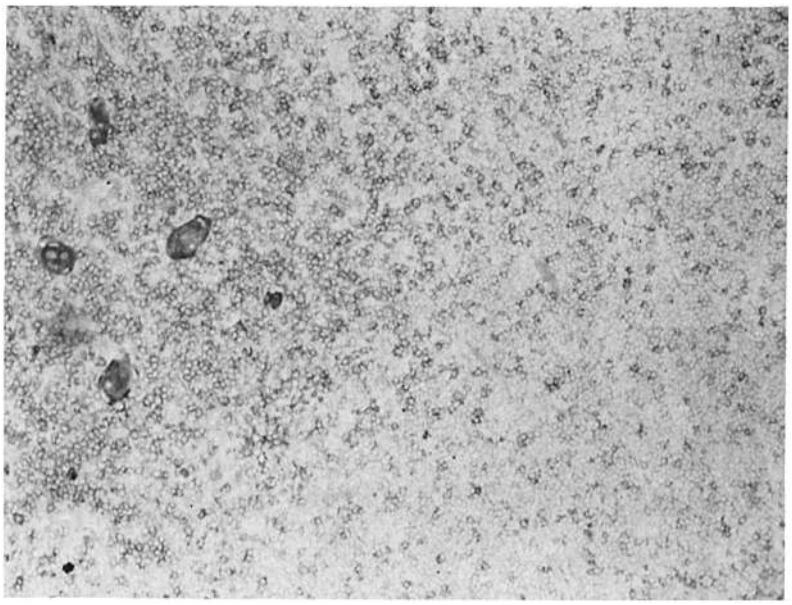

FIG. 5. Section of thymus stained with anti-T1 antibody by the PAP method. A majority of cells in the medulla (on the left) show intense membrane staining. Hassall's corpuscles are nonspecifically stained. A small number of intensely stained cells are present scattered in the cortex. In addition, many cortical cells show weak staining. × 160.

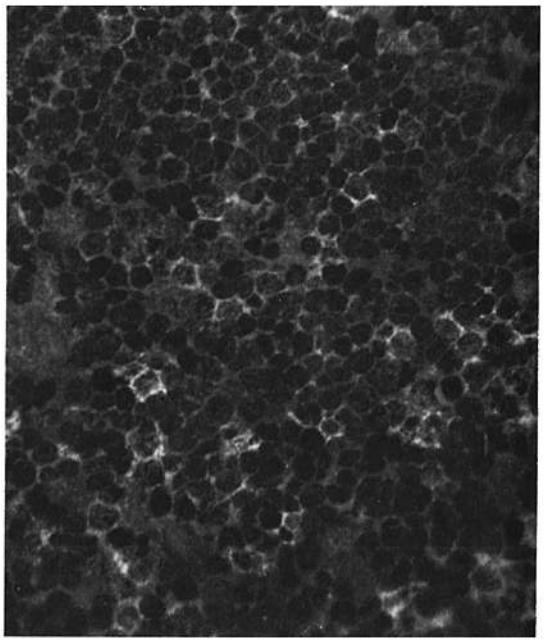

FIG. 6. Thymic cortex stained with anti-T3 antibody by the indirect immunofluorescence method. A small number of cells show bright peripheral fluorescence. × 500.

FIG. 7. Low-power view of thymus stained with anti-$\beta_2$-microglobulin antibody by the indirect immunofluorescence method. The cortex (on the right) shows peripheral staining of a majority of thymocytes. In addition, there are more-brightly stained cells scattered throughout the cortex (probably epithelial cells). Staining of endothelial cells is also seen. The medulla shows intense diffuse and membrane staining of almost all the cells. × 125

medulla (Fig. 7). However, it should be noted that some of the cells scattered throughout the cortex showed intense cytoplasmic staining. Moreover, endothelial cells lining vascular channels were reactive.

With anti-Ia antibody, predominantly diffuse cytoplasmic staining of large cells, presumably epithelial cells was seen in the medulla. Most of the medullary lymphocytes did not stain. In the cortex, two staining patterns were present; in some areas, groups of unstained cells were surrounded by a ring of staining (Fig. 8), whereas in other places, groups of cells showed peripheral staining around individual cells (Fig. 9). Endothelial cells lining vascular channels were also stained. It should be noted that $Ia^+$ cells have not been detected in cell suspensions prepared from human thymus (data not shown).

## Discussion

We have studied, by immunofluorescence and immunoperoxidase techniques, the presence and distribution of T cell-specific antigens, Ia antigens, and $\beta_2$-microglobulin in frozen tissue sections of human thymus using a series of monoclonal antibodies. The overall percentages of reactive cells appeared to correspond in general to the percentages obtained with thymocytes in suspension, as studied previously (5). Staining was more intense with the immunoperoxidase PAP technique than with the immunofluorescence technique, and this was particularly apparent in the medulla.

Although most cortical lymphocytes appeared to be reactive with antibodies to T4, T5, T6, T8, and T10, the intensity of staining seen with immunofluorescence varied, with $T6^+$ and $T8^+$ cells staining most brightly and $T4^+$ the least intensely. It is likely that the intensity of fluorescence was directly related to the density of the antigen defined by a given monoclonal antibody, because the antibodies were used in antibody excess, and identical fluorescein-labeled developing reagents were employed. With all

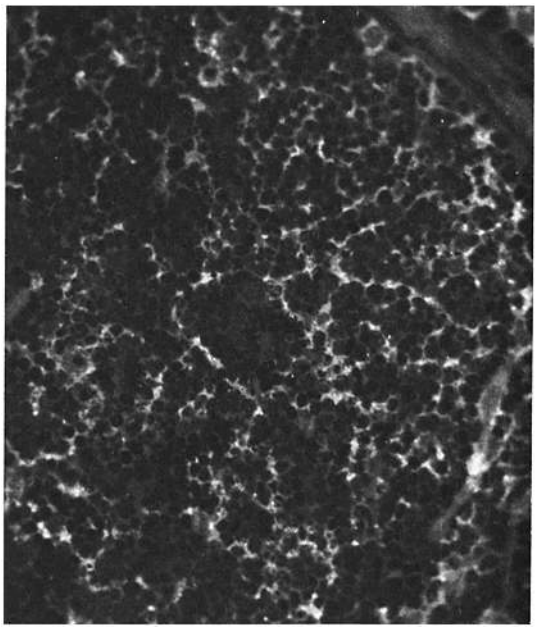

FIG. 8. Thymic cortex stained with anti-Ia antibody (anti-I1) by the indirect immunofluorescence method, showing a predominantly dendritic staining pattern. Groups of unstained thymocytes are surrounded by a ring of fluorescence. × 300.

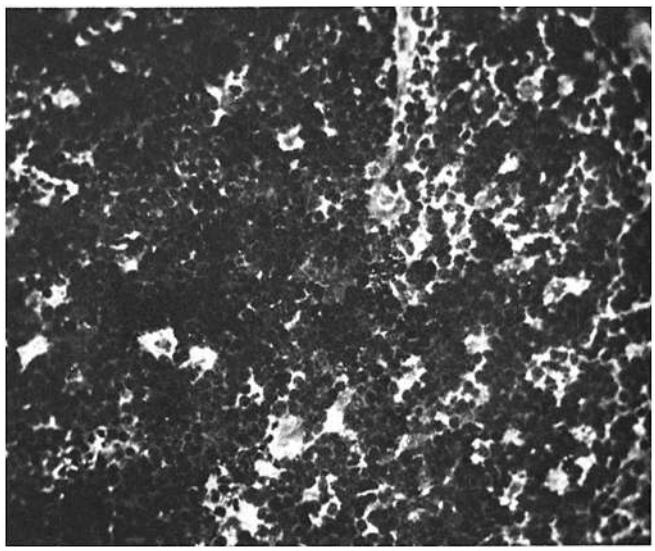

FIG. 9. Another view of cortex of thymus stained with anti-Ia antibody (anti-I1) by the immunofluorescence method. In addition to the dendritic staining pattern, individual thymocytes appear to be surrounded by a ring of fluorescence. × 300.

these antibodies, the intensity of staining in the medulla appeared to be appreciably less than in the cortex. However, there was no gradation of intensity of staining from outer cortex to cortico-medullary junction to suggest gradual loss of antigen from

cortex to medulla. These studies would support the earlier findings that the common thymocyte pool coexpresses T4, T5, T6, T8, and T10 antigens within the cortex (5).

Staining of cells in the medulla was most satisfactorily judged in sections prepared with the immunoperoxidase (PAP) technique. Many medullary cells showed reactivity with anti-T4 antibody, and a considerably smaller fraction stained with anti-T5, T6, T8, and T10 antibodies. In addition, anti-T1 and anti-T3 antibodies, which react with all peripheral T cells, intensely stained a majority (50–70%) of the medullary thymocytes. Given an earlier study demonstrating acquisition of T cell function with expression of T1 and T3 antigens by thymocytes (9), our findings support the conclusion that the most mature thymocytes are present largely in the medulla. Further support for this observation was obtained through the use of antibody to $\beta_2$-microglobulin, which showed more intense staining in the medulla than in the cortex. These observations are in keeping with studies in mice showing an increase in H-2 antigen expression with maturation (1, 3). The finding that a large percentage of medullary thymocytes were $T4^+$, whereas only a relatively small percentage were $T5^+$, suggests that divergence has already occurred in this lymphoid population.

These studies are consistent with earlier studies in the mouse showing that maturation generally proceeds from cortex to medulla. In the mouse, the outer cortex of the thymus is composed predominantly of large and medium rapidly dividing cells, whereas the mid-cortex and juxtamedullary cortex mainly contain small lymphocytes. Furthermore, as shown by Weissman (13), labeled cells appear in the deeper cortex and medulla 2–4 d after topical tritiated thymidine labeling of surface thymocytes. The administration of large doses of cortisone at the time of surface labeling, which results in destruction of most cortical thymocytes, prevents the subsequent appearance of labeled cells deep in the cortex or medulla. These findings suggest that at least some cells are derived from superficial cortical thymocytes. In addition, cortisone-resistant cells, which are located mainly in the medulla, account for the great majority of immunocompetent lymphocytes with peripheral T cell surface markers found within the thymus. Nevertheless, the often quoted conclusion that lymphocytes are exported largely or entirely from the medulla is not supported by direct evidence (2, 14). The possibility exists that some maturation of cells occurs preferentially in certain sites within the cortex, rather than proceeding uniformly from outer to inner cortex. Indeed, the present finding of single cells or clusters of cells that bear T1 and T3 antigens in the cortex suggests that maturation does occur within the cortex; it is possible that some emigration proceeds from these sites.

Because thymic cell suspensions are unreactive with antibodies to Ia antigens, it was not surprising to find that most lymphoid cells in the cortex and medulla did not stain with anti-I1. However, staining of a small number of cells was seen in both cortex and medulla, most probably representing epithelial cells. The staining seen in the cortex was present in two patterns; in one, groups of unstained lymphocytes were surrounded by a ring of staining (Fig. 8); and in the second, clusters of individual lymphocytes appeared to show peripheral staining (Fig. 9). Dendritic processes of epithelial cells are known to surround groups of thymic lymphocytes (15), which could account for the first pattern. With respect to the second pattern, it has been shown in electronmicroscopical studies in the mouse and rat (16, 17) that the thymus contains many large epithelial cells with numerous thymocytes present either within the cytoplasm or in deep invaginations of the epithelial cells; in the latter case, staining

of the epithelial cell membrane would result in a picture that would resemble peripheral staining of lymphocytes. We have found similar cells in suspensions of human thymus (data not shown). Macrophages or B cells, which also possess Ia antigens, are too infrequent in the thymus to account for the staining pattern observed in this study. Moreover, a monoclonal antibody capable of reacting with an antigen expressed on both $Ia^+$ and $Ia^-$ macrophages (18) stained only a few cells scattered throughout the cortex and the medulla (data not shown). It is probable that the absence of $Ia^+$ cells in suspensions prepared from the thymus results from the poor recovery of epithelial cells in suspensions.

It has been postulated that maturation and differentiation of thymic lymphocytes leading to development of H-2-restricted cells (in the mouse) involves close interaction of lymphocytes with histocompatibility antigens expressed on the surface of epithelial cells (4). In our study, the staining patterns observed for MHC antigens support a close relationship between thymocytes and epithelial cells.

## Summary

A series of monoclonal antibodies were used to study the intrathymic distribution of T cell-specific antigens, Ia antigens, and $\beta_2$-microglobulin in frozen sections of human thymus by immunofluorescence and immunoperoxidase techniques. Most of the cortical thymocytes reacted with anti-T4, anti-T5, anti-T6, anti-T8, and anti-T10 antibodies, thus indicating coexpression of multiple antigens on cortical lymphocytes. The staining of cells in the medulla was most satisfactorily judged in sections stained with the immunoperoxidase technique. Many medullary cells reacted with anti-T4— and a smaller fraction with anti-T5, anti-T6, anti-T8, and anti-T10 antibodies. In addition, T1 and T3 antibodies, which react with all peripheral T cells, stained a majority of medullary cells. The medullary cells were also more intensely stained with antibodies directed against $\beta_2$-microglobulin than the majority of cortical cells. Hence, the staining profile of medulla approximates the staining pattern of peripheral T cells, with large numbers of cells bearing $T1^+$, $T3^+$, and $T4^+$ antigens (helper/inducer cells) and a small number of cells bearing $T1^+$, $T3^+$, and $T5^+/T8^+$ antigens (suppressor/cytotoxic cells). This supports the conclusion that mature cells present in the medulla are derived from immature cells in the cortex. However, a small number of cells scattered throughout the cortex stained with T1 and T3 antibodies, which suggests that maturation of thymocytes can also occur in the cortex.

Antibody directed against Ia antigens resulted in a characteristic patchy pattern of staining in the cortex and in diffuse staining in the medulla, which was interpreted as resulting from staining of epithelial reticulum. The majority of thymocytes did not stain. The staining pattern suggests a close relationship between epithelial cells and thymocytes.

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