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DISTRIBUTION OF THYMOCYTES EXPRESSING γδ RECEPTORS IN THE MURINE THYMUS DURING DEVELOPMENT[1]

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We have examined the appearance of thymocytes expressing γδ TCR within the developing thymus by using immunohistochemical techniques and flow cytometry in conjunction with the mAb 3A10, which recognizes a determinant associated with the constant region of the δ-chain. γδ$^+$ Cells were first detected at day 16 of gestation, attained maximal levels at day 17 of gestation, and declined thereafter. By using the *Ulex europeus* agglutinin to identify medullary epithelial cells in situ, we observed a striking colocalization of γδ$^+$ thymocytes and *U. europeus* agglutinin-positive medullary epithelial cells during late fetal and neonatal periods of development. In the thymuses of adult mice, γδ$^+$ thymocytes were scattered throughout cortical and medullary areas of the thymus and most concentrated in the subcapsular areas of the thymus. Ultrastructural immunohistochemistry confirmed the close association between medullary thymic epithelial cells and γδ$^+$ thymocytes in the neonatal thymus and also showed that some TCR-γδ molecules were patched to areas of contact with medullary epithelial cells. In contrast to the cellular distribution of either CD3 molecules or the TCR-αβ, where extensive intracellular labeling of thymocytes has been observed, cytoplasmic accumulation of δ-chain was not detected.

Two populations of T cells can be identified according to the type of heterodimeric Ag receptor molecules they express. The Ag receptor molecule utilized by the majority of thymocytes and peripheral T cells (hereafter referred to as TCR2 cells) is a heterodimer consisting of α and β polypeptides and is noncovalently associated with the CD3 complex (1–9). The specificity repertoire of the αβ TCR is shaped during differentiation by interactions between immature T cells expressing αβ TCR molecules and the nonlymphoid cells comprising the thymic environment. Cellular interactions underlying this process remain poorly understood but are thought to be responsible for the both skewing of the TCR specificity repertoire toward recognition of Ag in the context of self major histocompatibility gene products and the establishment of self tolerance (10, 11).

The second population of T cells (TCR1 cells) expresses a TCR comprised of γ- and δ-chains that is also associated with the CD3 complex (12–16). These cells constitute a minor population of cells in the adult thymus or peripheral lymphoid tissue (17, 18) but are the dominant CD3$^+$ thymocyte population in the early fetal thymus (17, 19, 20) and preferentially localize to epithelial organs such as epidermis and gut[2] (20–24). No thymocytes or T cells expressing both TCR1 and TCR2 are detectable, suggesting that the γδ$^+$ T cells and αβ$^+$ T cells are generated through distinct cell lineages (17) (I. Ishida, S. Verbeek, M. Bonneville, A. Berns, and S. Tonegawa, manuscript in preparation).

Although it appears that the majority of peripheral TCR1 cells resemble TCR2 cells in that they represent a thymic dependent population (25) (I. Ishida, S. Verbeek, M. Bonneville, A. Berns, and S. Tonegawa, manuscript in preparation) the role of thymic stromal cells in the differentiation of TCR1 cells or the extent of their involvement in shaping the γδ receptor repertoire is not clear. The distinctive distribution patterns of TCR1 cells to epithelial tissues in the periphery and TCR2 cells to peripheral lymphoid tissue suggest that the contribution of the thymic environment to the differentiation of these distinct lineages of T cells may be qualitatively different.

In this report, we have utilized a recently developed hamster mAb specific for the δ-chain of the TCR1 molecule (17) to examine the distribution and ultrastructural features of TCR1 cells in the developing thymus. We report here that the expression of γδ molecules within the developing thymus differs in several respects from that previously described for αβ Ag receptors (26, 34) and that during fetal development, but not in the adult thymus, TCR1 cells show a strong tendency to localize in the vicinity of thymic epithelial cells destined to constitute the medullary epithelium in adult thymus tissue. These results are discussed in the context of V gene segment usage by TCR1 cells during development.

MATERIALS AND METHODS

Mice. Male and female BALB/c mice were purchased from Life Sciences (St. Petersburg, FL) and maintained in the Department of

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[2] Itohara, I., A. G. Farr, J. J. Lafille, M. Bonneville, Y. Takagaki, and S. Tonegawa. Preferential localization of TCR γδ-bearing T cells in epithelia; identification of distinct subpopulations in reproductive organs and tongue. *Submitted for publication.*

Biological Structure vivarium. For timed pregnancies, the appearance of a vaginal plug was considered to be day 0 of gestation. Adult tissue was obtained from 4- to 8-week-old mice.

Reagents. A recently developed hamster mAb, 3A10, was used to detect TCR1 molecules (17). This antibody can immunoprecipitate free δ-chains and can label a panel of TCR1$^+$ hybridomas irrespective of V gene segment utilization. Given that there appears to be a single Cδ gene segment in the mouse (16), it is likely that this antibody is a pan-δ-chain reagent. Anti-CD3 antibodies (clone 500AA2 (27)) were obtained from Dr. J. P. Allison. Biotinylated goat anti-hamster Ig antibodies (absorbed with mouse IgG) and streptavidin-PE[3] conjugates were purchased from Caltag (San Francisco, CA). Biotinylated UEA I was purchased from Vector Laboratories (Burlingame, CA). The specificity of this reagent for medullary thymic epithelial cells has been described previously (28). Avidin and horseradish peroxidase (type VI) was purchased from Sigma Chemical Co. (St. Louis, MO). Avidin-peroxidase conjugates were prepared according to the method of Wilson and Nakane (29).

Flow cytometry staining procedures. Cells were washed once in HBSS containing 1% BSA, 10 mM HEPES, and 0.2% sodium azide (labeling medium). Aliquots of cells (5×10^5) were incubated with biotinylated 3A10 antibodies and FITC-labeled 500 AA2 antibodies for 60 min on ice. After washing once with labeling medium, streptavidin-PE was added for another 60-min incubation. After two washes in labeling medium, the cells were suspended in PBS containing 2% paraformaldehyde for flow cytometric analyses.

Flow cytometry was performed with a FACStar cell sorter (Becton Dickinson Immunocytometry System, Mountain View, CA) by using a 5-W argon laser equipped with band pass filters at 530/30nm and 584/42nm (band pass/band width) for FITC and PE, respectively. In most experiments 2×10^4 cells were acquired for analysis. Dead cells were gated out with a combination of forward and right angle light scatter. Analyses of data utilized Consort 30 and Consort 40 software from Becton Dickinson.

Immunohistochemical procedures. The techniques used for light and electron microscopic immunohistochemistry have been described in detail (30). For light microscopy, frozen sections of thymuses were mounted on amino alkylsilane-treated slides (31) and allowed to air dry for at least 2 h before fixation (15 to 20 min in acetone at −20°C). After rinsing in PBS (0.05 M PO_4 and 0.9% NaCl, pH 7.4), the sections were subjected to sequential incubations in avidin (10 μg/ml) and biotin (50 μg/ml) with intervening washes in PBS to reduce levels of background staining. Sections were then incubated for 1 h at room temperature with unlabeled antibodies or biotinylated UEA (both diluted in PBS containing 1% BSA. After several washes in PBS, slides treated with biotinylated UEA were incubated with avidin-peroxidase conjugates at a concentration of 20 μg/ml. Slides treated with 3A10 antibodies were incubated for 15 min in 2% paraformaldehyde to stabilize antibody binding and then washed several more times with PBS. To localize the binding of hamster primary antibodies, sections were washed three times with PBS/BSA and incubated for 60 min with biotinylated goat anti-hamster IgG antibodies diluted in PBS/BSA containing 10% normal mouse serum. After washing repeatedly in PBS, the sections were next incubated with avidin-peroxidase conjugates. Peroxidase activity was demonstrated using 3,3′-diaminobenzidine as the electron donor (32). Slides were then dehydrated, mounted with coverslips, and photographed without additional counterstaining.

For ultrastructural examination, thymus tissue was fixed by brief vascular perfusion (33). The fixative consisted of 0.1 M cacodylate buffer, pH 7.4, containing 4% paraformaldehyde and 1 mM $CaCl_2$. Tissue was washed in PBS containing 5% sucrose (PBS/S) and sectioned at 50 μm with a Vibratome (Ted Pella, Tustin, CA). Tissue sections were incubated overnight with biotinylated antibodies diluted in PBS/BSA/S. After repeated washing in PBS/BSA/S, the tissue sections were incubated for 4 to 6 h with peroxidase-avidin conjugates. The sections were again washed repeatedly with PBS/BSA/S, and then subsequently washed in 50 mM Tris buffer, pH 7.6, before demonstration of peroxidase activity with 3,3′-diaminobenzidine as described previously (28). Sections were then exposed briefly (15 min) to 0.1 cacodylate buffer, pH 7.4, containing 1% glutaraldehyde, in order to preserve morphology. Tissue was then processed for conventional transmission electron microscopy. No counterstaining was used.

Quantitation of labeled cells. A grid reticule was placed in the microscope eyepiece and calibrated with a stage micrometer. One edge of the grid was oriented along the capsule of the thymus and labeled cells within each square of the grid were counted. Figures represent data collected from at least 32 mm^2 of tissue.

[3] Abbreviations used in this paper: PE, phycoerythrin; UEA, *Ulex europeus* agglutinin; S, sucrose.

RESULTS

Flow Cytometry. Thymocytes from mice of different gestational and postnatal ages were analyzed by two-color flow cytometry with fluoresceinated anti-CD3 antibodies and biotinylated anti-δ antibodies used in conjunction with phycoerythrin-streptavidin conjugates (Fig. 1). In our hands, the earliest stage of fetal development when CD3$^+$, γδ$^+$ cells could be detected was at day 16, when 2.4% of the thymocytes were labeled. At day 17 of gestation the percentage of γδ$^+$ cells in the fetal thymus reached maximal values (5%) and then progressively declined thereafter as the population of αβ thymocytes expanded. In the adult thymus, the percentage of cells reacting with the anti-δ antibody was on the order of

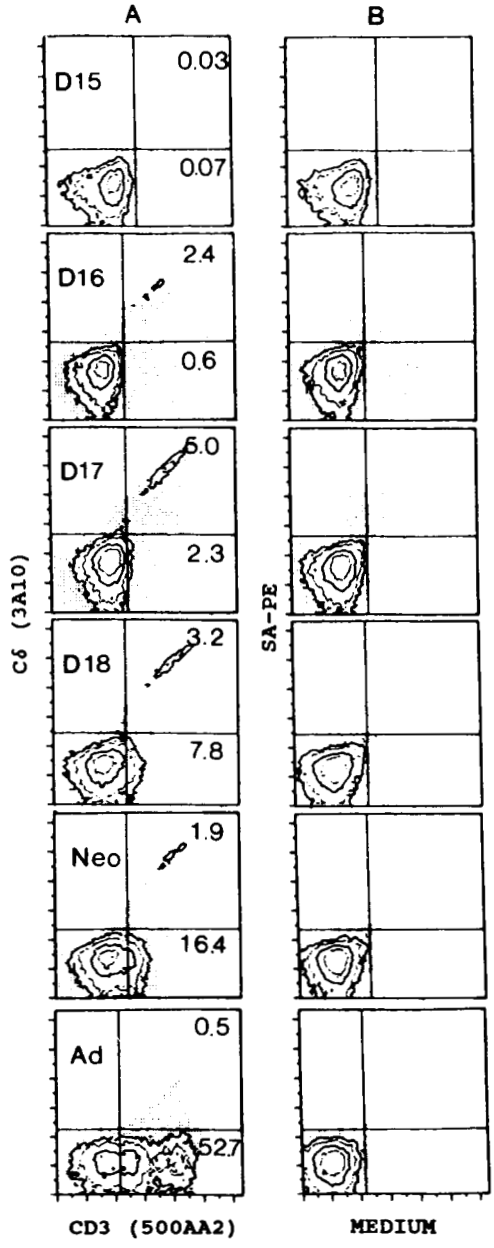

Figure 1. Ontogeny of γδ thymocytes in BALB/c mice. Thymocytes from mice of different ages were stained with biotinylated anti-δ (3A10) antibodies followed by streptavidin-PE (SA-PE) conjugates together with an anti-CD3 (500AA2)-FITC conjugate (*column A*) or with streptavidin-PE conjugates alone (*column B*). In calculating the percentages of labeled cells, values obtained in column B were subtracted from those in column A.

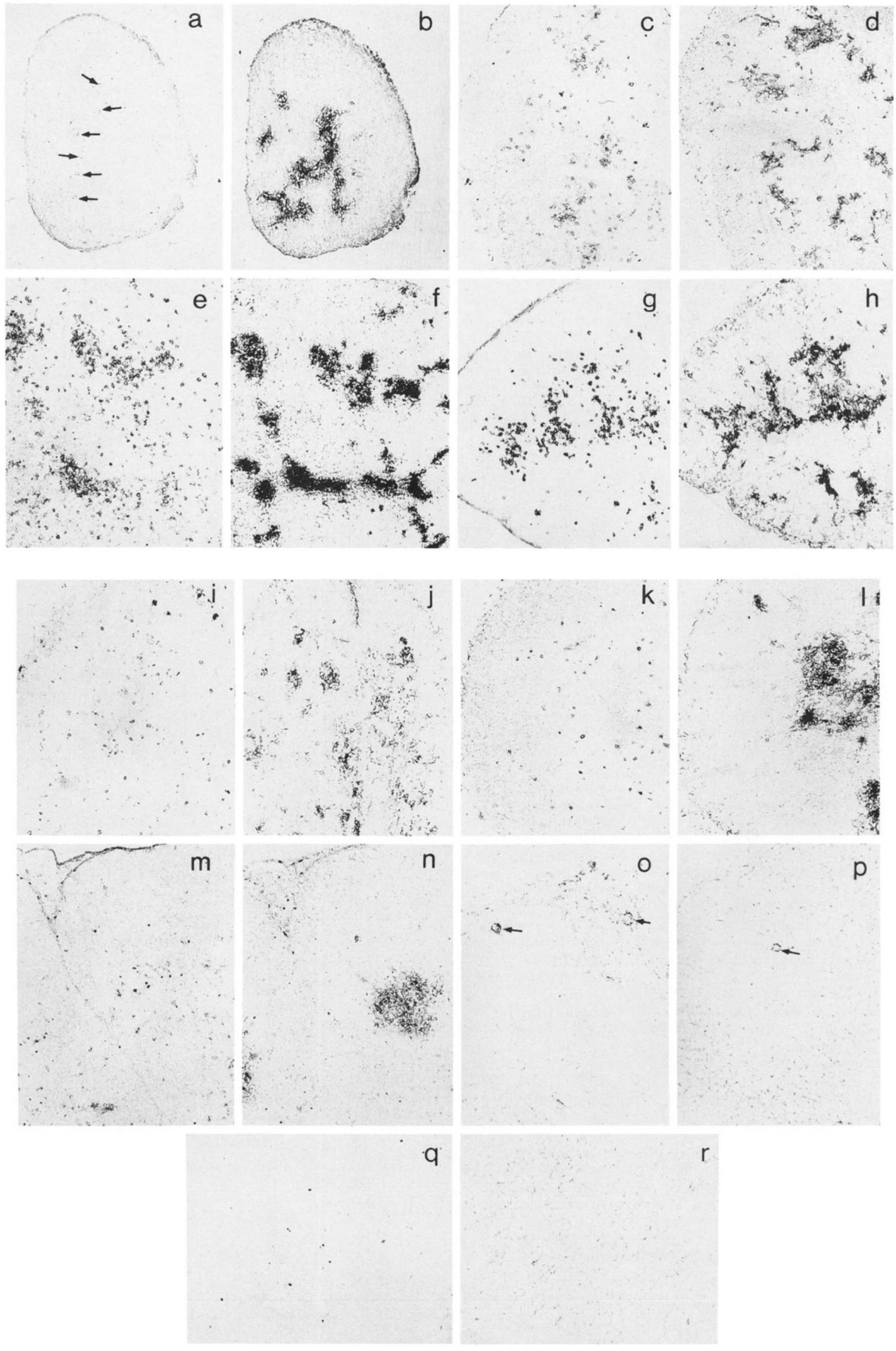

Figure 2. Distribution of thymocytes bearing TCR-γδ during thymus development. Adjacent serial sections of thymus tissue from mice of different ages was labeled with anti-Cδ reagent (*a, c, e, g, i, k, m, o,* and *p*) or with UEA (*b, d, f, h, j, l,* and *n*). Day 16, *a* and *b*. Lightly labeled cells indicated by *arrows*; day 17, *c* and *d*; day 18, *e* and *f*; neonate, *g* and *h*; day 4, postnatal, *i* and *u*; day 7 postnatal, *k* and *l*; adult, *m–p*. Degree of background labeling with omission of 1° antibody is indicated in panels *q* and *r*.

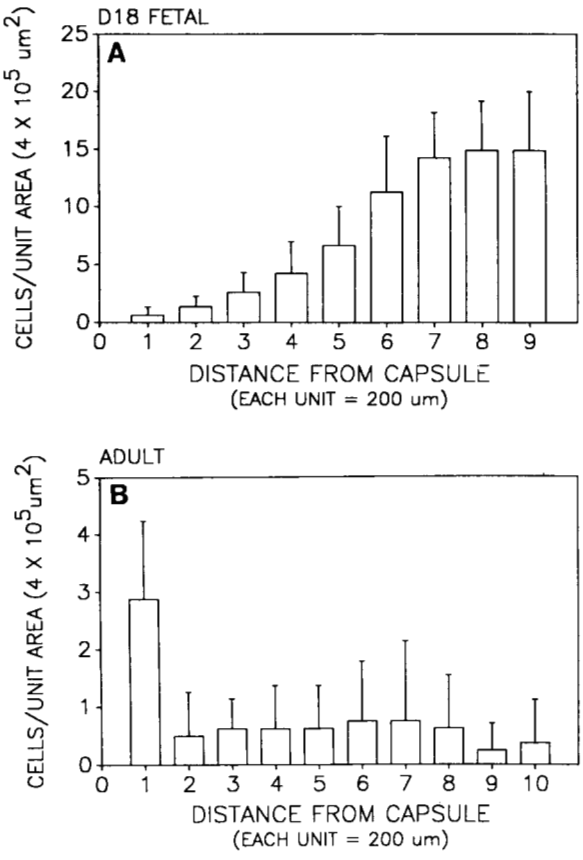

Figure 3. The distribution of cells reacting with 3A10 antibodies in fetal and adult thymus tissue. Labeled cells in sections of thymus tissue were quantitated as described in *Materials and Methods*.

0.5%. There were also qualitative changes in the labeling. Cells from day 16 or adult thymuses did not stain as brightly as day 17, day 18 and neonatal thymocyte suspensions. In contrast to the pattern of αβ receptor expression during fetal development, where the appearance of dully stained cells precedes the detection of brightly stained cells (33), cells expressing γδ receptors appeared as unimodal population of cells. The appearance of $CD3^+$, $\gamma\delta^-$ cells representing thymocytes expressing TCR-αβ increased gradually, beginning at day 17 of gestation and continuing after birth.

Distribution of TCR1 cells in the developing thymus. In order to relate the spatial distribution of TCR1 cells to the organization of the thymic environment, adjacent sections of thymus tissue were alternatively labeled with anti-δ antibodies or UEA, which we have previously shown to be a reliable marker for medullary thymic epithelial cells in the adult murine thymus (28). At day 16 of gestation, TCR1 cells were very rarely observed in the fetal thymus and were scattered throughout the thymus parenchyma. In agreement with flow cytometry data, these TCR1 cells were weakly labeled in tissue sections (Fig. 2*a* and *b*). Based on the distribution of medullary-type epithelium in adjacent sections, no particular affinity of TCR1 cells for medullary type epithelium was observed at this stage of development. At day 17 of gestation, TCR1 cells were localized predominantly in multiple foci within the thymus (Fig. 2*c* and *d*). Again, consistent with the flow cytometry data, the labeling intensity in the tissue sections was greater than that observed in day 16 tissue. When the distribution of TCR1 cells and UEA^+ medullary epithelial cells were compared in adjacent serial sections, the location of some of the foci corresponded to areas of medullary epithelium, whereas others did not. In general, these foci were located at some distance from the thymic capsule. At a later stage of fetal development (day 18) and at birth, the distribution of TCR1 cells within the thymus exhibited a close correlation with the distribution of UEA^+ medullary epithelium (Fig. 2*e* to *h*). However, by 4 days after birth this pattern of colocalization was less apparent (Fig. 2*i* and *j*), and by 7 days after birth TCR1 cells were located in areas adjacent to, but not encompassed by areas of UEA^+ epithelium (Fig. 2*k* and *l*). The labeling intensity of TCR1 cells in the postnatal mice was less than that observed in tissue from day 17 through neonatal mice. In the adult thymus TCR1 cells were rare, not heavily labeled, and usually observed as single cells in subcapsular and cortical areas of the thymus (Fig. 2*m* to *p*), although small clusters of labeled cells were also occasionally observed.

A quantitative analysis of the labeling of TCR1 cells in the fetal (day 18) and adult thymus revealed a striking shift in the intrathymic distribution of TCR1 cells (Fig. 3). The greatest density of TCR1 cells in the fetal thymus was located at a distance from the capsule in the medullary areas of the thymus. As expected in the adult thymus, the density of TCR1 cells was considerably less than in the fetal thymus. The subcapsular area of the adult thymus contained the highest density of TCR1 cells.

Immunoelectron microscopy. Ultrastructural immunohistochemical studies were performed to characterize the features of the thymic environment where TCR1 accumulated and to define the ultrastructural features of γδ receptor expression in situ. Neonatal thymus tissue was utilized in these studies for several reasons: the medullary distribution of TCR1 cells was still evident; the frequency of TCR1 cells at this age was still high enough to make detection of TCR1 cells feasible; and perfusion fixation of mouse embryos was found to be technically difficult. These ultrastructural studies confirmed the close proximity of TCR1 cells and epithelial cells exhibiting a medullary type morphology (Fig. 4*a* and *b*). Profiles of labeled cells not in apparent contact with epithelial cells were also observed (Fig. 4*c* and *f*), although serial sections would be necessary to rule out the possibility that such contact could be occurring at other areas of the cell surface.

Thymocytes expressing γδ receptors presented a varied morphology. Although many of the cells were large and possessed significant amounts of cytoplasm (Fig. 4*a* to *e*), some of the labeled cells exhibited a smaller nucleus to cytoplasm ratio and resembled small lymphocytes (Fig. 4*c* and *f*). Some of these smaller cells exhibited cytoplasm with increased electron density and altered patterns of chromatin condensation. This latter population was not unique to TCR1 cells, because other thymocytes with increased electron density were not labeled (data not shown). Labeling of γδ receptors was uniformly restricted to cell surfaces (Fig. 4 *a* to *c*, *e* and *f*). No label was seen associated with cytoplasmic structures, such as rough endoplasmic reticulum or the perinuclear envelope. This pattern was contrasted with that obtained with anti-CD3 antibodies by using tissue from the same animals, where many cortical cells exhibited strong perinuclear labeling which, in some instances, was accompanied by surface

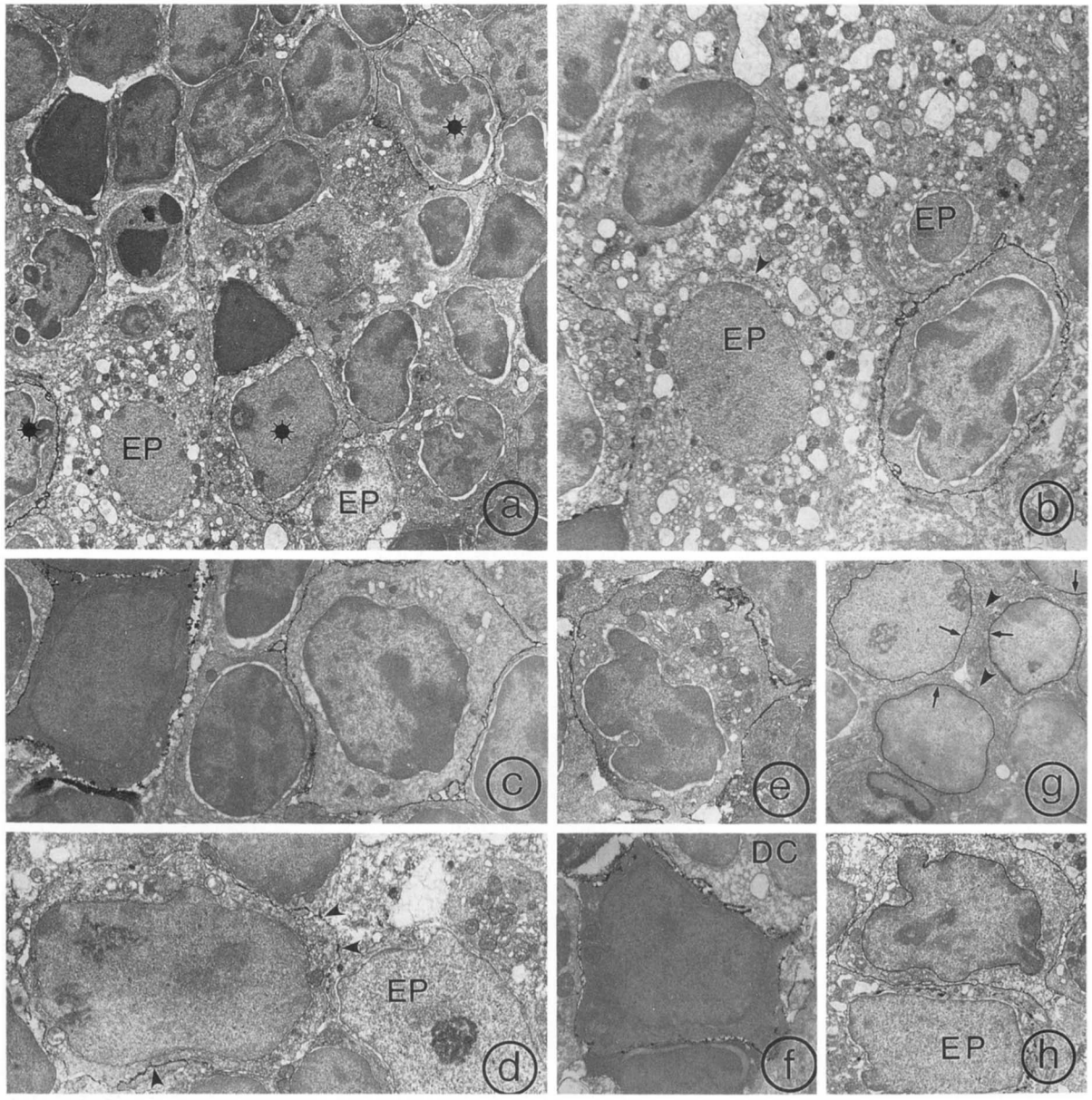

Figure 4. Ultrastructural localization of δ-chain in the neonatal thymus. *a*, Three labeled cells are indicated by *asterisks*. Two of the labeled cells are adjacent to a cluster of medullary epithelial cells (identified as such by the presence of membrane-bounded vacuoles with microvilli and perinuclear bundles of tonofilaments). Surface labeling is circumferential (× 2,600). *b*, Two labeled cells in contact with a cluster of epithelial cells. Surface labeling is circumferential (× 4,100). *c*, Labeled cells exhibited considerable morphologic heterogeneity; some have large cytoplasm to nuclear ratios whereas others do not (× 4,900). *d*, Patchy labeling of a cell in large thymocyte in contact with an epithelial cell (*arrowheads*). Some of the label is also localized to areas of contact with other thymocytes (× 4,800). *e*, Single labeled large thymocyte (× 5,100). *f*, A labeled small electron-dense thymocyte in contact with a dendritic cell (*DC*); labeling is circumferential (× 11,250). *g*, Distribution of CD3 molecules in tissue samples from the same animals. Note the prominent perinuclear cytoplasmic distribution of label (*arrows*) and the absence of detectable cell surface labeling (*arrowheads*) (× 3,150). *h*, Large thymocyte in contact with thymic epithelial cell labeled with anti-CD3 antibodies. Note intense perinuclear labeling in addition to cell-surface labeling (× 6,400).

labeling as well (Fig. 4*g* and *h*).

Although most of the labeling of γδ receptors was circumferential, a few TCR1 cells presented profiles where labeling was patchy and strongest in areas in contact with epithelial cells (Fig. 4*d*). This pattern of labeling was similar to the previously described patchy distribution of αβ TCR expressed by some thymocytes in contact with cortical epithelial cells (26, 34).

DISCUSSION

In this report we have characterized the pattern of TCR1 expression within the murine thymus during development. Similar to the expression pattern of γδ molecules in the developing chicken (P. Bucy, personal communication) and human (18) thymus, the pattern of γδ expression within the thymus differ in several respects from the expression of αβ molecules (26, 34–36). First, confirming the results of previous studies on the ontogeny of γ and δ gene rearrangements and receptor expression during fetal development (17, 19, 20, 37–39), the expression of γδ molecules precedes the appearance of TCR2 molecules by 1 or 2 days. Second, at the cellular level, TCR1 cells do not exhibit the extensive cytoplasmic labeling previously reported for TCR2 cells (27, 34). Third, the initial distributions of TCR1 and TCR2 cells within the fetal thymus are quite different. The location of TCR1 cells in the thymus during fetal development correspond closely to foci of thymic epithelial cells that

ultimately give rise to the medullary area of the thymus. In contrast, TCR2 cells first appear scattered throughout the thymic cortex (26, 35, 36). Finally, the intrathymic pattern of TCR1 distribution changes during late fetal and postnatal development. The predominantly medullary location of TCR1 cells in fetal and neonatal thymuses gives rise to a scattered distribution throughout the thymic parenchyma with a slightly increased density in the subcapsular area of the thymus in adolescent and young adult mice. In contrast, the distribution of TCR2 cells expands from an initial cortical location to include medullary and subcapsular areas of the thymus during late fetal and early postnatal development (26, 35).

Previous flow cytometric analyses of C57BL/6J thymocytes with the 3A10 antibody revealed that in contrast to TCR2 cells, which exhibit both low and high levels of TCR expression (40), TCR1 cells from fetal or adult sources were uniformly brightly stained (17). Although this study of BALB/c thymocytes confirms that the labeling intensity of γδ receptors is relatively homogeneous, it also indicates an interesting strain difference in the intensity of labeling exhibited by adult thymocytes, the basis for which is presently unclear.

In marked contrast to previous ultrastructural immunohistochemical examination of thymocyte expression of TCR β-chains in situ, where cytoplasmic labeling was prominent (26, 34), no ultrastructural evidence for cytoplasmic accumulations of δ-chain was observed. If cytoplasmic labeling reflects intracellular accumulation of unassembled TCR molecules, this inability to detect cytoplasmic δ-chain may indicate that the synthesis of δ-chain may be the rate-limiting step in the assembly and expression in TCR1 molecules. The extensive cytoplasmic labeling of β-chain in cortical TCR2 thymocytes may reflect the situation in which productive rearrangement of β-chain genes and synthesis of β chains precedes synthesis of α-chains, thus leading to the cytoplasmic accumulation of β-chain.

The ultrastructural similarities between the small subpopulation of TCR1 cells displaying electron dense cytoplasm and altered patterns of chromatin condensation and thymocytes undergoing anti-CD3 mediated apoptosis in thymus organ cultures (41) raises the possibility that some TCR1 cells may be eliminated intrathymically by a similar process.

The bases for the preferential association of late fetal/neonatal TCR1 cells with medullary thymic epithelium and changes in the intrathymic distribution of TCR1 cells during ontogeny are not known. Cytokines produced by epithelial cells of the thymus may contribute to the selective location/expansion of TCR1 cells. Medullary thymic epithelial cell lines secrete IL-1 (42) and thymic epithelial cells have been reported to produce factors chemotactic for T cell progenitors (43). Another nonexclusive possibility is that the repertoire of γδ receptor molecules expressed by TCR1 cells is shaped by thymic selection processes that are similar to those that occur during the differentiation of thymocytes expressing αβ receptors (10, 44) and that the association of TCR1 cells with medullary thymic epithelium reflects a specificity of the γδ receptors expressed by these cells for cell-surface molecules expressed by medullary epithelial cells. The observation that some TCR1 cells exhibit patchy γδ labeling at areas of contact with epithelial cells would be consistent with this notion. In this regard it may be significant that alterations in the pattern of V_γ gene expression in the thymus accompany the intrathymic changes in the distribution of TCR1 cells during development. In early fetal development (day 15) TCR1 cells utilizing $V_{\gamma5}$ gene segments predominate but are virtually absent from the thymus by day 18 of gestation (19). The γδ receptors preferentially expressed by newborn thymuses utilize $V_{\gamma6}$ gene segments (45), whereas γδ receptors found on adult cells are encoded by $V_{\gamma4}$ gene segments (45). Correlation of these data with the distribution pattern of TCR1 cells within the developing thymus suggests that TCR1 cells utilizing $V_{\gamma5}$ and $V_{\gamma6}$ gene segments appear to preferentially associate with medullary-type thymic epithelial cells, whereas TCR1 cells utilizing $V_{\gamma4}$ and $V_{\gamma7}$ gene segments in the adolescent and adult thymus do not exhibit the same colocalization with medullary epithelial cells and are scattered throughout the cortex and medulla. To address the possibility that the intrathymic distribution of γδ cells may be related to the specificity of the receptors they express, experiments are underway to examine the ability of medullary epithelial cell lines (42) to stimulate fetal and adult thymocytes in vitro and to define V_γ gene segment usage by these cells. The obvious possibility also exists that changes in the intrathymic distribution of TCR1 cells and changes in utilization of V_γ and V_δ gene segments during fetal development are concomitant yet unrelated processes.

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