Two Subsets of Epithelial Cells in the Thymic Medulla

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Summary

Information was sought on the features of epithelial cells in the murine thymic medulla. The expression of major histocompatibility complex (MHC) molecules on medullary epithelium was defined by light microscopy with the aid of bone marrow chimeras and MHC-transgenic mice. A proportion of medullary epithelial cells was found to show conspicuously high expression of conventional MHC (H-2) class I (K, D, L) and class II (I-A, I-E) molecules. These cells express a high density of the Y-Ae epitope, a complex of an Eα peptide and I-A^b molecules found on typical bone marrow–derived cells. MHC$^+$ medullary epithelial cells show limited expression of I-O molecules, a class of atypical nonpolymorphic MHC-encoded class II molecules present on B cells. Other medullary epithelial cells express a high density of I-O molecules but show little or no expression of typical MHC class I or II molecules. MHC and I-O expression thus appear to subdivide medullary epithelial cells into two phenotypically distinct subsets. This applies in adults. In the embryonic thymus most medullary epithelial cells express both types of molecules.

The thymus contains a dense network of epithelial cells, both in the cortex and medulla (1). Epithelial cells in the cortex appear to control positive selection of thymocytes; immature T cells with binding specificity for the MHC class I and II molecules on cortical epithelium are selected for survival and export to the periphery (2–4). Much less is known about the epithelial cells in the medulla. These cells are poorly characterized and are reported to have a different embryonic origin from cortical epithelium (5, 6).

Several markers have been described that distinguish between medullary and cortical epithelium. ER-TR4 (7), 6C3 (8), and related mAbs (9) react strongly with cortical epithelium but do not stain cells in the medulla. Conversely, ER-TR5 (7), Th-4 (10), MD-1 (11), and several other antibodies (9), as well as the fucose-specific lectin, UEA-1 (12), stain-medullary epithelium but not cortical epithelium (although some of the antibodies stain the thymic capsule). Selective staining of medullary but not cortical epithelium has also been described for an antibody specific for the β chain of a novel class of nonpolymorphic class II molecules, termed I-O (H-20) (13); I-O molecules are also expressed on B cells but, unlike normal class II molecules, are not detectable on macrophages or dendritic cells.

Some medullary epithelial cells are reported to express conventional class II MHC molecules (I-A and I-E molecules in the mouse, collectively referred to as Ia molecules) (12, 14–16). This raises the question whether Ia and I-O molecules on medullary epithelial cells are expressed on the same or different cells. Assessing MHC expression on medullary epithelium is difficult because of the dense network of Ia$^+$ bone marrow (BM)[1]-derived cells in the medulla. This problem can be avoided by examining the thymus of parent → F_1 bone marrow chimeras (BMC). As described elsewhere (17, 18), the Ia$^+$ BM-derived cells in the thymus and spleen of long-term parent → F_1 BMC prepared with supralethal irradiation are entirely of donor origin. Host Ia expression in the thymus of these chimeras is thus restricted to epithelial cells. With this and other approaches we show here that some medullary epithelial cells are strongly Ia$^+$ whereas others are Ia$^-$. Surprisingly, I-O expression on medullary epithelium is largely limited to the Ia$^-$ subset. Ia and I-O expression thus appears to subdivide medullary epithelium into two phenotypically distinct subsets. In the case of the Ia$^+$ subset,

[1] *Abbreviations used in this paper:* BM, bone marrow; BMC, bone marrow chimera; HRP, horseradish peroxidase; Mϕ, macrophage.

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evidence is presented that these epithelial cells closely resemble BM-derived cells in expressing a high concentration of the Y-Ae epitope, a complex of an Eα peptide bound to I-A^b molecules (19, 20).

Materials and Methods

Mice. Young C57BL/6 (B6), B6.PL (B6 with the Thy-1.1 allele), BALB/c, B10.A(5R), (B6 × CBA/Ca)F_1, C.B-17 SCID, B6 *nu/nu* (nude), and timed-pregnant BALB/c mice were obtained from the Research Institute of Scripps Clinic breeding colony. Transgenic mice designated 107–1 and 36–5 (21) were also bred at the Scripps facilities.

Reagents. The specificity and source of the mAbs and other reagents used to define epithelial cells are listed in Table 1. Anti-I-O antibody was prepared by injecting rabbits with a synthetic peptide corresponding to the cytoplasmic tail region of the mouse I-O β chain gene (13). Anti-I-O antibody, designated K507, was affinity purified before use.

Bone Marrow Chimeras. T cell–depleted B6, B6.PL, or 36.5 BM cells were injected intravenously into hosts exposed to heavy irradiation (1,100–1,300 rad) 3–4 h before, as described previously (17). Some of the chimeras were given secondary irradiation (900 rad) and BM reconstitution at 6 mo after initial reconstitution (17). The mice were analyzed 4–6 mo after the last reconstitution with BM cells.

Thymus Grafting. B10.A(5R) day 14 fetal thymuses were cultured with deoxyguanosine for 5 d in vitro (30) and then grafted under the kidney capsule of adult B6 nude mice (18). Mice were tested at 6 wk postgrafting.

Immunohistology. Fresh thymuses were immersed in OCT, quickly frozen, and 5–6-μm sections were cut with a cryostat (31). Sections were allowed to air dry for at least 30 min and then fixed in acetone for 1–2 min. After initial titration on control sections to determine optimal dilutions, biotinylated or unconjugated antibodies diluted in PBS supplemented with 2.5% gamma globulin–free horse serum was added directly onto the sections and incubated at room temperature for 60 min. After washing, sections were incubated either with horseradish peroxidase (HRP)-streptavidin (Jackson ImmunoResearch, West Grove, PA), HRP-anti-rabbit IgG, or biotinylated anti-rat mAb followed by streptavidin-HRP, washed, and incubated with the substrate 3-amino-9-ethylcarbazole (0.1 mg/ml in 0.05 M NaOAc pH 5.2 plus 0.01% H_2O_2) for 20–30 min. Some sections were then lightly counterstained with Meyer's hematoxylin. Two-color staining was performed by incubating tissue sections with a mixture of a biotinylated antibody and an unconjugated antibody raised in different species, which were then detected with a mixture of alkaline phosphatase–conjugated streptavidin and HRP-conjugated species-specific antibody. For example, two-color staining for I-A^k and I-O was performed by coincubating sections with optimal concentrations of biotinylated 10–2.16 (anti-I-A^k) plus unconjugated K507 (anti-I-O) antibody diluted in Tris saline with 2.5% horse serum and incubated for 60 min. After washing, the sections were incubated with an appropriately diluted mixture of alkaline phosphatase–conjugated streptavidin (Jackson ImmunoResearch) and HRP-anti-rabbit IgG for an additional 1 h. The sections were washed and incubated with an alkaline phosphatase substrate (0.2 mg/ml napthol AS MX phosphate + 1.0 mg/ml Fast Blue BB in 0.1 M Tris saline, pH 8.5), washed, and then incubated with the HRP substrate 3-amino-9-ethylcarbazole as described above.

Results

The general aim of the experiments presented below was to define the epithelial cells of the thymic medulla with respect to expression of conventional H-2 class I and II molecules vs. I-O molecules. Thymic epithelial cells were characterized by staining cryostat sections of thymus with the panel of reagents listed in Table 1. Unless stated otherwise, serial sections were stained. Except for Figs. 1 and 5 and the two-color sections, all sections were counterstained with hematoxylin.

Cortex of Normal Thymus. In accordance with previous findings (32), the reticular network of epithelial cells in the cortex is easily visualized with anti-Ia antibodies, i.e., with antibodies specific for I-A and I-E molecules (Fig. 1 *a*, and not shown). A similar reticular pattern of staining in the cortex is seen with antipancytokeratin antibody (Fig. 1 *f*), the cortical epithelium–reactive antibody 6C3 (Fig. 1 *d*), and with NLDC-145, an antibody that reacts strongly with cortical epithelium and weakly with dendritic cells (DC) (28) (Fig. 1 *g*). H-2 class I (K^b, D^b) expression on cortical epithelium is faint, but clearly detectable (D^b shown in Fig. 1 *c*). Staining with the anti-macrophage (Mϕ) antibody, F4/80, produces punctate staining scattered throughout the cortex (Fig. 1 *h*). I-O and UEA-1 expression in the cortex is very low or undetectable, and there is no cortical staining with MD-I antibody (see below).

Medulla of Normal Thymus. Unlike the cortex, the medulla shows confluent expression of Ia molecules and H-2 class I molecules (Fig. 1, *a* and *c*). Much of this staining can be attributed to the dense accumulation of NLDC-145$^+$ DC and F4/80$^+$ Mϕ in this region (Fig. 1, *g* and *h*). Staining with antikeratin antibody reveals the extensive network of epithelial cells in the medulla (Fig. 1 *f*). Many but not all of these cells stain with the medullary epithelium–specific antibody, MD-I (Fig. 1 *i*) and with a related antibody Th-4 (10; and data not shown). These antibodies produce a fusiform pattern of staining. I-O expression (Fig. 1 *b*) is similar, although under high power a reticular rather than a fusiform pattern of staining is seen (see below). UEA-1 expression is quite different (Fig. 1 *e*). This lectin produces very dense punctate staining, presumably on groups of adjacent cells, with only limited staining of cell processes. As described elsewhere (12), UEA-1 expression is restricted to a subset of medullary epithelial cells.

Fetal Thymus. The ontogeny of expression of some of the markers discussed above is shown in Fig. 2. MHC (I-A and L^d) expression in the fetal thymus is almost undetectable at day 14 of gestation. In the cortex, Ia (I-A) expression remains low throughout ontogeny and reaches adult levels only at 1–2 wk after birth (Fig. 2, *a*, *e*, *i*, and *m*); class I expression in the cortex is even lower and is difficult to visualize even at day 10 after birth (Fig. 2, *c*, *g*, *k*, and *o*). In the medulla, Ia and class I expression are first clearly detectable at around day 16 and increase progressively to reach adult levels by the time of birth.

In contrast to Ia and class I expression I-O and UEA-1 expression are clearly detectable at day 14 of gestation and

Table 1. *Reagents for Detecting Thymic Stromal Cells*

Reagents	Type, origin	Specificity	Reference
MHC class II			
BP107	mAb, mouse	$I\text{-}A^{b(d)}$	22
10-2.16	mAb, mouse	$I\text{-}A^{k(r,f,s)}$	23
14-4-4S	mAb, mouse	I-E	24
K507	Purified Ab, rabbit	I-O (β chain)	13
Y-Ae	mAb, mouse	$I\text{-}A^{b}/E\alpha$ peptide	19
MHC class I			
12-2-2S	mAb, mouse	K^{k}	24
Y3	mAb, mouse	K^{b}	25
28-14-8S	mAb, mouse	$D^{b}L^{d(q)}$	26
30-5-7	mAb, mouse	L^{d}	27
Epithelial cells			
MD-1	mAb, rat	Medullary EC	11
Th-4	mAb, mouse	Medullary EC	10
UEA-1	Lectin	Medullary EC	12
6C3	mAb, rat	Cortical EC	8
Antikeratin	Antiserum, rabbit	pan cytokeratin	Commercial (Dako Corp.)
Bone marrow–derived cells			
NLDC-145	mAb, rat	DC + cortical EC	28
F4/80	mAb, rat	Mϕ	29

increase progressively thereafter (Fig. 2, *b*, *f*, *j*, and *n*; and *d*, *h*, *l*, and *p*). Double staining for I-O (red) and UEA-1 (blue) produces overlapping staining in the neonatal period (Fig. 2, *d*, *h*, and *l*). However, by day 10 after birth a proportion of epithelial cells stain with only one of the two colors (Fig. 2 *p*) (note that Fig. 2, *a–d*, *l*, and *p* were photographed at a higher magnification than the other sections). This finding suggests that, after birth, UEA-1 and I-O might be expressed on different subsets of medullary epithelium. To assess this possibility, we examined the thymus of SCID mice.

SCID Thymus. In contrast to the normal thymus, the atrophied thymus of SCID mice contains only sparse collections of medullary epithelial cells (32a). These cells are less closely packed than in the normal thymus and are easily visualized at a single cell level. Double staining for UEA-1 (blue) and I-O (red) is shown in Fig. 3 *l*, *right*. It can be seen that, for the most part, UEA-1 and I-O expression on SCID medullary epithelial cells is mutually exclusive.

Thymus of Bone Marrow Chimeras. The experiments discussed below were designed to compare Ia vs. I-O expression on medullary epithelium. To avoid Ia staining of the BM-derived cells in the medulla, we used BMC to prepare chimeric thymuses containing $H\text{-}2^{k}$ epithelium and $H\text{-}2^{b}$ BM-derived cells. Sections were then stained for $H\text{-}2^{k}$.

BMC were prepared by repopulating heavily irradiated (1,300 rad) (B6 × CBA/Ca)F_1 ($H\text{-}2^{b}$ × $H\text{-}2^{k}$) mice with T cell–depleted B6 ($H\text{-}2^{b}$) BM cells. As mentioned earlier the Ia^{+} BM-derived cells of host origin disappear from these chimeras within 2–3 wk (17, 18). To ensure complete disappearance of these cells, the chimeras were left for 6 mo before use. At this stage Ia expression in sections of spleen, LN, gut, and skin is limited to low level staining of vascular endothelium and mild staining of the follicular dendritic cells of germinal centers (which are reported to be non-BM derived) (18); host Ia expression on B cells, DC, and Mϕ is undetectable.

In contrast to extrathymic tissues, the thymus of long-term parent → F_1 chimeras shows strong host Ia ($I\text{-}A^{k}$) expression, both in the medulla and the cortex (Fig. 3 *a*). In the medulla, host Ia expression is limited to dense clumps of cells with short processes; these cells are also conspicuous in BMC prepared with double irradiation amounting to a total of 2,200 rad (data not shown). Under high power the Ia^{+} cells in the medulla resemble epithelial cells and show double staining with an antikeratin antibody (not shown). The pattern of host Ia expression in the medulla closely resembles the staining seen with UEA-1 (Fig. 3 *e*); host class I (K^{k}) expression is also similar (Fig. 3 *i*). The close similarity in these three patterns of staining suggests that host Ia, host class I, and the UEA-1 ligand are expressed on the same class of cells, pre-

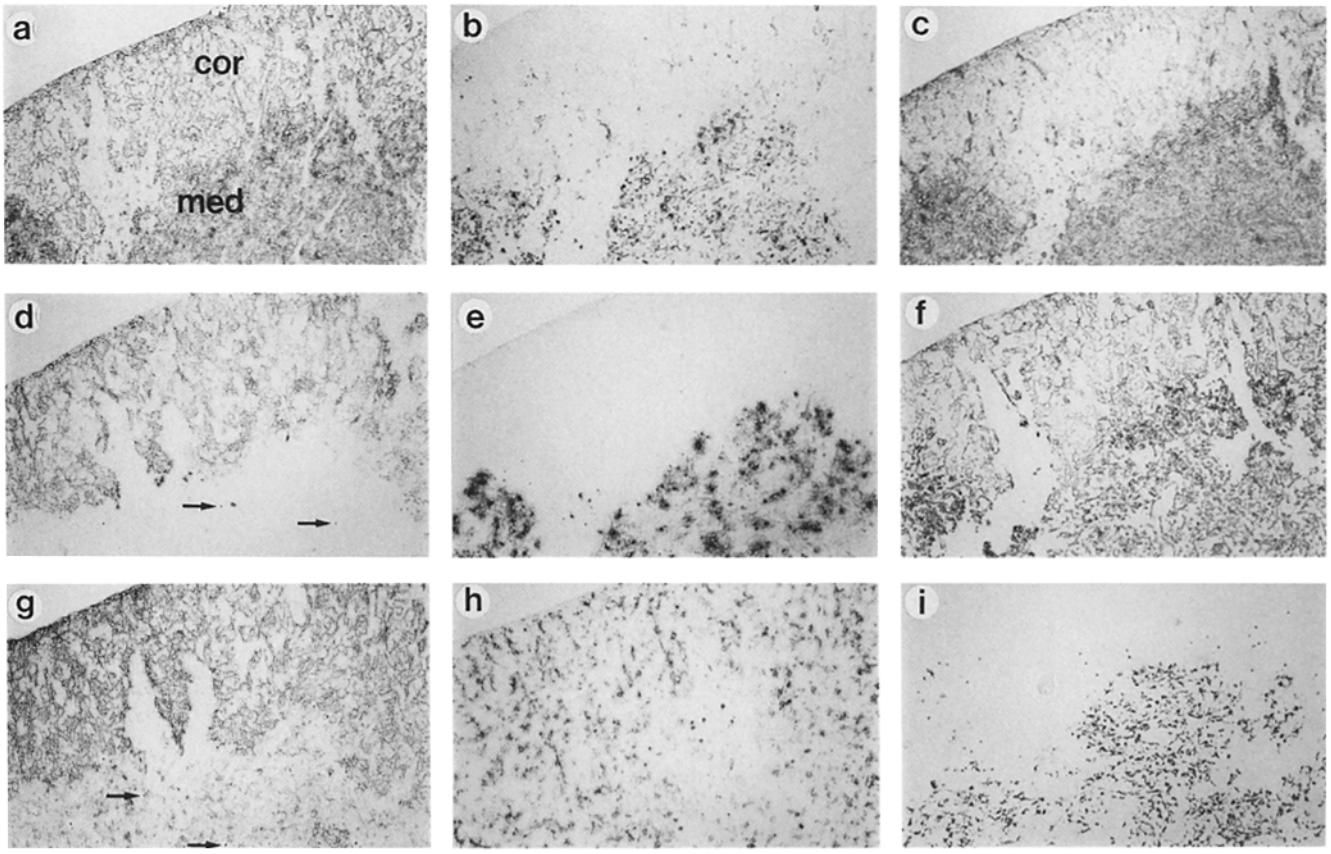

Figure 1. Normal adult thymus stained with some of the reagents listed in Table 1. Serial cryostat sections of a normal B6 (I-A^b) thymus were stained for the following markers as described in Materials and Methods. (*a*) I-A^b: there is confluent staining of BM-derived cells in the middle (*med*) and reticular staining in the cortex (*cor*) typical of epithelial cells. (*b*) I-O: the medulla shows prominent staining of a network of stellate cells that resemble epithelial cells under higher power (see Fig. 3 *g*): staining of the cortex is sparse. (*c*) K^b: as for I-A expression, there is strong staining of BM-derived cells in the medulla; reticular staining of cortical epithelium is weak but significant. (*d*) 6C3: staining is restricted to cortical epithelial cells. (*e*) UEA-1: staining is restricted to dense aggregates of epithelial cells in the medulla; by electron microscopy, UEA-1 staining is restricted to epithelial cells (12). (*f*) Pancytokeratin: staining is evident on the network of epithelial cells present in both the cortex and medulla. (*g*) NLDC-145: there is strong staining of cortical epithelial cells and weaker staining in the medulla, presumably of DC. (*h*) F4/80: scattered punctate staining of both cortex and medulla, indicative of Mϕ. (*i*) MD-1: staining of a lattice of stellate epithelial cells in the medulla. Eosinophils scattered in the medulla (which possess endogenous peroxidase activity) are depicted with arrows (see *d* and *g*). All sections were photographed at ×100.

sumably a subset of epithelial cells. It should be noted that quite different patterns of staining are observed with reagents detecting DC (NLDC-145) (Fig. 3, *j* and *k*), Mϕ (F4/80) (Fig. 3 *d*), and B cells (anti-B220) (Fig. 3 *c*). However, double staining for I-A^k (blue) and NLDC-145 (red) reveals two-color (brown) staining of cortical epithelium but only one-color staining of cells in the medulla (Fig. 3, *j* and *k*).

The extensive staining of the medulla with antikeratin antibody (Fig. 3 *h*) implies that only a proportion of medullary epithelial cells are Ia^+, class I^+ UEA-1^+. In this respect it is notable that I-O expression in the medulla of BMC is quite different from Ia, class I, and UEA-1 expression. I-O expression is more uniform and, under low power, is distributed on sheets rather than clumps of cells (Fig. 3 *b*). Under high power (Fig. 3 *g*), double staining for I-O (red) vs. I-A^k (blue) reveals that I-O is expressed on a network of cells with long processes. Most of these cells do not costain for I-A^k. Likewise, many of the I-A^{k+} cells do not costain for I-O. Although some cells appear to double stain, it is difficult to visualize with certainty whether such staining reflects conjoint expression of I-A^k and I-O rather than close proximity of single-positive cells or diffusion of the color substrates.

These findings on parent → F_1 BMC suggest that the epithelial cells in the medulla comprise two largely nonoverlapping populations of $Ia^+I\text{-}O^-$ and $Ia^-I\text{-}O^+$ cells (with perhaps also some $Ia^+I\text{-}O^+$ cells). To search for further heterogeneity of epithelial cells, the chimera thymuses were double stained for I-A^k and I-O expression using the same color. The staining pattern observed (not shown) appeared to be nearly identical to the pattern seen with anti–pan-keratin antibody, implying that few if any medullary epithelial cells are $Ia^-I\text{-}O^-$.

Thymus of the 36–5 Transgenic Line. Since the BMC discussed above were conditioned with heavy irradiation, it could

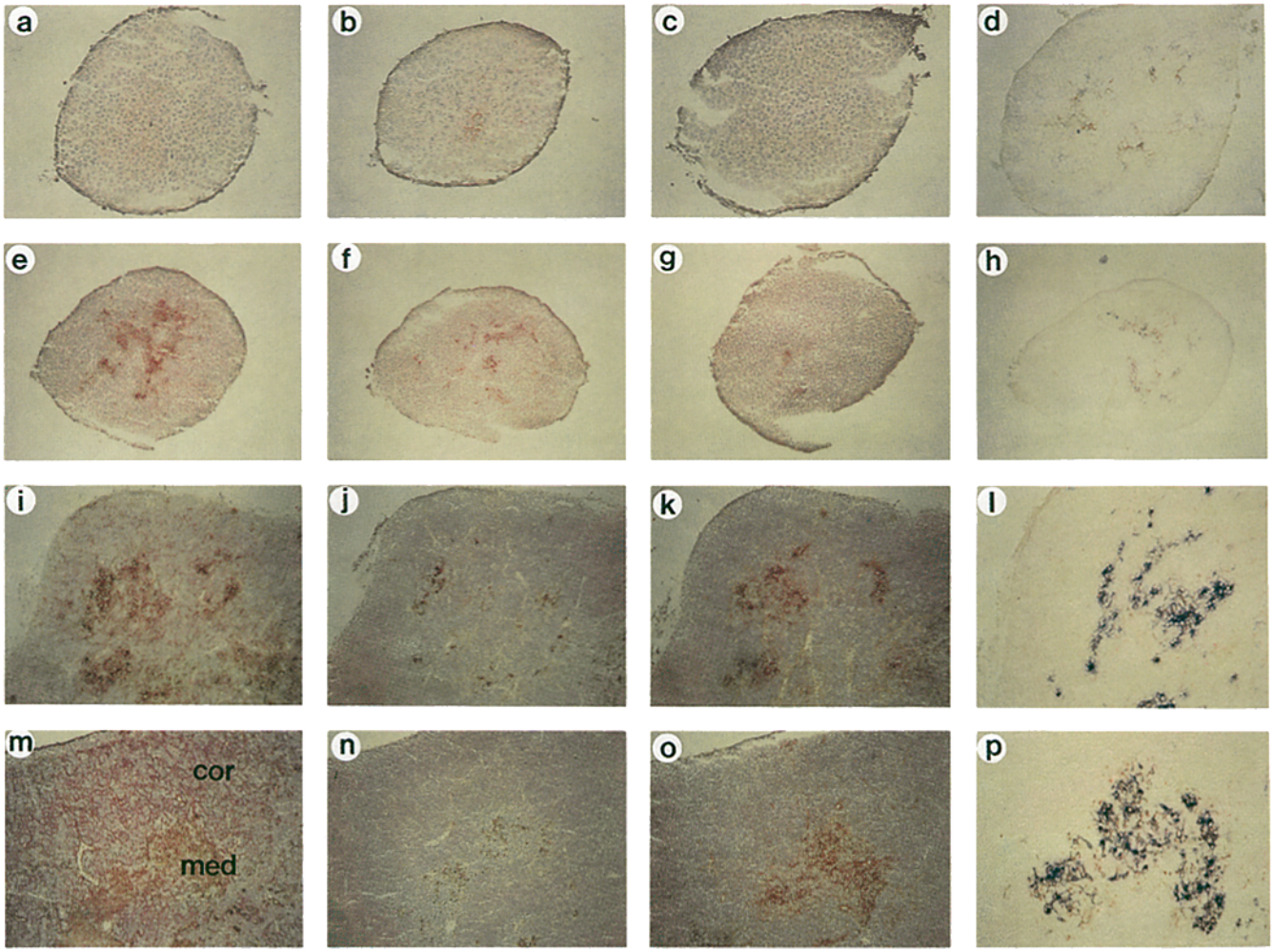

Figure 2. Ontogeny of H-2 class I, Ia, UEA-1, and I-O expression in the fetal thymus. Serial sections of BALB/c thymuses from day 14 fetus (*a–d*), day 16 fetus (*e–h*), newborn (*i–l*), and 10-d-old neonatal (*m–p*) mice were stained for the expression of Ia ($I\text{-}A^d$) (*a*, *e*, *i*, and *m*), I-O (*b*, *f*, *j*, and *n*), and class I (L^d) (*c*, *g*, *k*, and *o*) molecules, and counterstained with hematoxylin. Some of the sections were double stained for UEA-1 (in blue) vs. I-O (in red) (*d*, *h*, *l*, and *p*) as described in Materials and Methods; these sections were not counterstained with hematoxylin. I-A and class I expression (*a*, *e*, *i*, and *m*; and *c*, *g*, *k*, and *o*) is not clearly evident until about day 16 of gestation; staining is first detectable in the medulla and remains low on cortical epithelium until the time of birth (I-A) or after birth (class I). By contrast, UEA-1 (*d*, *h*, *l*, and *p*) and I-O (*d*, *h*, *l*, and *p*; and *b*, *f*, *j*, and *n*) is apparent on small central aggregates of cells as early as day 14 of gestation. Expression of these two markers increased progressively during ontogeny and reaches adult levels only after birth. Before birth, both markers seem to be expressed on the same cells (*d h*, and *l*). By 10 d after birth, a distinct population of $UEA\text{-}1^-$ $I\text{-}O^+$ cells is evident (*p*). All sections were photographed at magnification of 100, except *a–d*, and *p*, which were taken ×160.

be argued that the staining patterns observed were not representative of the normal thymus. To assess this possibility we examined the thymus of the 36–5 line of I-E transgenic mice (21). As discussed elsewhere, I-E expression in these mice is limited to the thymus (19, 21). In sections, I-E expression is prominent in the cortex, presumably on epithelial cells (Fig. 4 *a*). In addition, there is conspicuous staining of aggregates of cells in the medulla. This pattern of I-E expression in the medulla closely resembles the staining seen with UEA-1 (Fig. 4 *b*). The possibility that the $I\text{-}E^+$ cells in the medulla of 36–5 mice are BM derived seems unlikely for three reasons. First, $I\text{-}E^+$ cells in 36–5 mice are not seen in sections of spleen or LN (not shown). Second, reconstituting heavily irradiated (1,100 rad) B6 ($I\text{-}E^-$) mice with 36–5 BM cells does not lead to the appearance of $I\text{-}E^+$ cells in the host thymus (or elsewhere) (Fig. 4 *c*). Third, the aggregates of $I\text{-}E^+$ cells in the thymic medulla of 36–5 mice are retained when these mice are irradiated and reconstituted with B6 BM (Fig. 4 *d*). The only obvious difference in I-E (and UEA-1) expression in the thymus of normal 36–5 mice vs. long-term B6 → 1,100-rad 36–5 BMC is that the stained cells in the medulla show a more clumped appearance in the chimeras. As in the chimeras discussed earlier (Fig. 3 *b*), sheets of $I\text{-}O^+$ cells are scattered throughout the medulla (Fig. 4 *e*); most of the $I\text{-}O^+$ cells do not coexpress I-E molecules.

Another approach for examining non-BM-derived cells in

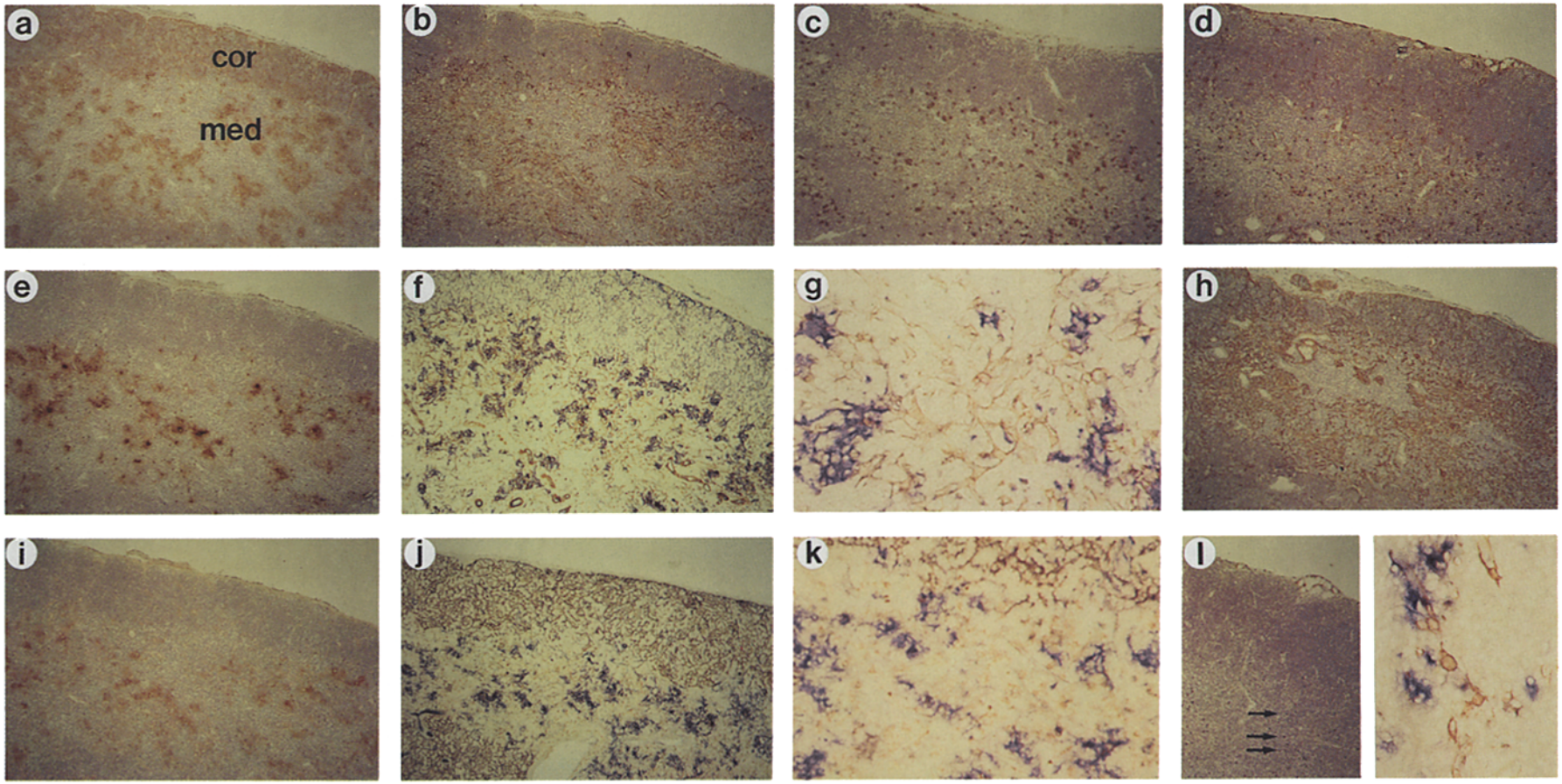

Figure 3. Host H-2 expression in thymic medulla of long-term parent → F_1 BMC prepared with supralethal irradiation. Serial sections of thymus from B6 (H-2^b) BM → 1,300-rad (B6 × CBA/Ca [H-2^k])F_1 BMC established 6 m before were stained for the expression of the following molecules using one- or two-color staining procedures. (*a*) I-A^k (host I-A): patches of dense staining are prominent in the medulla (*med*); there is also reticular staining of epithelial cells in the cortex (*cor*). (*b*) I-O: a lattice of stained cells is spread throughout most of the medulla; in accordance with previous findings (13) there is crossreactive staining of some of the blood vessels. (*c*) B220: scattered stained cells (presumably B cells) are evident in the cortical-medullary junction. (*d*) F4/80: scattered stained cells (presumably Mϕ) are visible in both the cortex and medulla. (*e*) UEA-1: the clumped staining in the medulla resembles the medullary staining for I-A^k. (*f*) Two-color staining for I-A^k (blue) and I-O (red): the cortex shows only blue (I-A^k) staining whereas the medulla shows both blue and red (I-O) staining. (*g*) Higher power (×400) view of *f*: a network of red-stained (I-O^+) cells is seen with most of these cells showing little or no blue staining; blue-stained (I-A^k) cells are more scattered; some of the blue-stained cells show little or no red staining whereas other cells appear to show both colors (although it is unclear whether the double staining is real or reflects intertwining of the processes of adjacent cells). (*h*) Cytokeratin: dense staining of epithelial cells in both cortex and medulla. (*i*) K^k (host class I): the clumped staining in the medulla resembles the staining patterns for UEA-1 and I-A^k. (*j*) two-color staining for I-A^k (blue) and NLDC-145 (red): double staining (which yields a brown color) is prominent in the cortex (indicative of NLDC$^+$ I-A^+ cortical epithelium) but not in the medulla. (*k*) High-power (×250) view of j (cortico-medullary region): distinct populations of blue-only (I-A^k medullary epithelium) and red-only (donor-derived DC) cells are evident; double-stained (brown) cells are very rare in the medulla but are easily seen in the cortex (*top*). (*l*, *left*) Background staining with an irrelevant antibody (anti-L^d) reveals endogenous peroxidase–containing cells scattered in the medulla (*arrows*). (*l*, *right*) High-power (×400) view of C.B-17 SCID thymus stained for UEA-1 (blue) and I-O (red), separate populations of UEA-1$^+$ and I-O$^+$ epithelial cells are clearly apparent with almost no double-positive cells. Except for the double-stained sections, cell sections were counterstained with hematoxylin. Except for *g*, *k*, and *l* (*right*), all sections were photographed at ×100.

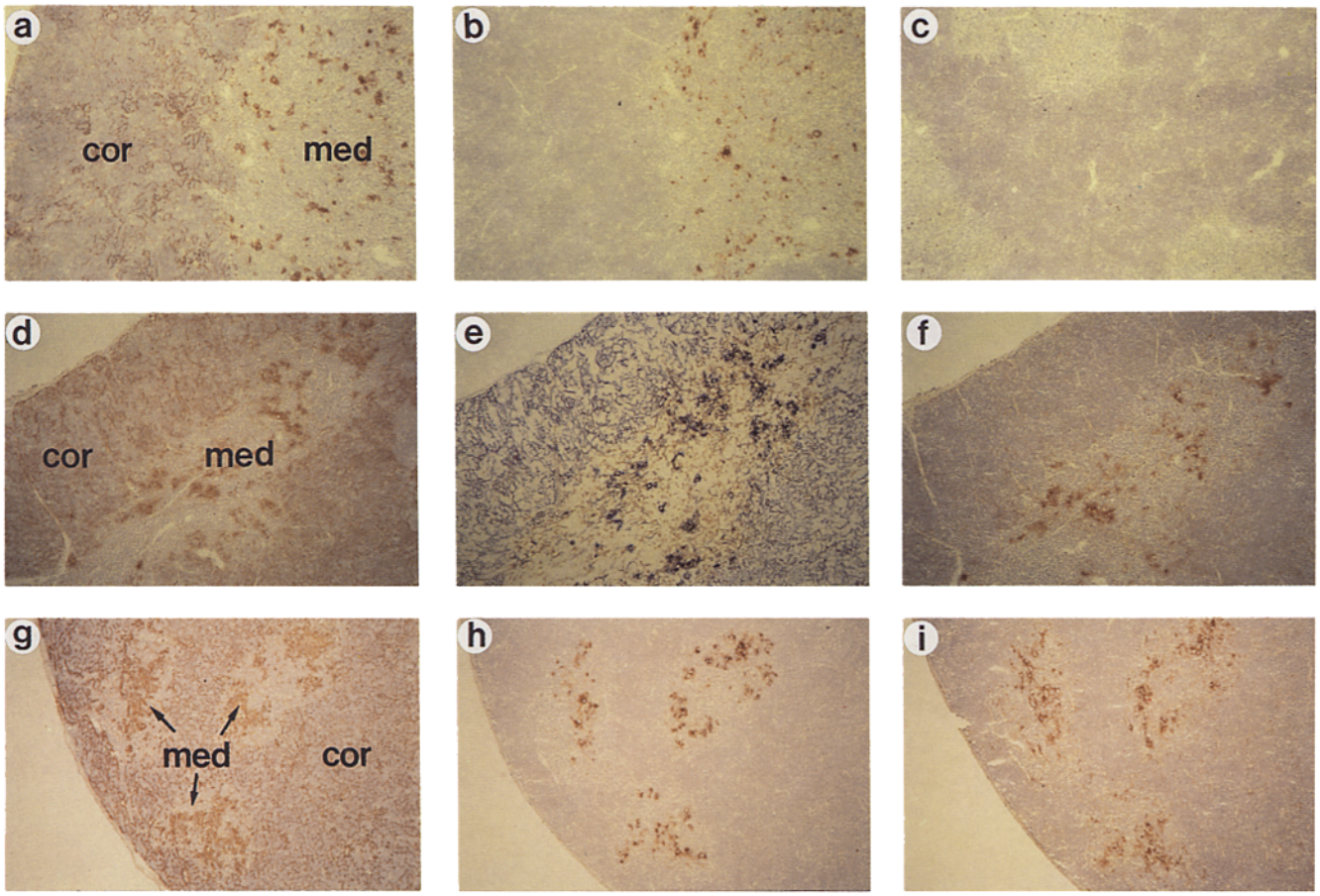

Figure 4. I-E expression on medullary epithelial cells in the thymus from the 36–5 line of I-E transgenic mice and from nude mice given MHC-mismatched deoxyguanosine-treated fetal thymus grafts. Serial sections were stained; except for *e*, all sections were counterstained with hematoxylin. (*a*) I-E expression in normal 36–5 mice: there is scattered dense staining in the medulla and reticular staining in the cortex; as documented elsewhere (19, 21), I-E expression in 36–5 mice is restricted to the thymus. (*b*) UEA-1 expression in normal 36–5 mice: the scattered dense staining in the medulla closely resembles the pattern of I-E staining (compare with *a*). (*c*) I-E expression in 36–5 BM → 1,100-rad B6 mice: no staining is evident. (*d*) I-E expression in B6 ($I\text{-}E^-$) BM → 1,100-rad 36–5 BMC (tested at 6 mo postreconstitution): staining is very similar to the normal 36–5 thymus, except that the stained cells in the medulla are more aggregated (compare with *a*). (*e*) I-E-(blue) vs. I-O (red) staining in B6 BM → 36–5 BMC: many $red^+/blue^-$ cells are evident in the medulla. (*f*) Y-Ae expression in B6 BM → 36–5 BMC: the pattern of dense aggregates of stained cells in the medulla is similar to I-E staining in the medulla (compared with *d*); staining of the cortex is very weak (see Fig. 5). (*g*) I-E expression in the thymus grafts taken from B6 nude mice given $I\text{-}E^+$ B10.A (5R) fetal thymuses (FT) treated with deoxyguanosine (dGua) in vitro: aggregation of the stained cells in the medulla is even more pronounced than in B6 BM → 36-5 BMC (compare with *d*). (*h*) UEA-1 expression in dGua 5R FT → B6 nude mice: the clumped staining in the medulla resembles I-E staining (compare with *g*), although UEA-1 staining is less intense; the more extensive staining for I-E than UEA-1 is probably artifactual, reflecting greater diffusion of the substrate for I-E staining. (*i*) Y-Ae expression in dGua 5R FT → B6 nude mice: the dense aggregates of stained cells in the medulla resembles the staining for I-E and UEA-1 (compare with *g* and *h*); there is minimal staining of the cortex. All sections were photographed at ×100.

the thymic medulla is to graft MHC-mismatched mice with fetal thymuses treated with deoxyguanosine in vitro; this treatment depletes the thymus of BM-derived cells but leaves epithelial cells intact (30). The sections shown in Fig. 4, *g*, *h*, and *i* were prepared from the thymus grafts taken from B6 nude ($I\text{-}E^-$) mice given deoxyguanosine-treated B10.A(5R) ($I\text{-}E^+$) day 14 fetal thymuses 6 wk before. Very dense aggregates of $I\text{-}E^+$ cells are prominent in the medullary region of these thymus grafts (Fig. 4 *g*); UEA-1 expression is similar (Fig. 4 *h*), though less intense than for I-E expression (see figure legend).

The above studies with BM chimeras, the 36–5 transgenic line, and thymus-grafted mice essentially rule out the possibility that the $Ia^+/UEA\text{-}1^+$ subset of medullary epithelial cells is of BM origin. It is striking, however, that the density of Ia on medullary epithelium is conspicuously high, probably as high as on BM-derived APC. This raises the question whether the Ia molecules on medullary epithelium and BM-derived cells are qualitatively similar. As discussed below, both cell types express high levels of a unique peptide/Ia complex defined by the Y-Ae mAb (19).

Y-Ae Expression. As mentioned earlier (see Introduction),

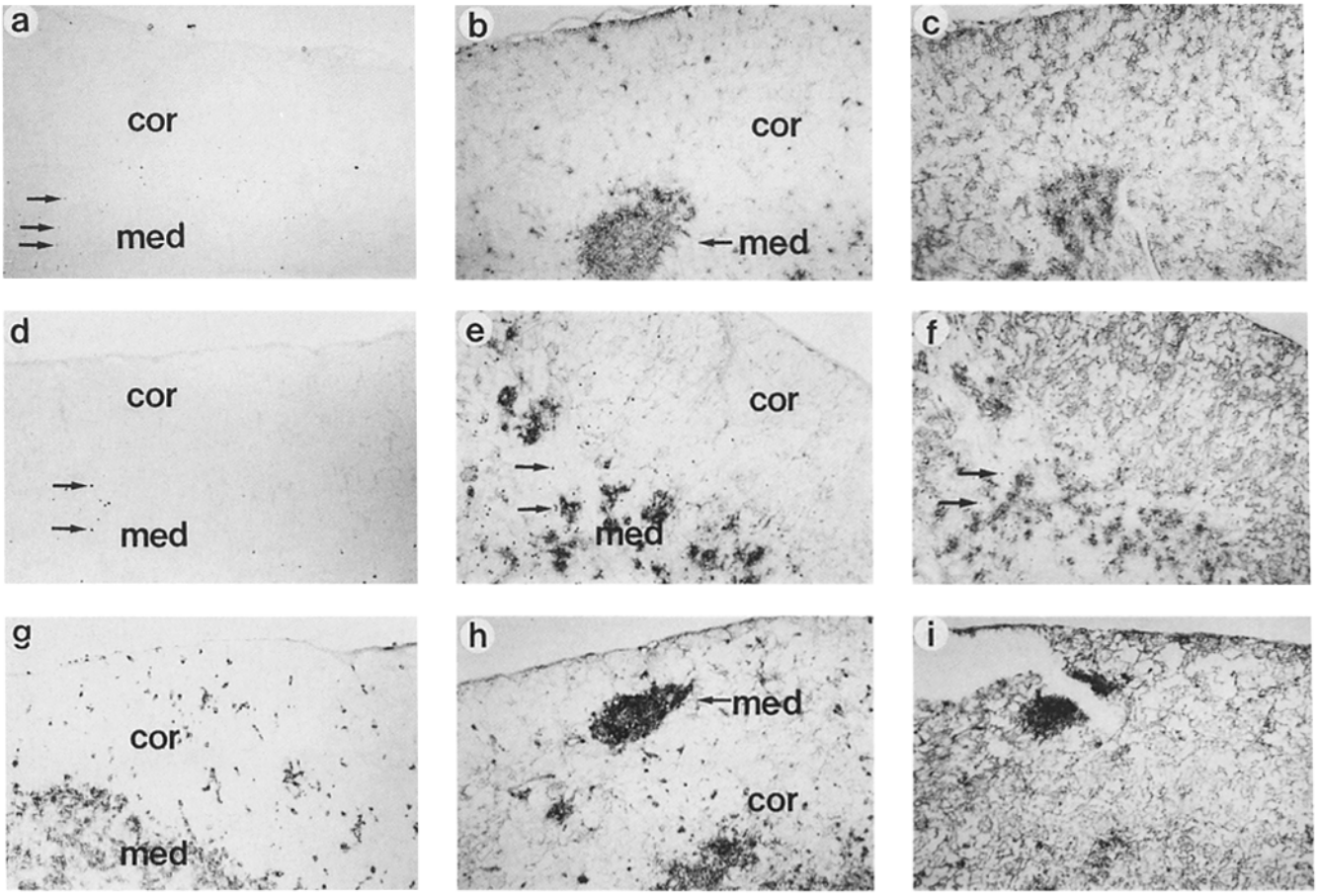

Figure 5. Y-Ae expression on medullary epithelial cells. Serial sections of thymus from various strains, including the 36–5 (I-E$^+$ only in the thymus) and 107–1 (normal I-E expression) transgenic lines, were stained: counterstaining with hematoxylin was omitted. (*a*) Y-Ae staining of a control Y-Ae$^-$ CBA/J (H-2^k) thymus: no staining is evident except for faint background staining of blood vessels; small dark granular staining is due to endogenous peroxidase–positive cells (*arrows* also prominent in *d–f*). (*b*) Y-Ae staining of Y-Ae$^+$ B10.A(5R) thymus: there is strong staining throughout the medulla (*med*) and on scattered cells (presumably Mϕ) in the cortex (*cor*): there is also very weak but significant staining of cortical epithelial cells (compare with *a* and *d*). (*c*) I-E staining of 5R thymus: typical confluent staining of the medulla and reticular staining of the cortex is seen. (*d*) Y-Ae staining of a control Y-Ae$^-$ B6 (H-2^b) thymus: no staining except for weak background staining of blood vessels and on cells with endogenous peroxidase activity. (*e*) Y-Ae expression in 36–5 thymus: there is strong staining on clumps of cells in the medulla (*med*), and weak staining of epithelial cells in the cortex (*cor*). (*f*) I-E expression in 36–5 thymus: staining of cortical epithelium is much stronger than for Y-Ae (compare with *e*), whereas staining of the medulla is similar. (*g*) Y-Ae expression in thymus from B10.A(5R) BM → 1,100-rad B6 BMC: there is strong confluent staining in the medulla (*med*) and punctate staining of scattered cells (probably Mϕ) in the cortex (*cor*); there is no staining of cortical epithelial cells. (*h*) Y-Ae staining of 107 thymus: there is strong confluent staining of the medulla (*med*) and of scattered cells (presumably Mϕ) in the cortex (*cor*); there is also appreciable staining of the cortical epithelial cells (note that the 107 transgenic mouse has a higher copy number of I-E-genes than normal I-E$^+$ mice (reference 19). (*i*) I-E expression in 107 thymus: there is strong staining of both cortex and medulla (compare with *h*). All sections were photographed at ×100.

the Y-Ae antibody detects complexes of an Eα peptide bound to I-A^b molecules (19, 20). Y-Ae expression is limited to strains expressing both I-A^b and I-E (Eα), e.g., B10.A(5R). In these strains, Y-Ae expression is prominent on B cells and DC but not on cortical epithelium. The original paper on Y-Ae expression (19) involved staining of two lines of I-E transgenic mice, 36–5 and 107–1; unlike 36–5, the 107–1 line shows a normal tissue distribution of I-E molecules with prominent expression on BM-derived cells as well as on thymic epithelium. In both lines, Y-Ae expression was found to be negative in the cortex (19). The significant finding was that, in contrast to 107–1 mice, there was no detectable Y-Ae expression in the medulla of the 36–5 line. These findings were taken to imply that Y-Ae expression in the thymus is limited to BM-derived cells, both cortical and medullary epithelium being negative. This conclusion needs to be modified because, with newer preparations of conjugated antibody and a more sensitive staining technique, both populations of epithelial cells show significant Y-Ae expression. The evidence is as follows.

Y-Ae vs. I-E expression in the thymus of B10.A(5R), 36–5, and 107–1 mice is shown in Fig. 5; all sections were stained

under the same conditions. To increase the sensitivity of staining, sections were not counterstained with hematoxylin. In comparison with the undetectable level of staining in Y-Ae$^-$ control CBA/J and B6 mice (Fig. 5, *a* and *d*), the cortical epithelium of 5R mice shows very faint but significant staining for Y-Ae (Fig. 5 *b*). This Y-Ae staining of cortical epithelial cells is not artifactual because no staining of epithelial cells is seen in the cortex of 5R → B6 BMC (Fig. 5 *g*). Note that the scattered punctate staining in the cortex of 5R mice and 5R → B6 BMC presumably reflects staining of BM-derived cells. Y-Ae expression on cortical epithelium is stronger in 36–5 mice (Fig. 5 *e*) and even more prominent in 107–1 (Fig. 5 *h*). Note, however, that even in 107–1 mice Y-Ae expression is clearly much less intense than I-E expression (Fig. 5 *c*, *f*, and *i*).

In the medulla, both 5R and 107–1 mice show confluent and dense Y-Ae expression, presumably reflecting staining of BM-derived cells (Fig. 5, *b* and *h*). If this staining were restricted to BM-derived cells, the medulla of 36–5 mice would be expected to be Y-Ae$^-$. The same prediction would apply to deoxyguanosine-treated 5R thymus grafts placed in B6 nude mice. In fact, in both situations (Fig. 5 *e* and Fig. 4 *i*), and also in B6 → 36–5 BMC (Fig. 4 *f*), the medulla shows strong Y-Ae expression. Y-Ae expression in the medulla is restricted to clumps of cells and closely resembles I-E and UEA-1 expression (compare Fig. 5 *e* vs. *f*, Fig. 4 *i* vs. *h*, and Fig. 4 *f* vs. *d*).

These data indicate that, in addition to BM-derived cells, a subset of medullary epithelial cells is strongly Y-Ae$^+$. Based on staining patterns, Y-Ae expression on medullary epithelium is probably restricted to the Ia$^+$, UEA-1$^+$ subset. In this respect, double staining for Y-Ae vs. I-O has shown that most I-O$^+$ cells in the above thymuses are Y-Ae$^-$ (data not shown).

Discussion

Epithelial cells in the cortex of the thymus are well characterized and appear to play a key role in controlling positive selection of T cells (see Introduction). Much less is known about medullary epithelial cells. These cells are poorly characterized and their function is obscure. Since T cell selection in the thymus reflects contact with MHC molecules, it is important to know whether medullary epithelial cells show a normal distribution of MHC molecules. In fact, the experiments in this paper indicate that MHC expression on medullary epithelium is highly unusual. Some medullary epithelial cells show a conspicuously high density of typical MHC class I and II (Ia) molecules. However, other cells display little or no conventional MHC expression but instead show high expression of I-O molecules, a novel class of atypical nonpolymorphic MHC-encoded molecules present on B cells.

Ia Expression. Although Ia expression on a proportion of medullary epithelial cells has been demonstrated by electron microscopy (15, 16), the dense network of Ia$^+$ BM-derived cells in the medulla makes it very difficult to visualize Ia expression on medullary epithelium by conventional light microscopy. There is also the possibility that Ia molecules on medullary epithelium are absorbed from adjacent BM-derived cells. These problems can be avoided by replacing the BM-derived cells in the thymus with cells of a different Ia haplotype, thereby creating a chimeric thymus.

Using this approach we show here that the medulla contains a prominent population of Ia$^+$ epithelial cells. These cells are easily visualized in parent → F_1 chimeras prepared with supralethal irradiation (1,300 rad) and remain conspicuous even after a double dose of irradiation (1,300 rad followed 6 mo later by another 900 rad). The possibility that the cells are radioresistant host BM-derived cells thus seems most unlikely. Moreover, comparable populations of Ia$^+$ (I-E$^+$) cells in the medulla are seen in the 36–5 transgenic line (which does not express I-E molecules outside the thymus) and in thymus grafts pretreated with deoxyguanosine in vitro. In these various situations the Ia$^+$ cells in the medulla have short reticular processes and tend to be arranged in clumps, especially in the irradiated thymus. The pattern of Ia staining in the medulla corresponds closely with UEA-1 staining, which suggests that most Ia$^+$ medullary epithelial cells coexpress the UEA-1 ligand; Farr and Anderson (12) reached a similar conclusion using electron microscopy. The Ia$^+$/UEA-1$^+$ cells in the medulla stain with an antipankeratin antibody and also appear to express class I molecules.

Although the function of the Ia$^+$ subset of medullary epithelial cells is unknown, their strong expression of typical MHC class I and II molecules suggests that these cells may play a role in thymic selection, especially negative selection (tolerance induction) (17). A number of groups have concluded that thymic epithelial cells can contribute to tolerance induction (reviewed in reference 2), but it is unclear whether this process reflects contact with medullary epithelium, cortical epithelium, or both. The best support for the notion that medullary epithelium can contribute to tolerance induction has come from two recent studies with MHC-transgenic mice. First, Hoffman et al. (33) have reported partial tolerance to class I (K^b) antigens when nude (K^k) mice are grafted with embryonic thymuses (branchial clefts) taken from a transgenic line expressing K^b exclusively in medullary epithelium. Second, Burkly and Lo (manuscript in preparation) have found that, after irradiation and reconstitution with I-E$^-$ B6 BM, a transgenic mouse line expressing I-E molecules selectively in medullary epithelium shows significant (50%) deletion of I-E-reactive Vβ5$^+$ T cells. Despite this evidence, it is generally agreed that full tolerance induction in the thymus requires contact with BM-derived cells (2).

The failure of medullary epithelial cells to induce more than partial tolerance of T cells is surprising because MHC expression on medullary epithelium is conspicuously high, perhaps as high as on BM-derived cells. Why medullary epithelium (and thymic epithelium in general) is much less tolerogenic than BM-derived cells is unclear. One possibility is that epithelial cells and BM-derived cells express a different range of MHC-associated self-peptides (34). Thymic epithelium might be fully capable of tolerizing T cells reactive to the particular peptide/MHC complexes expressed on epithelial cells, but would be unable to tolerize T cells reactive to other

self-peptides, i.e., peptides expressed exclusively on BM-derived cells. Interest in this idea has been heightened by the discovery that, unlike BM-derived cells, cortical epithelial cells show only limited expression of the Y-Ae epitope, a complex of an Eα peptide bound to Ia (I-A^b) molecules (20). In the medulla, Y-Ae expression was originally thought to be restricted to BM-derived cells with little or no expression on medullary epithelium (19). One could then make the case for differential expression of self-peptides on medullary epithelium vs. BM-derived cells. Under optimal staining conditions, however, we now find that the Ia$^+$ subset of medullary epithelial cells shows strong Y-Ae expression. Y-Ae expression is also demonstrable on cortical epithelium but is clearly much weaker on these cells than on medullary epithelium. This finding implies a qualitative difference in the capacity of cortical and medullary epithelium to process and present self-peptides (though whether this difference applies to peptides other than Eα remains to be determined). By the same token, the high Y-Ae density on medullary epithelial cells suggests that these cells more closely resemble BM-derived cells than cortical epithelium in their antigen-processing functions.

The finding that medullary epithelial cells and BM-derived cells share Y-Ae expression argues against the notion that these two cell types express a different range of self-peptides. Why medullary epithelial cells fail to cause more than partial negative selection thus remains a paradox. Perhaps the most likely possibility is that medullary epithelial cells show limited expression of certain cell surface accessory molecules or mouse mammary tumor virus products. The tolerogenicity of medullary epithelium would then be restricted to high-affinity T cells.

I-O Expression. With regard to heterogeneity among medullary epithelial cells, the staining patterns for Ia, UEA-1, Y-Ae, and H-2 class I expression are all very similar, especially in chimeras, and it seems very likely that these markers are expressed on the same class of cells. I-O expression is quite different. In contrast to the clumped distribution of Ia$^+$ medullary epithelial cells, the I-O$^+$ component of medullary epithelium forms a uniform network of cells throughout the medulla. Based on double staining for I-O vs. Ia, most I-O$^+$ cells appear to be Ia$^-$. Conversely, at least some of the Ia$^+$ cells are I-O$^-$. Other cells appear to stain for both markers, although it is difficult to know whether such staining is real or artifactual. Nevertheless, it seems reasonable to conclude that, for the most part, the expression of Ia and I-O molecules on medullary epithelium is mutually exclusive and that distinct subsets of Ia$^+$I-O$^-$ and Ia$^-$I-O$^+$ cells exist. The relationship between these cells is unclear. In the fetal thymus, it is notable that most medullary epithelial cells are Ia$^+$I-O$^+$, which suggests that the Ia$^+$I-O$^-$ and Ia$^-$I-O$^+$ subsets in the adult thymus might differentiate from Ia$^+$I-O$^+$ precursors.

Since we used a relatively limited panel of reagents to define medullary epithelium, it is quite likely that other reagents will reveal further complexity of these cells. In this respect, preliminary work with another marker for medullary epithelium, MD-1 (11), has shown that MD-1 expression closely resembles I-O expression. Surprisingly, however, double staining for MD-1 vs. I-O indicates that expression of these two markers is largely nonoverlapping (C.D. Surh, and J. Sprent, unpublished data). Comparable experiments with other markers are underway. It should be mentioned that the physiological significance of I-O expression on medullary epithelial cells is obscure. Resolving this issue will probably hinge on defining the function of I-O molecules and explaining why the post-thymic expression of these molecules seems to be limited to a single cell type, namely B cells.

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