Immunohistochemical and Topographic Studies of Dendritic Cells and Macrophages in Human Fetal Cornea

Claudia M. Diaz-Araya,* Michele C. Madigan,† Jan M. Provis,*‡ and Philip L. Penfold†

Purpose. To investigate the distribution and phenotype of major histocompatibility complex (MHC) class II-positive dendritic cells and macrophages in normal human fetal cornea in the age range 10 to 25 weeks gestation.

Methods. Peroxidase and gold immunohistochemistry were used to visualize MHC class II and macrophage antigen (S22) immunoreactive cells. Cell distributions were analyzed quantitatively, and topographic maps were produced.

Results. Immunoreactive cells, concentrated centrally, were present at 10 weeks gestation in the corneal epithelium and stroma. Average densities increased steadily up to 25 weeks gestation. Two morphologic forms of MHC class II and S22 immunoreactive cells were observed—large, dendritiform cells and small, rounded cells with short processes. Electron microscopy revealed that most MHC class II-positive cells were morphologically consistent with previous ultrastructural descriptions of corneal Langerhans cells. Immunoreactive cells were more numerous in immunogold-labeled specimens than in peroxidase-labeled specimens of similar ages. However, quantitative analysis of both techniques revealed that S22-positive cells comprised 30% to 50% of MHC class II-positive cells.

Conclusions. This study provides a detailed description of heterogeneous populations of MHC class II and S22 immunoreactive cells in the human fetal cornea. In contrast to the adult cornea, which is typically devoid of MHC class II-positive cells, immunoreactive cells in the fetal cornea are concentrated centrally and increase in density up to at least 25 weeks gestation. These results indicate that reduction in Langerhans cell numbers to adult levels must occur after 25 weeks gestation. The presence of dendritic cells and macrophages in the fetal cornea has important implications for the understanding of corneal immunology.


It has been established that Langerhans cells (LC) are a population of dendritic cells (DC) that mediate antigen presentation in vitro and in vivo in the skin and cornea. Constitutive expression of major histocompatibility complex (MHC) class II antigens is a characteristic feature of DC, including LC in the skin, the conjunctivum, and the adult corneal epithelium of several animal species and humans. Although the presence of LC throughout the epithelium of the central cornea in normal infants has previously been reported using nucleotidase reactivity, little is known about these cells in the developing human cornea, and the identity of MHC class II-positive cells in the corneal stroma remains controversial.

Subsets of macrophages and DC have been identified previously using monoclonal antibodies in normal human tissues, including brain and skin. In addition, populations of resident DC and macrophages have been reported in the rat ciliary body and iris, the rat choroid, and in the human retina. The presence of heterogeneous populations of DC and macrophages in the human fetal cornea, however, has not been previously documented. In the present study, we have used antibodies to MHC class II and macrophage (S22) antigens to study the morphology and distribution of DC and macrophages in wholemounted fetal cornea.

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Dendritic Cells and Macrophages in Fetal Cornea

MATERIALS AND METHODS

Specimens and Tissue Preparation

Human tissues used in this study were obtained (after obtaining informed consent) according to the tenets of the Declaration of Helsinki and approval by the Human Ethics Committee at the University of Sydney, Australia. Normal human fetal eyes (n = 74) in the age range 10 to 25 weeks gestation were obtained within 15 minutes of pregnancy termination. Fetuses at 21 weeks gestation or more died of natural causes, usually after premature delivery. Approximate gestational ages were determined from medical records and ultrasound examinations. Specimens were fixed in either 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) or in a periodate-lysine-paraformaldehyde solution containing 3% paraformaldehyde, 1.8% lysine, and 0.3% periodate (pH 7.4) for 24 hours at 4°C and subsequently washed in 0.1 M Tris-buffered saline (TBS). Eyes were dissected into two portions by an incision at the ora serrata. Cornea with the conjunctivum attached, the latter used as a positive control, were dissected away from the ciliary body and lens.

Immunohistochemistry

Dendritic cells and macrophages in fetal corneas and conjunctivum were identified using immunohistochemical procedures. Specimens were soaked in 0.2% Triton X-100 (BDH, Sydney, Australia) in TBS (pH 7.6) for peroxidase labeling or in 0.4% saponin in TBS for immunogold labeling for 1 to 6 days at 4°C (depending on corneal size and thickness) before incubation in primary antibody for 3 to 6 days at 4°C. Monoclonal mouse anti-human class II HLA-DR, CR3/43, 1:50 dilution (Dako, Sydney, Australia) and mouse anti-human macrophage (S22 antigen), 1:5 dilution (Amersham, Sydney, Australia) antibodies were used in this study. Primary and secondary antibodies were diluted in TBS-0.4% saponin—2% normal sheep serum (Dako) for peroxidase labeling or in TBS-0.4% saponin—2% normal goat serum (Dako) for immunogold labeling. Specimens were then rinsed in TBS.

Avidin-Biotin-Peroxidase Labeling

Specimens were incubated in a biotinylated secondary antibody, anti-mouse, 1:50 dilution (Amersham) for a further 24 hours at 4°C. After another rinse in TBS, bound antibody was detected with an avidin–biotin–peroxidase labeling technique (Vecastain, Vector Laboratories, Burlingame, CA) using a nickel-enhanced 3,3′diaminobenzidine tetrahydrochloride solution.26 Radial cuts were made in whole corneas to assist flattening; specimens were wholemounted, epithelium upward, onto gelatinized slides, placed in a humidified chamber overnight at 4°C to avoid sudden dehydration, air dried, dehydrated, and mounted in DePeX (BDH).

Immunogold Labeling

Specimens were labeled using 1-nm gold particles enhanced with silver for light microscopy. Specimens were incubated overnight at 4°C in 1 nm gold conjugated to goat F(ab′)2 anti-mouse IgG + IgM (BioCell Research Laboratory, Cardiff, UK), diluted 1:50. After further washing in 0.4% saponin–TBS, TBS, and 0.1 M sodium cacodylate buffer (pH 7.4), tissue was fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 hour at room temperature or overnight at 4°C. After rinsing in distilled water, bound gold particles were visualized using silver enhancing solution (BioCell Research); the reaction was stopped by washing in distilled water, and specimens were flattened onto glass slides and mounted in glycerol.

Controls

Delay between death and fixation was less than 6 hours for all specimens used in this study. However, to examine for the effect of postmortem delay on MHC class II expression, fixation of some human fetal corneas was intentionally delayed at intervals of 45 minutes and at 6, 12, and 18 hours (at 4°C), and the intensities of staining were compared qualitatively between samples. In all cases, the intensity of MHC class II staining was similar. Nonspecific antibody binding was excluded by substituting the primary antibody with a nonimmune monoclonal mouse IgG isotype, 1:50 dilution (Dako). In addition, the primary and secondary antibodies were excluded in some experiments to control for nonspecific binding of secondary antibodies and the possible expression of endogenous peroxidase activity.

Electron Microscopy

Pieces of periodate-lysine-paraformaldehyde-fixed cornea (10 to 20 weeks gestation; n = 6) were cut 1 to 2 mm wide in a cross-section from the limbus to the central cornea. These pieces were immunogold labeled for MHC class II and macrophage antigens as described above. After fixation in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), specimens were postfixed in 2% osmium tetroxide, dehydrated through a series of alcohols and acetone, embedded in epon–araldite resin, and cured at 60°C. Semithin sections (0.5 μm) were stained with toluidine blue and examined with a 100X objective. Ultrathin sections were examined at 100 kV in a Hitachi (Tokyo, Japan) H500 transmission electron microscope.

Analyses

The morphology, size, and distribution of immunoreactive cells in cornea and conjunctiva were assessed by...
TABLE 1. Avidin–Biotin Peroxidase Labeled Cells

<table>
<thead>
<tr>
<th>Age (weeks gestation)</th>
<th>MHC Class II</th>
<th>S22</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>10</td>
<td>3.4 ± 2</td>
<td>4.4 ± 0.7</td>
</tr>
<tr>
<td>13</td>
<td>6.5 ± 4</td>
<td>4.4</td>
</tr>
<tr>
<td>14</td>
<td>9.5 ± 3</td>
<td>4.4</td>
</tr>
<tr>
<td>15</td>
<td>11.3 ± 2</td>
<td>4.4</td>
</tr>
<tr>
<td>16</td>
<td>15.7 ± 2</td>
<td>4.4</td>
</tr>
<tr>
<td>17–18</td>
<td>17.2 ± 2</td>
<td>4.4</td>
</tr>
<tr>
<td>19</td>
<td>17.8 ± 3</td>
<td>4.4</td>
</tr>
<tr>
<td>20</td>
<td>19.5 ± 4</td>
<td>4.4</td>
</tr>
<tr>
<td>25</td>
<td>31.6 ± 1</td>
<td>4.4</td>
</tr>
</tbody>
</table>

Density distributions of MHC class II and S22-positive cells (number per mm²) were mapped by light microscopy using a squared ocular graticule at a final magnification of ×125. Counts of positive cells were made in consecutive squares, completely sampling the wholemounted cornneas. Positively labeled cells either within the graticule square or touching its upper and left borders were included in the counts, and the data were plotted onto an outline of the wholemount drawn to scale from which the corneal area was also estimated. The full thickness of each cornea was surveyed in two planes of focus (superficial and deep) for peroxidase-labeled specimens and in approximately four planes of focus for immunogold labeled specimens; the final densities were obtained by adding the counts from each plane of focus.

These data were analyzed in two ways. For population analyses, the total number of immunoreactive cells for each specimen was determined, and the mean density per mm² was calculated. Data for all specimens in each age group were pooled, and the average density per mm² and the average total population were calculated (Tables 1 and 2). For topographic analyses, distribution maps of MHC class II and S22-positive cells in each corneal wholemount were prepared using

<table>
<thead>
<tr>
<th>Age (weeks gestation)</th>
<th>MHC Class II</th>
<th>S22</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>12</td>
<td>2.6</td>
<td>4.0</td>
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<td>13–14</td>
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<tr>
<td>15</td>
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<td>17</td>
<td>14.2</td>
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</tr>
<tr>
<td>18</td>
<td>17.0</td>
<td>17.0</td>
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</table>

TABLE 2. Immunogold Labeled Cells

<table>
<thead>
<tr>
<th>Age (weeks gestation)</th>
<th>Average Area (mm²)</th>
<th>Density/mm² (range)</th>
<th>Average Density/mm² (range)</th>
<th>Total Number of Positive Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC Class II</td>
<td>2.6</td>
<td>10–35</td>
<td>40</td>
<td>104</td>
</tr>
<tr>
<td>3.3</td>
<td>15–190</td>
<td>167</td>
<td>550</td>
<td></td>
</tr>
<tr>
<td>10.8</td>
<td>148–2311</td>
<td>181</td>
<td>1956</td>
<td></td>
</tr>
<tr>
<td>13.9</td>
<td>15–190</td>
<td>92</td>
<td>1283</td>
<td></td>
</tr>
<tr>
<td>S22</td>
<td>4.0</td>
<td>12–17</td>
<td>16</td>
<td>63</td>
</tr>
<tr>
<td>4.5</td>
<td>20–56</td>
<td>28</td>
<td>124</td>
<td></td>
</tr>
<tr>
<td>3.1</td>
<td>25–48</td>
<td>52</td>
<td>164</td>
<td></td>
</tr>
<tr>
<td>11.2</td>
<td>60–114</td>
<td>88</td>
<td>983</td>
<td></td>
</tr>
<tr>
<td>16.6</td>
<td>40–147</td>
<td>97</td>
<td>1613</td>
<td></td>
</tr>
<tr>
<td>17.0</td>
<td>32–191</td>
<td>156</td>
<td>2650</td>
<td></td>
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</tbody>
</table>

Downloaded From: http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933409/ on 10/16/2017
FIGURE 1. Semithin sections (0.5 μm) of human fetal cornea reacted for MHC class II antigens (16 weeks gestation, A, C, E) and macrophage (S22) antigens (20 weeks gestation, B, D, F). Immunoreactive cells were distributed in a nonlaminar fashion throughout fetal corneas. Immunoreactive cells with distinct silver-particle labeling of the cell surface are indicated (arrows) in anterior stroma (A, B), mid-stroma (C, D), and posterior stroma (E, F). Ep = epithelium; En = endothelium; K = keratocytes. (Immunogold histochemistry; toluidine blue.) Scale bar = 30 μm.

the Magellan program for Macintosh (Apple; Cupertino, CA).

RESULTS
Morphology
In all fetal corneal wholemounts flattened onto glass slides, the anterior cornea lies uppermost and the posterior cornea underneath. We will, therefore, subsequently refer to the anterior cornea as the superficial cornea and to the posterior cornea as the deep cornea.

MHC class II and S22-positive cells were present in all specimens studied, displaying uniformly intense reactivity. Immunoreactive cells were distributed throughout the fetal corneal epithelium and stroma, and in the immunogold preparations they were dis-
diameters ranging from 13 to 81 μm. Positive cells with a more rounded, condensed morphology were also present, particularly in specimens prepared using immunogold histochemistry (Fig. 2F). At 25 weeks gestation, MHC class II-positive cells, evident at the junction of the peripheral cornea and conjunctiva, exhibited long streamer-like processes oriented perpendicularly to the limbus (not illustrated).

Generally, the average diameters of the cell fields and somata of MHC class II and S22-positive cells were similar at all gestational ages. Positive cells had field diameters ranging from 13 to 81 μm and average field diameters of 36 ± 9 μm at 12 weeks gestation, 38 ± 10 μm at 15 weeks gestation, 36 ± 9 μm at 18 weeks gestation, and 36 ± 9 μm at 20 weeks gestation. Soma diameters of positive cells ranged from 4 to 15 μm, and average soma diameters were 8.5 ± 2 μm at 12 weeks gestation, 9 ± 2 μm at 15 weeks gestation, 8 ± 2 μm at 18 weeks gestation, and 7.5 ± 2 μm at 20 weeks gestation. Average field and soma diameters of MHC class II or S22-positive cells at four representative gestational ages are presented in Figure 3. The data indicate that, with the exception of MHC class II cells at 12 weeks gestation, the field diameters of both S22 and MHC class II-positive cells were larger in the superficial cornea than the deep cornea (P < 0.0001; unpaired t-test). Furthermore, S22-positive cells consistently had larger fields (P < 0.0001; unpaired t-test) and larger somata (P < 0.0001; unpaired t-test) than MHC class II-positive cells. There was no statistically significant difference in cell soma diameter in the superficial versus the deep cornea.

Conjunctival immunoreactive cells had a ramified morphology similar to the immunoreactive cells of the cornea (Fig. 2G). In contrast to immunoreactive cells of the cornea, however, many conjunctival immunoreactive cells were elongated parallel to the corneal circumference, displaying larger average field diameters than immunoreactive cells of the cornea. Class II-positive cells of the conjunctiva had an average soma diameter of 6.1 ± 1.4 μm (range, 4.4 to 10.6 μm) and an average field diameter of 44.2 ± 9 μm (range, 20 to 71 μm). No staining was observed in corneas in which a nonspecific IgG1 isotype control was substituted for the primary antibody (Fig. 2H). Similarly, no staining was apparent in corneas in which primary and secondary antibodies were excluded.

At the electron microscopic level, silver-enhanced immunogold labeling was detected on the surface of cells distributed throughout the full thickness of the cornea (Fig. 4). Immunoreactive cells had monolobed or bi-lobed nuclei with condensed heterochromatin in the periphery. The Golgi apparatus and rough endoplasmic reticulum were well developed. Similar morphologic forms were apparent throughout fetal corneas. There was no evidence of MHC class II or S22 reactivity on keratoctyes or endothelial cells, although some epithelial cells appeared to display MHC class II or S22 reactivity.

**Corneal Area**

Corneal area increased steadily with increasing gestational age. Mean corneal area for MHC class II and S22-labeled specimens at each gestational age are listed in Tables 1 and 2. These data were pooled to obtain average corneal areas for all specimens analyzed at each gestational age (Fig. 5), and revealed a sixfold increase in corneal area over the period of development studied. Analysis of corneal areas in fellow eyes at 13, 15, and 17 weeks gestation, in which one eye was prepared using avidin–biotin–peroxidase immunohistochemistry and the other using immunogold histochemistry, indicated shrinkage of the peroxidase-labeled corneas of 17.7%, 15.8%, and 14.4%, respectively.
FIGURE 3. Average field and soma diameters of MHC class II and macrophage antigen S22-positive cells in human fetal cornea. Average diameters were estimated from eight corneal wholemounts prepared using immunogold histochemistry (12, 15, 18, and 20 weeks gestation) in superficial (anterior) cornea and deep (mid and posterior) cornea.

Topography in Wholemounts

Immunoreactive cells were distributed across the full corneal area and throughout the conjunctiva in all preparations (Fig. 6).

**Peroxidase.** In peroxidase-reacted, corneas anti-MHC class II and anti-S22 labeled cells were visualized at two planes of focus in the superficial and deep cornea (Figs. 2A to 2D and 6). Greater numbers of cells were evident in the superficial cornea than in the deep cornea. Quantitative data from corneas in which MHC class II (n = 50) and S22 (n = 8) immunoreactivity were visualized with peroxidase are summarized in Table 1 and illustrated in Figure 7. The average density of MHC class II immunoreactive cells increased from 2 cells/mm² at 10 weeks gestation to 245 cells/mm² at 25 weeks gestation; the total numbers of MHC class II immunoreactive cells were 10 at 10 weeks gestation and 7627 at 25 weeks gestation. The average density of S22 immunoreactive cells increased from 14 cells/mm² at 12 weeks gestation to 86 cells/mm² at 18 weeks gestation; the total numbers of immunoreactive cells increased from 33 at 12 weeks gestation to 1456 at 18 weeks gestation.

Total densities (superficial plus deep counts) and distributions of anti-class II-positive peroxidase-reacted cells are shown in five representative maps covering the range of fetal ages studied (Fig. 8). Similarly, four representative maps displaying the distributions of peroxidase-reacted anti-S22-positive cells are shown in Figure 9. These distribution maps indicate that for all specimens, the densities of immunoreactive cells were higher in the central cornea than in the peripheral cornea, and they increased with gestational age. Further, the densities of anti-S22-positive cells were generally less than half of anti-class II-positive cells at similar ages.

**Immunogold.** Immunogold labeling for MHC class II and macrophage S22 reactivity in 10 corneas revealed positive cells of both dendritic and rounded form (Figs. 2E, 2F). Using immunogold techniques, positive cells were distributed throughout the corneal epithelium and stroma in a nonlaminar fashion. Many of these immunogold-labeled cells (S22-positive and MHC class II-positive), present in the stroma, had a few short processes and a rounded morphology (Fig. 2F). In contrast to corneas prepared using peroxidase techniques, immunogold-labeled corneas displayed greater numbers of immunoreactive cells in the deep cornea than in the superficial cornea (not illustrated). Quantitative analyses indicated that...
greater numbers of immunoreactive cells were present in corneas processed using immunogold histochemistry compared with avidin–biotin–peroxidase histochemistry.

Data from the 10 specimens prepared using immunogold histochemistry are presented in Table 2. The average density of MHC class II immunoreactive cells was 40 cells/mm² at 12 weeks gestation and 181 cells/mm² at 15 weeks gestation, and the total numbers of MHC class II immunoreactive cells were 104 cells at 12 weeks gestation and 1956 cells at 15 weeks gestation. The average density of S22 immunoreactive cells was 16 cells/mm² at 12 weeks gestation and 156 cells/mm² at 18 weeks gestation, and total numbers of immunoreactive cells were 63 at 12 weeks gestation and 2650 cells at 18 weeks gestation. Generally, the total numbers and average densities of immunogold labeled S22-positive cells were 50% or less than those of MHC class II-positive cells at similar ages.

DISCUSSION
Morphology
The present study indicates that MHC class II and S22 immunoreactive cells are present in the epithelium and stroma of the normal human fetal cornea. Previous studies have described isolated MHC class II antigen-bearing cells in the corneal stroma of adult guinea pigs and humans. Rounded MHC class II-positive cells, assumed to be of lymphoid origin, have been reported in the peripheral stroma of guinea pig cornea. Others have identified MHC class II-positive cells of dendritic morphology in corneal stroma as interstitial DC, rare MHC class II antigen-bearing keratocytes, ‘spindle cells’ (possible precursors of epithelial DC) or ‘indeterminate cells.’ However, many reports have suggested that MHC class II-positive cells in the corneal stroma may be LC. Langerhans cells have been shown to express high levels of MHC class II antigens. MHC class II-positive cells in the adult human peripheral cornea and limbus have also been shown to contain Birbeck granules, ultrastructural features considered specific for LC. MHC class II-positive ‘indeterminate cells’ of the corneal stroma have been reported to be morphologically similar to LC except for the absence of Birbeck granules. Andersson et al and Karás et al, however, reported that examination of epidermal serial sections in combination with

FIGURE 5. Mean corneal area, estimated from all specimens analyzed (n = 68) at each gestational age using peroxidase and immunogold histochemistry. A sixfold increase in average corneal area was evident during the period of development studied. Error bars = standard deviations.

FIGURE 6. Photomicrographs of a human fetal corneal wholemount (19 weeks gestation) reacted for MHC class II reactivity (peroxidase histochemistry). Immunoreactive cells were distributed evenly in the full corneal area and were evident at planes of focus through the corneal epithelium (A) and the posterior stroma (B). Variation in cell morphologies was apparent: (A) anteriorly immunoreactive cells displayed dendritic morphology; (B) posteriorly immunoreactive cells were dendritic, elongated, or rounded with short processes. Scale bar = 200 μm.
were consistently present in numbers comprising 30% to 50% of the population of MHC class II-positive cells. The presence of Birbeck granules in the so-called indeterminate cells must be interpreted as evidence of a relationship with LC. The ultrastructural identification of Birbeck granules as the definitive feature of LC remains contentious; confident identification of Birbeck granules necessitates meticulous serial sectioning of tissue, and such a task is difficult and impractical in any extensive study. No attempt was made in the present study to identify Birbeck granules because all electron microscopic specimens had been fixed and stained for optimal immunohistochemical reactivity, and, as such, ultrastructural preservation was inadequate for confident identification of these small subcellular granules. The present study indicates that the majority of the MHC class II-positive epithelial and stromal cells are morphologically and phenotypically consistent with previous descriptions of LC.32,33

We observed two morphologically distinct populations of MHC class II and S22-positive cells throughout the developing cornea: large, dendritic cells and smaller, rounded cells; a larger proportion of the smaller, rounded cells were present in deep stroma. These observations suggest that there is a heterogeneous population of MHC class II immunoreactive cells in the developing human cornea. Although macrophages are expected to express both MHC class II and macrophage (S22) antigens, dendritic LC are expected to be MHC class II positive but macrophage (S22) antigen negative.4 Our data suggest that in the developing human cornea, macrophages represent approximately 30% to 50% of the population of MHC class II immunoreactive cells. The remaining MHC class II immunoreactive cells, we suggest, represent dendritic LC. Dendritic cells can be distinguished from macrophages by their morphology, more potent accessory cell function, minimal phagocytic capacity, and lower levels of lysosomal enzymes.36 The observation that dendritic morphology is not indicative of cell origin or function37 is confirmed by the results of the present study. Cells of rounded and dendritic morphologies are evident in human fetal cornea labeled with either MHC class II or anti-S22 antibodies, suggesting that corneal LC and macrophages can occur in “dendritic” and rounded forms.

Comparison of Immunohistochemical Techniques

Avidin–biotin–peroxidase and immunogold techniques indicated a greater number of MHC class II-positive cells than S22-positive cells in corneas of similar ages. However, in specimens of similar ages and incubated in the same primary antibodies, the immunogold technique consistently labeled more cells than the avidin–biotin–peroxidase technique. Furthermore, differences in apparent lamination of immunoreactive cells, prepared using the peroxidase or immunogold techniques, suggest better penetration of the tissue by the immunogold F(ab′)2 secondary antibody compared with the biotinylated (IgG) secondary antibody used for the peroxidase labeling.

Comparison of corneal areas from the two techniques suggests that dehydration of corneas in the peroxidase method produced a shrinkage of 10% to 20%. Densities of positive cells from peroxidase-reacted corneas are, therefore, slightly overestimated. Taking account of the improved penetration using the immunogold procedure and approximately 15% shrinkage of peroxidase-labeled specimens, we estimate that the peroxidase-labeled specimens may underestimate actual numbers of LC and macrophages by approximately 40% to 50%. Despite these discrepancies, both methods reveal similar observations in cell morphologies, general topographies, and overall populations (see below).

Topography

Leukocyte lineage cells have been reported to migrate from the bone marrow as monocyte-like cells to peripheral structures through the circulating blood.31,38 The results of the present study indicate that DC and macrophages are normal constituents of the human cornea from as early as 10 weeks gestation and further suggest a continued influx of MHC class II and S22 immunoreactive cells up to, and most likely beyond, 25 weeks gestation. These findings are consistent with a study of LC ontogeny in human fetal skin which reports MHC class II-positive LC to be present during early gestation (6 to 7 weeks gestation).39 Fetal corneas are relatively thin, and the wholemount technique allows direct analysis of immunoreactive cells in all.
layers. Previous studies have used corneal sections or flatmounts of corneal epithelium (separated from the underlying stroma) to examine LC distribution in adults and infants.\textsuperscript{8,9,15,19} Nucleoside phosphatases, such as adenosine triphosphatase (ATPase), adenosine diphosphatase, and inosine diphosphatase, have been used as markers of retinal microglia, macrophages, and epidermal LC.\textsuperscript{37,40} Quantification of ATPase-positive LC in one human fetal cornea (23 weeks gestation) reported densities considerably lower than those described in the present study (48 LC/mm\textsuperscript{2} in central cornea and 75 LC/mm\textsuperscript{2} in peripheral cornea).\textsuperscript{15} We estimate an LC density in the central corneal epithelium (and immediately adjacent anterior stroma) in specimens of 20 to 25 weeks gestation of 118 to 241 LC/mm\textsuperscript{2}, which is greater than those reported by Chandler et al.\textsuperscript{15} The higher LC densities observed in the present study do not simply reflect differences in technique but more likely arise from difficulties in comparing data from a single cornea\textsuperscript{15} with data averaged from a number of corneas (n = 11, 20 to 25 weeks gestation, present study).

Studies of adult corneas in guinea pig, hamster, mouse, and man have established that LC are present in the epithelium of the conjunctiva and peripheral third of the cornea but are absent from the central cornea.\textsuperscript{34,1,42} In contrast, LC have been reported to be present in the epithelium of the central fetal and infant cornea.\textsuperscript{15} We observed immunoreactive cells in the full corneal area distributed throughout the epithelium and stroma and concentrated in the central cornea at all gestational ages. The increase in average density of immunoreactive cells with increasing gestational age observed in the present study indicates that MHC class II and S22 immunoreactive cells are not simply included pro rata in relation to an increase in corneal area. The presence of maximum average densities of MHC class II immunoreactive cells centrally at 25 weeks gestation (present study) and in newborn infants\textsuperscript{15} indicates that LC exit the cornea well after 25 weeks gestation and that the characteristic adult topography, in which central corneal LC densities approach 0/mm\textsuperscript{2}, occurs sometime after birth.

Streamer-like dendrites have been described in guinea pig\textsuperscript{43,44} and mouse corneas,\textsuperscript{45} and it has been suggested that these elongated cells may be a migrating or activated form of LC.\textsuperscript{48} Although we cannot conclude that the streamer-like cells observed at 25 weeks gestation in the present study (not illustrated) are migrating, their similarity to previously reported streamers suggests that, by 25 weeks gestation, LC may have commenced their movement away from the central concentration of LC observed during development.

We also observed that the average density of MHC class II immunoreactive cells centrally increases 120-fold over the period studied, whereas corneal area increases 6-fold. The elevated densities of LC in infant corneas have been suggested to be the result of the smaller eye allowing greater numbers of LC to reach the central corneas because of limited mobility of LC from blood vessels.\textsuperscript{15} However, this is inconsistent with our data showing that the highest densities occur centrally, the region most distant from the limbal vasculature. The susceptibility of LC to ultraviolet radiation has also prompted the suggestion that ultraviolet radiation may be responsible for the decreased LC densities in the adult cornea.\textsuperscript{15} However, although acute ultraviolet radiation has been shown to reduce epidermal and corneal LC temporarily,\textsuperscript{43,44} LC numbers are not reduced with age or in response to chronic ultraviolet exposure.\textsuperscript{31} Exposure of the infant eye to ultraviolet radiation does not appear to be the principal reason for clearing LC from these corneas. The absence of LC in central adult cornea is more likely the result of the production of factors by adult corneal epithelial FIGURE 8. Total densities (superficial plus deep counts) and distributions of MHC class II-positive cells in human fetal corneas (peroxidase histochemistry) are shown in five representative maps covering the range of fetal ages studied. Immunoreactive cells were distributed at all corneal locations, and densities increased steadily as gestation progressed, with a tendency for cells to concentrate centrally. Dot size is proportional to cell density; density range is indicated to the left of each map. Within the outline for each specimen, the limbus is indicated by a broken circle.
FIGURE 9. Total densities (superficial plus deep counts) and distributions of macrophage antigen S22-positive cells in human fetal corneas (peroxidase histochemistry) are shown in four representative maps covering the range of fetal ages studied. S22 immunoreactive cells were also distributed at all corneal locations, and densities increased steadily as gestation progressed, with a tendency for cells to concentrate centrally (compare with Fig. 8). Dot size is proportional to cell density, and density range is indicated to the left of each map. Within the outline for each specimen, the limbus is indicated by a broken circle.

or stromal cells that inhibit the migration or reduce the life span of LC. Furthermore, other possible explanations to account for the reduced numbers of LC in the central adult cornea may include cell migration of central LC toward the limbus, undetectable levels of expression of MHC class II antigens by LC, or possibly apoptotic deletion of centrally located LC. An important objective of future studies will be to determine the precise stimulus, time course, and mechanism of reduction of LC numbers in prenatal and postnatal corneas.

Immunologic Significance

This study describes in detail the presence of large numbers of DC and macrophages throughout the human fetal cornea, consistent with observations in other tissues. Resident populations of DC and macrophages have been described in normal tissues including spleen, bone marrow, thymus, lymph node, liver, brain, lung, kidney, colon, and skin. In addition, heterogeneous populations of resident DC and macrophages have been reported in the rat ciliary body, iris, and choroid and in the human retina. In the human retina, it has been estimated that a subpopulation of microglia express macrophage antigens. Functional tests indicate that in the gut and lung, macrophages may inhibit the activity of dendritic antigen-presenting cells, thereby modulating the local immune responses. Accordingly, it seems reasonable to suggest that in most tissues, including cornea, similar functional capacities related to the occurrence of both DC and macrophages may exist. A complete picture of the means by which tolerance to self-antigens is established is not presently available; however, T cells, B cells, and specialized populations of DC have been implicated. Similarly, DC in the developing human cornea may have a role in establishing tolerance to corneal antigens.

A similar study of fetal epidermal LC demonstrated their presence before the full activation of the immune system and suggested that fetal epidermal LC may have the capacity to function as antigen-presenting cells but that parts of the immune system may be too immature to cooperate. Because LC are powerful initiators of T cell-mediated immune responses, their presence in fetal and adult cornea, even in small numbers, has important implications for transplantation. The transplantability of the cornea has been suggested to be related to the absence, or reduced numbers, of class II-positive immunocompetent cells in the corneal epithelium. The presence of class II-positive cells in the central stroma of adult corneas appear to be related to the relatively low corneal graft survival rate in vascularized and inflamed eyes. Retention into adulthood of a proportion of the class II-positive cells observed in human fetal corneal stroma may explain the presence of some stromal class II-positive cells in normal human adult cornea and corneal grafts. The present results confirm, therefore, the unsuitability of fetal and infant tissue for corneal transplantation. The significance of the existence of a transient population of LC and macrophages in the fetal corneal stroma and epithelium and the implications for the immunologic status of this tissue remain to be established fully.

Key Words
cornea, dendritic cells, Langerhans cells, macrophages, MHC class II

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