Bone Marrow–Derived Cells in Normal Human Corneal Stroma

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Objective: To examine the normal human corneal stroma for the presence of bone marrow–derived cells.

Methods: Thirty-four corneas from donors aged 56 to 71 years were used. The stroma of the donor corneas was examined immunohistochemically by fluorescent microscopy. CD45-positive and -negative cells were separated from collagenase-digested stroma by magnetic beads, and the expression of toll-like receptor 4 was analyzed.

Results: CD45-positive cells were mainly found in the anterior stroma of the central and paracentral cornea as well as all stromal layers of the peripheral cornea (n=5). These cells uniformly expressed CD11b, CD11c, CD14, and HLA-DR antigen but not CD3, CD19, CD56, or CD66, indicative of bone marrow–derived monocyte lineage cells, which can include monocytes, macrophages, or dendritic cells. CD45-positive cells isolated with magnetic beads accounted for 6.0% of total stromal cells (n=20). Stromal CD45-positive cells, but not CD45-negative cells, expressed toll-like receptor 4 by flow cytometry and reverse-transcriptase polymerase chain reaction.

Conclusion: Our findings demonstrate that DR antigen–positive bone marrow–derived monocyte lineage cells exist in the anterior and peripheral posterior stroma of normal human cornea.

Clinical Relevance: These cells may play a role in the innate and adaptive immune responses in the human cornea.


MACROPHAGES AND their precursors, peripheral blood monocytes, are widely distributed populations of myeloid cells. Resident tissue macrophages display extensive functional and phenotypic heterogeneity, as do monocytes recruited to sites of inflammation and immune stimuli. Monocytes can differentiate into macrophages and immature dendritic cells (DCs) after stimulation by cytokines. Both macrophages and DCs are professional antigen-presenting cells and are essential regulators of the innate and acquired arms of the immune response, although DCs are much more potent at initiating and expanding secondary immune responses than macrophages. The DCs capture and process antigens, migrate to lymphoid organs, and secrete cytokines to initiate an immune response. During this process, DCs lose their antigen-capturing capacity and become mature cells that express CD80, CD86, and CD40, which activate naive T cells recirculating through the T cell–dependent areas of secondary lymphoid organs. When macrophages and DCs phagocytose pathogenic type I transmembrane proteins, toll-like receptors (TLRs) on these cells are required for microbial recognition. There are 10 members of the TLR family, and each is involved in recognizing a variety of microorganism-derived molecules. Among them, TLR2 and TLR4 are receptors for gram-positive and gram-negative bacteria, respectively, in a CD14-dependent fashion.

In studies on the human cornea, major histocompatibility complex (MHC) class II (HLA-DR) antigen expression has been used as a marker of DCs, which have been found in the normal human corneal stroma. These MHC class II antigen–positive cells are decreased in the corneal stroma after a week of preservation in organ culture and corneal preservation media. In contrast, many authors have found no MHC class II antigens in the normal human corneal stroma. Also, the phenotype of leukocytes in the human corneal stroma has not been determined.

In this study, the stroma of human donor corneas was examined by 1- and...
METHODS

DONOR HUMAN CORNEAS AND PREPARATION FOR IMMUNOHISTOCHEMICAL STUDY

This study was conducted in accordance with the Declaration of Helsinki. Thirty-four corneas (donors aged 56-71 years) were obtained from the Rocky Mountain Lions’ Eye Bank at 4 to 8 days post mortem and were kept in Optisol GS (Bausch and Lomb, Rochester, NY) storage medium at 4°C until used. Corneas with diffuse or epithelial opacity or with a history of corneal disease in the eye bank report were excluded from this study. For immunohistochemical examination of whole corneal cross-sections, the corneas (5 corneas at 4 to 5 days’ post mortem for the immunohistochemical study and CD45-positive cell counting and 6 corneas at 4 days’ post mortem for the Optisol GS preservation experiments) were embedded in optimal cutting temperature compound and cut on a cryostat at a thickness of 8 µm. For flat-mount analysis of the corneal stroma by confocal microscopy, the epithelium was carefully removed from the stroma by scraping the outer surface of the cornea, while the endothelium and Descemet membrane were peeled away in a sheet from the peripheral to the center of the inner surface of the cornea with fine forceps according to the procedure described previously. Then the denuded corneal stroma (n = 3) was mounted flat in optimal cutting temperature compound and sliced with a cryostat into 40-µm sections. The thin cross-sections and thick flat mounts of the corneal stroma were dried in air, and immunostaining was done without fixation.

IMMUNOHISTOCHEMICAL STUDY

Unfixed specimens were prepared for the immunohistochemical studies. Frozen specimens were cut into 8-µm transverse cross-sections and 40-µm flat-mount sections on a cryostat, air dried for 10 minutes, and washed in phosphate-buffered saline (PBS). Because monocytes or macrophages express Fc receptors, the sections were blocked with anti-Fc receptor blocker (dilution 1:4; Miltenyi Biotec, Bergisch Gladbach, Germany) and isotype-matched immunoglobulin for each antibody (5 µg/mL of mouse IgG1, 5 µg/mL of IgG2a, and 5 µg/mL of IgG2b; Dako, Carpinteria, Calif) diluted in PBS for 30 minutes to prevent nonspecific staining. Then each fluorescein isothiocyanate conjugated (FITC) or phycoerythrin (PE)-conjugated mAb, or the FITC or PE-conjugated isotype-matched control antibody (Table), was applied for 30 minutes. The specimens and corneal stroma were covered with a mounting medium (Vector Laboratories, Burlingame, Calif) after 4 washes in PBS and analyzed under a fluorescent microscope (BH2-RFL-T3 or BX50; Olympus, Tokyo, Japan) or a confocal microscope (Leica TCS 4D; Lasertechnik, Heidelberg, Germany). The sections stained with FITC anti-CD45 mAb were coverslipped using an antifading mounting medium that contained propidium iodide (PI) (Vectorshiel1; Vector Laboratories). All staining procedures were done at room temperature. On each cross-section, the central area was defined as being within 1.5 mm from the center of the cornea. The paracentral region was the area between 1.5 and 3.5 mm from the center, while the periphery was defined as being

Table. List of Monoclonal Antibodies Used in Labeling*

<table>
<thead>
<tr>
<th>Antibody (Clone)</th>
<th>Specificity</th>
<th>Antibody Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3-FITC (UCHT1)</td>
<td>T lymphocytes</td>
<td>Mouse IgG1, κ</td>
</tr>
<tr>
<td>CD11b-PE (ICRF44)</td>
<td>Activated lymphocytes, monocytes, granulocytes, a subset of NK cells</td>
<td>Mouse IgG1, κ</td>
</tr>
<tr>
<td>CD11c-PE (B-ly6)</td>
<td>NK cells, a subset of B and T cells, monocytes, granulocytes, macrophages</td>
<td>Mouse IgG1, κ</td>
</tr>
<tr>
<td>CD14-FITC and PE (M5E2)</td>
<td>Monocytes, macrophages, DCs, LCs</td>
<td>Mouse IgG2a, κ</td>
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<tr>
<td>CD19-FITC (SJ25C1)</td>
<td>Mature and immature B cells</td>
<td>Mouse IgG1, κ</td>
</tr>
<tr>
<td>CD45-FITC (HI30)</td>
<td>Pan leukocytes</td>
<td>Mouse IgG1, κ</td>
</tr>
<tr>
<td>CD56-PE (B159)</td>
<td>Pan NK cells</td>
<td>Mouse IgG1, κ</td>
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<td>CD66-FITC (B6.2/CD66)</td>
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<tr>
<td>CD68-PE (Y1/82A)</td>
<td>Monocytes or macrophages, DCs, granulocytes, myeloid progenitor cells</td>
<td>Mouse IgG2b, κ</td>
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<td>CD80-FITC (L307.4)</td>
<td>B7-1, costimulatory molecules</td>
<td>Mouse IgG1, κ</td>
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<tr>
<td>CD86-FITC (FUN-1)</td>
<td>B7-2, costimulatory molecules</td>
<td>Mouse IgG1, κ</td>
</tr>
<tr>
<td>CD163 (GH/61)</td>
<td>Scavenger receptor</td>
<td>Mouse IgG1, κ</td>
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<td>HLA-DR-FITC (B46-6)</td>
<td>HLA-DR antigens</td>
<td>Mouse IgG2a, κ</td>
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<td>Mouse IgG1, κ</td>
</tr>
<tr>
<td>CD1a-PE (HI149)</td>
<td>LCs, DCs</td>
<td>Mouse IgG1, κ</td>
</tr>
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<td>Mouse IgG1, κ</td>
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<tr>
<td>Mouse IgG2a, κ-FITC (6155-178)</td>
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<td>Mouse IgG2a, κ</td>
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<tr>
<td>Mouse IgG2b, κ-PE (27-35)</td>
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<td>Mouse IgG2b, κ</td>
</tr>
<tr>
<td>TLR4 (HTA125)</td>
<td>TLR4</td>
<td>Mouse IgG2a, κ</td>
</tr>
</tbody>
</table>

Abbreviations: DC, dendritic cell; FITC, fluorescein isothiocyanate conjugated; LC, Langerhans cell; NK, natural killer; PE, phycoerythrin; TLR, toll-like receptor.

*Monoclonal antibody sources are as follows: HLA-DR-FITC (TAL.1B5), Chemicon International, Temecula, Calif; TLR4, Monosan, Uden, the Netherlands. All other monoclonal antibodies were purchased from BD Biosciences, San Diego, Calif.

ANTIBODIES

The primary monoclonal antibodies (mAbs) used for the immunohistochemical and flow cytometric staining procedures, their specificity, and their respective control antibodies are shown in the Table. Two mAbs (clones G46-6 and TAL.1B5) were used to examine HLA-DR antigens.
within a 3.5- to 5.5-mm radial distance from the center. The central, paracentral, and peripheral areas of each cornea were assessed separately. Each area was outlined on the surface of the coverslip with a measure and a marker. The percentage of CD45-positive cells in each corneal stromal area was determined by counting CD45-positive cells and PI-positive cells in 3 sections and then dividing the number of CD45-positive cells by the number of PI-positive cells. The border between the anterior and posterior stroma was defined as the center of the full-thickness stromal layer under the microscope. Data are reported as the average for 5 corneas (4-5 days’ post mortem). The stromal flat mounts for confocal microscopy were quadrisected corneas from different donors (n = 3 at 4-5 days’ post mortem).

### ISOLATION OF CD45-POSITIVE CORNEAL STROMAL CELLS

Before isolation of corneal stromal cells, the peripheral cornea (including the limbal region) was dissected away from the corneal stroma to avoid possible contamination by corneal limbal epithelial cells. Then the stroma was cut into small pieces about 1 mm in diameter, which were incubated overnight at 37°C in serum-free basal medium containing 0.02% collagenase (Sigma-Aldrich, St Louis, Mo). After washing 3 times with PBS, single cells were dissociated by trituration with a fire-polished Pasteur pipette. CD45-positive cells were positively isolated with a magnetic-activated cell sorter (MACS; Miltenyi Biotec) according to the manufacturer’s instructions. For separation of CD45-positive and negative cells, 3 to 5 corneal stromas were processed in a group and the average total number of cells per cornea and the percentage of CD45-positive cells were determined (5 groups and 20 corneas in total at 5-8 days’ post mortem). The separated cells were subjected to flow cytometry and reverse transcriptase polymerase chain reaction. For detection of HLA-DRα antigen messenger RNA, a 3-mm diameter circle of the central cornea was trephined and CD45-positive and negative cells were isolated as described earlier.

### FLOW CYTOMETRY

For flow cytometry, the cells isolated by MACS were blocked in 3% normal human serum before incubation for 30 minutes with antihuman TLR4 mAb (HTA125; Monosan, Uden, the Netherlands) or the isotype control (mouse IgG2a, or PE-conjugated isotype control antibodies (IgG1, IgG2b) was not detected after pretreatment with anti-Fc receptor blocker and the isotype-matched immunoglobulin for each antibody (data not shown). Immunofluorescent microscopy of cross-sections of the stroma was performed with FITC anti-CD45 mAb (a panleukocyte marker) and nuclear staining was done with PI. CD45-positive cells were seen in the anterior stroma of the central cornea (Figure 1A) and the paracentral cornea (Figure 1B), as well as throughout the stroma of the peripheral cornea (Figure 1C). A few CD45-positive cells were detected in the posterior central and paracentral stroma (not shown). In the peripheral cornea, CD45-positive cells were less common in the posterior stroma than in the anterior stroma, as shown in Figure 1C. Confocal microscopy of 40-µm flat mounts of the corneal stroma showed that the CD45-positive cells were mainly round or spindle form (Figure 1D). To determine the percentage of CD45-positive cells in each corneal stromal area, the average percentage among the PI-positive cells was calculated for 5 donor corneas. As shown in Figure 1E, there was a trend for a higher percentage of CD45-positive cells in the peripheral area. The mean ± SD percentage of CD45-positive cells in the posterior stroma compared with those in the anterior stroma was, respectively, 10% ± 5%, 18% ± 6%, and 45% ± 12% (n = 5 each) in the central, paracentral area, and periphery of the corneal stroma.

### RESULTS

#### CD45-POSITIVE CELLS IN THE CORNEAL STROMA

In all layers of the stroma, positive staining with the FITC or PE-conjugated isotype control antibodies (IgG1, IgG2a, and IgG2b) was not detected after pretreatment with anti-Fc receptor blocker and the isotype-matched immunoglobulin for each antibody (data not shown). Immunofluorescent microscopy of cross-sections of the stroma was performed with FITC anti-CD45 mAb (a panleukocyte marker) and nuclear staining was done with PI. CD45-positive cells were seen in the anterior stroma of the central cornea (Figure 1A) and the paracentral cornea (Figure 1B), as well as throughout the stroma of the peripheral cornea (Figure 1C). A few CD45-positive cells were detected in the posterior central and paracentral stroma (not shown). In the peripheral cornea, CD45-positive cells were less common in the posterior stroma than in the anterior stroma, as shown in Figure 1C. Confocal microscopy of 40-µm flat mounts of the corneal stroma showed that the CD45-positive cells were mainly round or spindle form (Figure 1D). To determine the percentage of CD45-positive cells in each corneal stromal area, the average percentage among the PI-positive cells was calculated for 5 donor corneas. As shown in Figure 1E, there was a trend for a higher percentage of CD45-positive cells in the peripheral area. The mean ± SD percentage of CD45-positive cells in the posterior stroma compared with those in the anterior stroma was, respectively, 10% ± 5%, 18% ± 6%, and 45% ± 12% (n = 5 each) in the central, paracentral area, and periphery of the corneal stroma.

### CHARACTERIZATION OF THE CD45-POSITIVE CELLS

To investigate the characteristics of the CD45-positive cells, we performed staining of the cross-sections with various markers and double staining of each positive marker with CD45. In the donor corneas examined at 4 days’ post mortem, positive staining was seen with mAbs for CD11b, CD11c, and CD14 (monocyte or macrophage or pre-DC), whereas staining with the mAbs for CD3 (T cell), CD19 (B cell), CD56 (natural killer cell), CD66 (granulocyte), CD80 (B7.1), and CD86 (B7.2) were not detected in the corneal stroma (not shown). These findings...
showed that the cells could not be T cells, B cells, granulocytes, natural killer cells, or activated DCs. As shown in Figure 2A-C, all of the CD45-positive cells were stained with CD11b in the central (Figure 2A-C), paracentral, and peripheral (not shown) areas of the cornea. These CD45-positive cells were all stained by PE-conjugated anti-CD11c mAb (Figure 2D) and anti-CD14 mAb (Figure 2E) in the central, paracentral, and peripheral areas, indicating that the CD45-positive cells were also CD11b-, CD11c-, and CD14-positive cells. These cells were negative for mAbs directed against CD1a (Langerhans cell) and CD68 (Y1/82A, monocyte or macrophage, DCs).

To better characterize the CD45-, CD11b-, CD11c-, and CD14-positive cells, we stained the corneal stroma with anti–HLA-DRα antigen (MHC class II) mAb. The HLA-DRα antigen was detected in CD11b– (Figure 3A and B) and CD11c–positive cells using anti–DR antigen mAb (clone G46-6), and staining was confirmed by using another anti–DR antigen mAb (clone TAL.1B5) (not shown), indicating that the CD45–, CD11b–, CD11c–, and CD14–positive cells were all positive for DRα antigen. The HLA-DRα antigen messenger RNA was detected in CD45–, but not CD45+ stromal cells derived from the central 3-mm-diameter region (Figure 3C).

Donor corneas were bisected at 4 days’ post mortem (n=6), and half of each cornea was kept in Optisol GS for a further 3 or 10 days. After corneas were preserved in Optisol GS for 14 days (n=3), stromal staining for CD45, CD11b, CD11c, and DRα antigen (clone TAL.1B5 but not clone G46-6) was decreased but still detectable. CD14–positive cells showed a 50% decrease in the donor corneas preserved for 7 days’ post mortem compared with those preserved for 4 days (n=3). There was an approximately 70% decrease of CD14–positive and DRα antigen–positive (clone G46-6) cells in donor corneas preserved for 2 weeks compared with those stored for 4 days (n=3).
COUNTING OF CD45-POSITIVE CELLS IN THE CORNEAL STROMA

We counted the total number of stromal cells and CD45-positive cells isolated with a magnetic-activated cell sorter in 20 corneas (5 groups). The mean±SD total number of cells per cornea was 17.5±5.4×10⁴, and the mean±SD number of CD45-positive cells per cornea was 10.5±0.9×10³. The mean±SD percentage of CD45-positive cells among total stromal cells was 6.0%±0.5%.

TLR4 EXPRESSION BY FLOW CYTOMETRY AND REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION

Toll-like receptor 4 expression was examined in CD45-positive and -negative cells separated from the corneal stroma by magnetic beads. Using flow cytometry, TLR4 protein was detected in CD45-positive cells, but not CD45-negative cells (Figure 4A). Using reverse-transcriptase polymerase chain reaction, TLR4 messenger RNA was also only found in CD45-positive cells (Figure 4B).

COMMENT

We identified CD45-, CD11b-, CD11c-, CD14- and HLA-DR antigen–positive and CD3-, CD19-, CD56-, and CD66-negative bone marrow–derived monocyte lineage cells in the stroma of healthy human donor corneas. These cells can include monocytes, macrophages, or DCs. Confocal microscopy of flat mounts of the corneal stroma showed that the bone marrow–derived cells were mainly round or spindle form. These CD14-expressing cells may efficiently develop...
derived HLA-DR antigen–positive antigen-presenting cells exist in corneal grafts suggests that graft antigens may be directly presented to host T cells after human corneal transplantation, as has been shown to occur in a mouse corneal transplantation model.21 Moreover, the presence of bone marrow–derived cells may be related to a variety of inflammatory conditions that lead to corneal stromal opacity, such as herpetic disciform keratitis, diffuse lamellar keratitis after laser in situ keratomileusis, and interstitial keratitis.

Toll-like receptors recognize pathogen-associated molecules and induce antimicrobial immune responses.22,23 Ten members of the TLR family (TLR1-10) have been identified in humans.24 Each TLR recognizes a distinct microbial component and elicits different, but sometimes overlapping, immune responses.24-26 Several studies have shown that immunocompetent cells differentially express the various TLR family members and their expression is regulated in a cell type–specific manner.27 A major mediator of the immune response is endotoxin and lipopolysaccharide, a component of the cell wall of gram-negative bacteria. Toll-like receptor 4 mutations are associated with hyporesponsiveness to endotoxin in humans.28 In the human eye, TLR4 and the coreceptor CD14 have been detected in the uvea, retina, sclera, conjunctiva, and corneal epithelium.29–31 CD14 expression has been demonstrated in normal human corneal stroma.30 These findings suggest that eyes possess a potent defense mechanism against gram-negative bacterial infections throughout the eyeball and that the TLR4, expressed mainly in the anterior stroma,
may support the corneal epithelium, which is exposed to the outside environment.

CD45-positive cells accounted for 6% of those obtained with magnetic beads. This is consistent with the percentage of CD45-positive cells among Pl-stained cells in transverse cross-sections calculated by our immunohistochemical study. Among corneal stromal cells mixed with CD45-negative resident stromal cells and CD45-positive bone marrow–derived cells, we have identified multipotential precursors in the stroma of the normal adult human cornea with a sphere-forming assay. Interestingly, undifferentiated sphere colonies can be formed from CD45-negative, but not CD45-positive, cells (S. Yamagami, unpublished data, 2004), suggesting that CD45-positive cells lack any proliferative capacity and continuously renew from the bone marrow.

In the normal mouse cornea, MHC class II–negative immature DCs and CD80- and/or CD86-positive mature DCs are present in the anterior and peripheral corneal stroma, respectively. Both MHC class II–positive and –negative monocyte and macrophage lineage cells are also observed in the stroma. Direct comparison between human and mouse leukocytes is difficult because the standards for classifying human leukocytes by surface markers do not completely correspond to those for mice. Monocytes and macrophages and CD14-positive precursor-type DCs are present in both human and mouse corneal stroma. The findings that all of the bone marrow–derived cells in normal human corneal stroma are MHC class II positive and CD80 and CD86 negative (immature cells) and do not contain CD14-negative myeloid DCs are, however, different from those in mouse corneal stroma.

The technique applied in our study was as follows. First, we did not fix our specimens with a fixative solution such as acetone, paraformaldehyde, or methanol for immunohistochemical study because the fixation process may lead to degeneration of target antigens and decrease the sensitivity of antigen detection. Second, an Fc-receptor blocker for protection against nonspecific staining of antigen-presenting cells mouse IgG1, IgG2a, or IgG2b of the same subclass was used before the staining procedure. Moreover, direct rather than indirect immunostaining was performed using FITC or PE-conjugated mAbs. These techniques all reduce the possibility of nonspecific staining. Third, we compared the staining patterns obtained with various markers after donor corneas were preserved in Optisol GS for 4, 7, and 14 days. In contrast to the well-maintained expression of CD45, CD11b, CD11c, and DRα antigen (TAL1.1B), expression of CD14 and DRα antigen (G46-6) both decreased after 7 and 14 days of storage. Thus, the storage period of donor corneas should be considered when immunohistochemical studies are done.

In summary, we found MHC class II–positive bone marrow–derived monocyte lineage cells in the stroma of donor corneas. These cells were localized in both the anterior and peripheral posterior stroma and expressed TLR4, suggesting a role in host defenses against gram-negative bacteria. Our findings provide new data on the innate and adaptive immune responses of the human cornea.

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January 2006 Web Quiz Winner

Congratulations to the winner of our September quiz, Heather Beck, fourth-year medical student, Georgetown University School of Medicine, Washington, DC. The correct answer to our September challenge was lipid keratopathy. For a complete discussion of this case, see the Clinicopathologic Reports, Case Reports, and Small Case Series section in the October ARCHIVES (Loeffler KU, Seifert P. Unusual idiopathic lipid keratopathy: a newly recognized entity? Arch Ophthalmol. 2005;123:1435-1438).

Be sure to visit the Archives of Ophthalmology Web site (http://www.archophthalmol.com) and try your hand at our Clinical Challenge Interactive Quiz. We invite visitors to make a diagnosis based on selected information from a case report or other feature scheduled to be published in the following month’s print edition of the ARCHIVES. The first visitor to e-mail our Web editors with the correct answer will be recognized in the print journal and on our Web site and will also be able to choose one of the following books published by AMA Press: Clinical Eye Atlas, Clinical Retina, or Users’ Guides to the Medical Literature.