

## ORIGINAL ARTICLES—Laboratory science

## Endotoxins modulate the autocrine function of organ cultured donor corneas and increase the incidence of endothelial cell death

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### Abstract

**Background/claims**—Bacterial endotoxin is a potent inflammatory stimulator, the local and systemic responses thereby elicited being mediated via the release of cytokines from diverse cell types. Under physiological conditions, the corneal endothelium is protected from these toxins by the epithelial and vascular barriers, but in organ culture these safeguards are no longer operative, and such substances will therefore have ready access to this cell layer. The consequences of such exposure may take the form of overt damage to the endothelium and/or a more discreet influence on the cornea's immunological status, the effects of which may be realised only after transplantation, by its poor performance. The media bathing organ cultured donor corneas were monitored for the presence of various cytokine mediators of the inflammatory response before and after incubation with endotoxin, and these data compared with those pertaining to endothelial cell morphology and numerical density.

**Methods**—Six pairs of fellow donor corneas were cultured for an initial equilibration period of 10 days and then transferred to fresh medium; thereafter, one of each pair was incubated in the absence, and the other in the presence, of endotoxin (50 µg/ml = 25 000 units/ml), and culturing continued for a further 10 days. Samples of medium were withdrawn at regular intervals throughout the 20 days and screened for the cytokines IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, GM-CSF, and TNF by ELISA; endothelial cell morphology and area density were assessed on days 0, 10, and 20.

**Results**—Spiking of organ culture media with endotoxin led to a substantial increase in the level of IL-8, and a smaller one in that of IL-6, but none of the other cytokines were detected. In five of the six stimulated corneas, these changes coincided with an increased incidence of endothelial cell loss, compared with that incurred by the fellow control, and the

surviving population also evinced signs of degeneration not seen in the latter.

**Conclusion**—Endotoxin induced increases in the levels of IL-6 and IL-8 appear to be correlated with endothelial cell loss. Since no adverse effects of this toxin on long term cultured monolayers of human corneal endothelial cells have been previously observed, the damage incurred in corneal organ culture may well be attributable to the influence of cytokines produced by other corneal cells or a non-intrinsic (passenger) cell population, such as macrophages, Langerhans cells, or lymphocytes present under these latter conditions.

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Bacterial endotoxins or, more specifically, lipopolysaccharides, are known to trigger both local and systemic inflammatory responses by stimulating the production of cytokines. Many blood cells (including neutrophils, lymphocytes, monocytes, and platelets), as well as vascular endothelial cells, respond directly to endotoxins either via surface bound or soluble CD14 receptors,<sup>1</sup> or possibly by means of lipid-lipid interactions.<sup>2</sup>

Human corneal cells are also known to interact with endotoxins, which stimulate either directly or indirectly, via cytokines, the secretion of interleukins (IL). The participation of corneal epithelial cells in the regulation of local inflammatory responses is known to be mediated by IL-1; indeed, this substance was originally referred to as corneal epithelial cell derived thymocyte activating factor.<sup>3</sup> Within the corneal stroma and endothelium, the expression of IL-8 mRNA has been reported to be enhanced by either IL-1 or tumour necrosis factor (TNF),<sup>4</sup> and all indigenous corneal cells (epithelium, endothelium, stromal keratocytes) secrete increased amounts of IL-6 after exposure to lipopolysaccharides.<sup>5</sup>

In vivo, corneal tissue is usually protected from exposure to endotoxins by the epithelial and vascular barriers; but in organ culture, this is no longer the case. The potential threat of contamination with such substances is,

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moreover, particularly relevant in the case of the cornea which, unlike other transplanted tissue, is explanted from the body surface, where bacteria and fungi abound. These organisms, once carried over into storage media, will have ready access to the corneal stroma and endothelium, with potentially dire consequences. Since organ culture media contain antibiotics, as a matter of course, contamination with replicating bacteria is, in actuality, extremely rare.<sup>6,7</sup> However, even in the absence of bacterial growth, endotoxins may none the less be present at low concentrations; they have, indeed, been detected in up to 50% of sterile organ culture media, irrespective of the storage technique employed.<sup>8</sup>

Relatively little is known about the effects of endotoxins on cultured donor corneas or the possible implications for grafting. But this may be readily conceived to be multifarious when one considers the complexity of the biological responses in which endotoxins and cytokines are implicated; a situation which will, moreover, be exacerbated by the diversity of cell types involved (including neutrophils, lymphocytes and macrophages, as well as the indigenous population).

In the current investigation, we focused on endothelial cell viability, and endeavoured to establish whether the incidence of necrosis was associated with the presence in culture media of any particular cytokine formed by setting the endotoxin induced reaction cascade in train.

## Materials and methods

### EVALUATION OF DONOR CORNEAS AND ORGAN CULTURING CONDITIONS

Donor globes excluded for transplantation because of viral infection of the donor (hepatitis and/or HIV) were examined with the slit lamp and excluded if corneal scars or other pathologies were manifested (for full details of selection criteria, see Boehnke,<sup>9</sup> Eye Bank Association of America,<sup>10</sup> and Frueh and Boehnke<sup>11</sup>). They were disinfected with povidone-iodine, and the corneoscleral buttons excised using a microsurgical technique under laminar flow conditions. Tissue discs were immersed in MEM (Seromed) and endothelial cell morphology evaluated by inverted phase contrast microscopy according to standard criteria.<sup>12</sup> Corneal buttons were then transferred to wells containing buffered saline (Alcon), in order to induce swelling of the intercellular spaces; the endothelium was photographed using a high resolution video camera, and central endothelial cell numerical densities calculated from video prints using the fixed frame technique.

Corneal discs, maintained in a vertical position by means of a standard holding device (Storz; no 93-100),<sup>13</sup> were cultured in 60 ml of medium (MEM supplemented with 2% fetal calf serum, glutamine, HEPES buffer, antibiotics, and amphotericin B (all purchased from Seromed)) contained within conventional tissue culture bottles (Falcon; no 3013E); they were incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide; the

medium was changed after 10 days. Twelve hours before the final evaluation on day 20, deswelling of the corneal stroma was achieved by introducing Dextran T500 (Pharmacia) to a final concentration of 6%.

Endothelial cell numerical densities were determined before organ culture (see above) and, thereafter, on days 10 and 20.

### EXPERIMENTAL SET UP

Corneal discs from the fellow eyes of six donors were evaluated in this investigation, the incidence of endothelial cell loss incurred after exposure to endotoxin being correlated with the presence of various cytokines released into the culture medium.

Samples of fluid were withdrawn for analysis between days 0 (baseline value and negative control) and 20. On day 10, the corneas were transferred to fresh media: one of each pair was incubated in the absence (left corneas), and the other (right corneas) in the presence, of *E coli* endotoxin (Sigma; 50 µg/ml (equivalent to 25 000 endotoxin units/ml)), and culturing continued for a further 10 days.

Aliquots of medium were screened for cytokines IL-1 (R&D), IL-4, IL-5, IL-10, GM-CSF (Pharmingen), IL-2, IL-6, IL-8, and TNF (Genzyme) by employing the sandwich ELISA technique. The detection level for each cytokine, except IL-1 (4 pg/ml), was 30 pg/ml.

All fluid samples before day 10, and control ones thereafter, were tested for the presence of donor derived endotoxins using the QCL-1000 Chromogenic Limulus Amoebocyte Lysate Assay (BioWhittaker), the lower level for a positive result being set at 0.600 endotoxin units/ml.

### STATISTICAL EVALUATION

ELISA results and endothelial cell numerical densities were compared statistically using Student's *t* test.

## Results

Figure 1 depicts changes in the cumulative levels of cytokines detected in culture fluid during the initial 10 day equilibration period and, following transfer of tissue to fresh media spiked (right corneas) or unspiked (left corneas) with 25 000 units/ml of *E coli* endotoxin, during the ensuing 10 days.

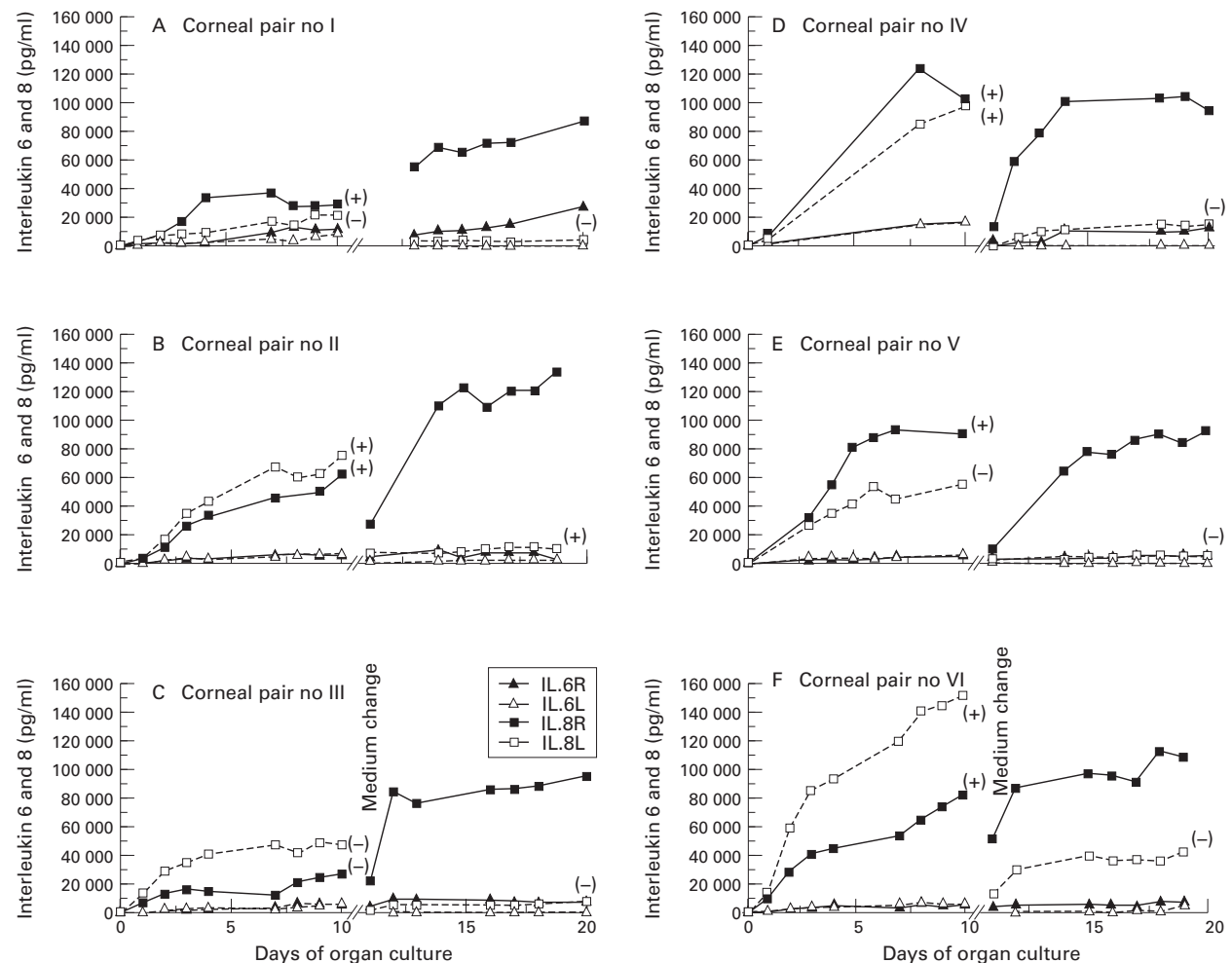
During the initial 10 days of culture, all media contained low levels of IL-6 and considerably higher ones of IL-8; none of the other cytokines (IL-1, IL-2, IL-4, IL-5, IL-10, GM-CSF, and TNF) were detected during either this (1 to 10 day) or the second (11 to 20 day) phase of incubation. The final concentration of IL-6 achieved by day 10 varied between 5800 and 17600 pg/ml; that of IL-8 between 27 600 and 151 000 pg/ml. In controls (left corneas), the levels of IL-6 and IL-8 attained after the medium change were much lower (IL-6: 620 to 2500 pg/ml; IL-8: 5800 to 43 000 pg/ml) than those achieved before it. Spiking of media with endotoxin elicited a marginal increase in the monitored level of IL-6, compared with that attained during the initial 10 day phase; and a marked one in that of IL-8,

**Table 1** Mean concentration of endotoxin detected in the media bathing six (I to VI) fellow pairs (left and right) of cultured donor corneas during an initial 10 day equilibration phase and a subsequent experimental one of equal duration, the onset of which was distinguished by change of medium and spiking of right corneal cultures with 25 000 units/ml of *E coli* endotoxin. Media were considered as registering endotoxin negative (–) if the concentration fell below 0.600 units/ml and as endotoxin positive (+) if the level exceeded this value

| Corneal pair no | Endotoxin (units/ml)                       |           |                                     |       |
|-----------------|--|-----------|-------------------------------------|-------|
|                 | Initial 1 to 10 day phase of organ culture |           | 11 to 20 day phase of organ culture |       |
|                 | Left                                       | Right     | Left                                | Right |
| I               | 0.505 (–)                                  | 1.060 (+) | 0.423 (–)                           |       |
| II              | 1.066 (+)                                  | 1.012 (+) | 1.021 (+)                           |       |
| III             | 0.506 (–)                                  | 0.530 (–) | 0.588 (–)                           |       |
| IV              | 0.759 (+)                                  | 0.818 (+) | 0.360 (–)                           |       |
| V               | 0.425 (–)                                  | 0.750 (+) | 0.445 (–)                           |       |
| VI              | 0.850 (+)                                  | 0.837 (+) | 0.568 (–)                           |       |

the final concentration achieved for the latter being higher in four instances ((I, II, III, VI/right cornea; Fig 1A, B, C, F);  $p = 0.032$ ), and virtually the same in the other two (IV, V/right cornea; Fig 1D, E). The proportional increase in the levels of IL-6 and IL-8 realised in right relative to those attained in fellow control (left) corneal media were, however, 5.5 ( $p = 0.009$ ) and 10-fold higher ( $p \leq 0.001$ ) after spiking than during the initial 10 days of culture.

During the initial 10 day phase, eight of 12 corneal cultures registered positive for donor derived endotoxin (Table 1). The data pool is too small to ascertain with any degree of certainty whether this had a desensitising effect on the susceptible cell population since, of the six endotoxin spiked cultures, five had contained these substances during the initial period of equilibration. There is, however, some support for this contention. When the level of IL-8 achieved after spiking with endotoxin is expressed as a ratio of that attained before the medium change, then the proportional increase realised in the single instance in which donor derived endotoxin had not been present (III/right; 3.4-fold; Fig 1C), was one and a half times greater than the highest yielded (II/right; 2.1-fold; Fig 1B) among the five that did. That this donor derived endotoxin did indeed contribute to cytokine production, at least of IL-8, during the initial 10-day phase, is borne out by a comparison of the mean final concentration attained within media lacking and containing these substances, the level of IL-8 in the former (86 800 pg/ml;  $n = 8$ ), being approximately double that in the latter (40 000 pg/ml;  $n = 4$ ; ( $p = 0.029$ )). And



**Figure 1** Levels of IL-6 (triangles) and IL-8 (squares) accumulating in the media of cultured corneas, data pertaining to each of the six fellow pairs (I to VI) being represented in (A) to (F), respectively. Traces through solid symbols correspond to right corneal media; those through open symbols to left ones. After an initial 10 day equilibrium phase, the medium bathing each cornea was replaced (break in x axis), and those containing right corneas spiked with 25 000 units/ml of *E coli* endotoxin; left corneas served as controls. Symbols in parentheses indicate whether media registered negative (–) or positive (+) for donor derived endotoxin (see Table 1).

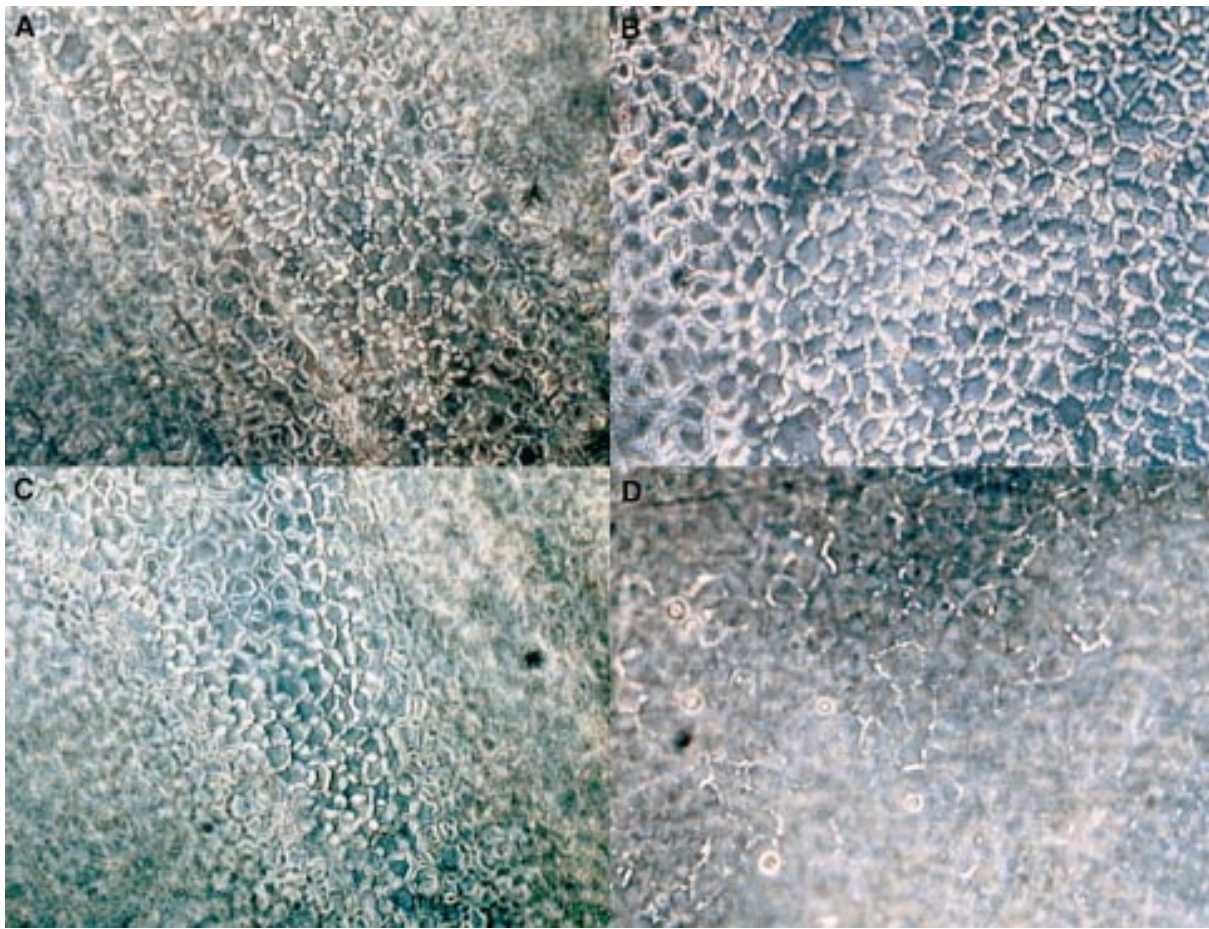


Figure 2 Appearance of left (A, B) and right (C, D) corneal endothelial cells (case no V), as seen in the phase contrast microscope, after an initial 10 day equilibration phase (A, C) and upon termination of the subsequent experimental one of equal duration (B, D), the onset of which was distinguished by change of medium and spiking of the right corneal culture with 25 000 units/ml of *E coli* endotoxin (D); the left corneal culture served as control (B). Left corneal endothelial cells exhibited a normal appearance over the entire 20 day culture period, as did right ones during the initial 10 day phase; but right corneal endothelial cells exposed to 25 000 units/ml of endotoxin manifested an increased incidence of polymorphism, cell enlargement, and incomplete intercellular border swelling (after immersion in buffered saline).

when one considers that the presence of donor derived endotoxin at less than 1 unit/ml (Table 1) sufficed to elicit the production of IL-8 at a mean level which was equivalent to that achieved by spiking with 25 000 units/ml, then

one can readily appreciate how sensitive the responsive cell population is to detecting the presence of this toxin.

With respect to controls, five of the six cultures registered negative for endotoxin during the 11 to 20 day period. And the removal of these substances by change of medium may have partially accounted for the reduction in the levels of IL-6 and IL-8 produced during this phase; but not entirely. In the one case in which donor derived endotoxin persisted after transferring corneas to fresh media (II/left; Fig 1B), the level of IL-8 (11 000 pg/ml) did not exceed those attained in the ones in which it did not persist ( $\leq 17\ 100$  pg/ml). This intimates that the responsive cells may not belong primarily to the intrinsic population, but to one that is readily dislodged and washed away—that is, to the passenger cell pool (see Discussion).

In five of the six endotoxin spiked cultures, the incidence of endothelial cell loss was considerably higher than in the fellow controls ( $p = 0.002$ ; (Table 2)). Surviving cells within the former group had also undergone morphological changes (Fig 2D); these included an increased incidence of pleomorphism, cell enlargement, and incomplete intercellular

Table 2 Endothelial cell numerical densities and losses incurred ( $\Delta$ ) by six (I to VI) fellow pairs (left and right) of donor corneas after an initial 10 day equilibration phase and a subsequent experimental one, the onset of which was distinguished by change of medium and spiking of right corneal cultures with 25 000 units/ml of *E coli* endotoxin; left corneal cultures served as controls

| Corneal pair no | Cell density ( $\text{mm}^2$ ) |                                |                                | Total cell loss |
|-----------------|--------------------------------|--------------------------------|--------------------------------|-----------------|
|                 | Before organ culture           | After 10 days of organ culture | After 20 days of organ culture |                 |
| I               |                                |                                |                                |                 |
| L               | 2400                           | 2100 ( $\Delta$ 300)           | 1750 ( $\Delta$ 350)           | $\Delta$ 650    |
| R               | 2350                           | 2000 ( $\Delta$ 350)           | 1050 ( $\Delta$ 950)           | $\Delta$ 1300   |
| II              |                                |                                |                                |                 |
| L               | 2300                           | 2100 ( $\Delta$ 200)           | 1650 ( $\Delta$ 550)           | $\Delta$ 650    |
| R               | 2450                           | 2100 ( $\Delta$ 350)           | 1400 ( $\Delta$ 700)           | $\Delta$ 1050   |
| III             |                                |                                |                                |                 |
| L               | 2200                           | 2000 ( $\Delta$ 200)           | 1350 ( $\Delta$ 200)           | $\Delta$ 400    |
| R               | 2200                           | 2000 ( $\Delta$ 200)           | 1800 ( $\Delta$ 650)           | $\Delta$ 850    |
| IV              |                                |                                |                                |                 |
| L               | 2050                           | 1500 ( $\Delta$ 550)           | 1650 ( $\Delta$ +150)          | $\Delta$ 400    |
| R               | 2050                           | 1700 ( $\Delta$ 350)           | 1600 ( $\Delta$ 100)           | $\Delta$ 450    |
| V               |                                |                                |                                |                 |
| L               | 2200                           | 2000 ( $\Delta$ 200)           | 1950 ( $\Delta$ 50)            | $\Delta$ 250    |
| R               | 2050                           | 2000 ( $\Delta$ 50)            | 1250 ( $\Delta$ 750)           | $\Delta$ 800    |
| VI              |                                |                                |                                |                 |
| L               | 2150                           | 1900 ( $\Delta$ 250)           | 1800 ( $\Delta$ 100)           | $\Delta$ 350    |
| R               | 2150                           | 1900 ( $\Delta$ 250)           | 1150 ( $\Delta$ 750)           | $\Delta$ 1000   |

border swelling (after immersion in buffered saline), all of which are considered to be signs of degeneration.

### Discussion

Phlogistic reactions, whether of extraocular or intraocular origin, tend to be down regulated by the avascular, and hence immune privileged, cornea. And this feature is recognised as being essential for a favourable graft prognosis. In situ, corneal tissue is protected from potential inflammatory stimulants, such as endotoxins, by the epithelial and vascular barriers; but in vitro, the situation is different. Under these conditions, contamination of culture media with endotoxins could have untoward effects; these may be manifested either directly, by overt changes in cell morphology, and/or by a more discreet influence on the tissue's immunological status, the consequences of which may be realised only after transplantation, by a compromised graft performance.

In the current study, we monitored the media of organ cultured corneas for a number of cytokine mediators of the inflammatory response, both before and after spiking with endotoxin; these data were then compared with those pertaining to endothelial cell morphology and numerical density. Spiking of culture media with endotoxin markedly enhanced the release of IL-8 from corneal tissue and increased that of IL-6 to a lesser degree; these events coincided with degenerative changes in endothelial cells and a higher incidence of actual loss among this population. Increases in the production of IL-6 by corneal endothelial, as well as epithelial and stromal, cells have been reported also by Sakamoto and Inada,<sup>5</sup> but to our knowledge they did not ascertain whether there were concomitant changes in the morphological status and integrity of the different cell populations. The mechanisms underlying this causal relation between endotoxin induced increases in IL-6 and IL-8 production by corneal tissue and endothelial cell loss must remain speculative at present. However, since the viability of long term cultured monolayers of human corneal endothelial cells is not affected by exposure to endotoxins,<sup>14</sup> the losses incurred in organ culture are most likely actuated by one or several coexisting cells present under the latter conditions. Moreover, in the one control instance in which donor derived endotoxin persisted after the change of medium (II/right; Fig 1B), the level of IL-8 did not increase to anything like the degree observed during the initial 10 day phase. This suggests that at least a portion of the responsive cells may be readily dislodged and washed away, thereby implicating the passenger pool—for example, macrophages, Langerhans cells, or lymphocytes. Alternatively, the susceptible cell population (intrinsic or otherwise) may have become refractory. And this state of unresponsiveness is likely to be overcome only if the concentration of endotoxin is elevated substantially above the existing (that is, donor derived) level, as indeed will be the case in cultures spiked with this substance.

IL-6 is expressed by a number of cell types, and plays an important role in host defence and immune responses. It influences the differentiation of B lymphocytes and the subsequent antibody producing capacity of plasma cells; it regulates T lymphocyte function; and increases the expression of Fc receptors on macrophages as well as stimulating phagocytic activity, generally. In endotoxin induced uveitis, macrophages are known to be the main protagonists of the immune response. The ensuing inflammation and the resulting tissue damage are believed to be precipitated by circulating prostaglandins and cytokines<sup>15-18</sup> rather than by endotoxin itself, which does not accumulate in ocular structures after systemic administration.<sup>19</sup> Indeed, IL-6, in particular, has been shown to elicit more damage than endotoxin when injected intravitreally, and this cytokine, in turn, increases the expression of HLA class II antigens, which findings may be of particular relevance in corneal grafting.<sup>20</sup> That IL-6 is involved in the local pathogenesis of human uveitis is further supported by the detection of a 50 000-fold higher concentration of this cytokine in aqueous humour samples of uveitis patients,<sup>21</sup> and its elevated level in those of healthy human volunteers receiving systemic administration of endotoxin.<sup>22</sup>

In some cell types, particularly monocytes, endotoxins also promote the production of other cytokines, such as TNF and IL-1, which can act in an autocrine capacity, further enhancing the expression of IL-6 and IL-8. Indeed, stimulation of human corneal endothelial and stromal cells with TNF and IL-1 has been shown to increase the expression of IL-8 mRNA.<sup>4</sup> We found no detectable levels of either TNF or IL-1 in endotoxin spiked culture media, but this does not absolutely exclude the possibility of their acting in the heretofore mentioned capacity. The secretion of IL-8 by organ cultured corneas may play a role in directing the migration of leucocytes towards this tissue from neighbouring ones. Infiltration of the cornea with such cells is a characteristic of both infectious and non-infectious ocular inflammation, including keratitis, uveitis, and allograft rejection. And the production of IL-8 by corneal tissue in culture may thus have important clinical consequences.

Up to 50% of organ culture media may contain endotoxins, despite their being sterile, after receiving corneal buttons.<sup>8</sup> Instances of endothelial cell death may thus be partially attributable to these substances being carried over with excised tissue and their subsequent stimulation of cytokine production. These latter mediators of inflammation may, in turn, elicit an increased expression of HLA class II antigens, which, by upregulating the recipient's local immune response, may lead to graft rejection and ultimately to its failure. In order to arrest these events, it may be expedient to supplement corneal preservation media with agents which either block the action of endotoxins directly or interrupt the sequence of events set in train by them.

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