Mononuclear cells in the corneal response to endotoxin

Edward L. Howes, Virginia K. Cruse, and Marion T. Kwok

A severe keratitis can be produced after the direct injection of bacterial endotoxin, or lipopolysaccharide (LPS), in rabbits. Corneal inflammation can progress to scarring and vascularization within a 2 to 3 week period. Pretreatment with systemic adrenal corticosteroids (triamcinolone) prevents this response. Limbal cellular and vascular events were studied during the first 20 hr after injection of LPS in treated and nontreated rabbits. Perivascular limbal inflammatory cells were counted and limbal vascular permeability was assessed by extravasation of 125I-albumin and 125I-fibrinogen in the cornea. Corticosteroids decreased but did not prevent the early protein extravasation and profoundly altered the inflammatory cell population around blood vessels at the limbus. Mononuclear cells, particularly mononuclear phagocytes, were sharply reduced. It is proposed that these cell types play an important role in the perpetuation and amplification of the inflammatory response in this reaction. (INVEST OPHTHALMOL VIS SCI 22:494-501, 1982.)

Key words: endotoxin, bacterial lipopolysaccharide, cornea, mononuclear cells, mononuclear phagocytes, 125I-albumin, 125I-fibrinogen, corticosteroids

The intracorneal injection of bacterial endotoxin (lipopolysaccharide, LPS) produces a prolonged acute inflammation, which may lead to corneal vascularization and scarring within a 2 to 3 week period. This reaction is characterized by a perpetuation of the acute inflammatory process and the formation of "corneal rings" around the injection site during the first week. These rings are composed of granulocytes, and immunofluorescent studies indicate the importance of activation of properdin in the alternate complement pathway.1

The present study was undertaken to examine some of the factors possibly contributing to perpetuation of this acute inflammation. Cellular and vascular changes occurring during the first 20 hr after corneal injection of endotoxin were examined in normal and corticosteroid-treated animals. A method was devised to measure the extravasation of radioactively labeled proteins in the cornea as an estimate of altered vascular permeability of limbal blood vessels and an index of ongoing inflammation during the first 24 hr of the response. The cellular response was determined in a semiquantitative manner by examining the perivascular limbal cell population.

The results suggest that the presence of mononuclear cells at the limbus may be important in the amplification of acute prolonged inflammation in this experimental model.

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Table I. Protein exudation in cornea and limbal tissues

<table>
<thead>
<tr>
<th>Experimental groups (n)</th>
<th>Protein quantities*</th>
<th>125I-Albumin</th>
<th>125I-Fibrinogen</th>
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<tr>
<td></td>
<td>4 hr after protein</td>
<td>R 238 ± 124</td>
<td>490 ± 213</td>
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<tr>
<td></td>
<td></td>
<td>L 87 ± 39</td>
<td>139 ± 53</td>
</tr>
<tr>
<td>A. Normal rabbits (8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Triamcinolone-treated (9) (25 mg for 3 days)</td>
<td>4 hr after protein</td>
<td>R 154 ± 66</td>
<td>277 ± 133</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L 58 ± 21</td>
<td>76 ± 30</td>
</tr>
<tr>
<td>C. Triamcinolone-treated (6) (25 mg 3 hr before)</td>
<td>4 hr after protein</td>
<td>R 135 ± 43</td>
<td>266 ± 105</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L 51 ± 15</td>
<td>96 ± 20</td>
</tr>
<tr>
<td>D. Normal rabbits (6)</td>
<td>20 hr after protein</td>
<td>R 218 ± 76</td>
<td>478 ± 206</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L 72 ± 22</td>
<td>137 ± 40</td>
</tr>
<tr>
<td>E. Normal rabbits (9)</td>
<td>20 hr after protein</td>
<td>R 447 ± 191</td>
<td>1019 ± 461</td>
</tr>
<tr>
<td>F. Triamcinolone-treated (9) (3 days before)</td>
<td>20 hr after protein</td>
<td>R 105 ± 40</td>
<td>146 ± 92</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L 49 ± 24</td>
<td>28 ± 20</td>
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</table>

R = right eye (endotoxin-treated, 25 μg/50 μl); L = left eye (saline-treated, 50 μl).

*Data expressed as mean cpm/0.10 gm of ocular tissue ± S.D.

Materials and methods

Albino rabbits of both sexes weighing approximately 2 kg were used throughout these studies. An Escherichia coli endotoxin (055: B5, Boivin extraction, Difco Laboratories, Inc., Detroit, Mich.) was injected in the center of the right cornea via a 30-gauge needle after local anesthesia with 0.1% proparacaine drops (Ophthaine; E. R. Squibb & Co., Princeton, N.J.). After initial trials with different quantities of this endotoxin, it was found that 25 μg in 50 μl of saline gave consistent results in terms of producing a prolonged inflammation, which leads to peripheral corneal vascularization and actual corneal scarring over a period of 2 to 3 weeks. Saline alone was injected in the left cornea.

Albumin labeled with 131I (131I-human serum albumin, Albumotope 1-131, specific activity 20 μCi/mg; Squibb) and fibrinogen labeled with 125I (125I-human fibrinogen, specific activity 180 μCi/mg; Abbott Laboratories, North Chicago, Ill.) were injected via a marginal ear vein (10 μCi each). Two experimental groups of six or more rabbits were used. In the first group, the labeled proteins were injected just after the injection of endotoxin and the rabbits were killed at 4 hr. Animals in the second group were injected with labeled proteins 4 hr after LPS injection and were killed at 20 hr, thus allowing 16 hr of the circulation of these proteins.

A technique was evolved so that isotopes could be quantitated and histopathologic assessment of limbal blood vessels could be performed on the same tissues. For this purpose, rabbits were killed and the eyes were enucleated, trimmed of excess tissue, and placed in 2% phosphate-buffered glutaraldehyde for 10 min. The cornea and 1 mm of a surrounding rim of conjunctival and limbal tissue was dissected away from the globe, washed three times in buffer, and placed in the fixative in a gamma-well counting vial. Fixation was carried out for at least 2 hr and sometimes overnight in the vials while the quantity of isotopes was being determined. After counting, the tissues were processed and embedded in plastic embedding media and sections were cut at 1 to 2 μm thickness, stained with methylene blue and azure II, and examined by light microscopy. The brief initial fixation and the washing steps were shown in preliminary studies to produce more uniform results.

The quantities of protein in the cornea-limbal tissues were determined by measurement of the isotopes in a gamma-well counter. Counts in 1 ml of cardiac plasma were determined as well. Results are expressed as average counts per minute per 0.10 gm of ocular tissue ± standard deviation. Averages in different groups were compared by a Student’s t test to determine significance levels.

To determine whether adrenal corticosteroid might have a differential effect on the parameters being examined, triamcinolone (Kenalog; Squibb) was employed in two regimens. The corticosteroid was given for 3 days prior to endotoxin injection on day 4 and also as a single injection 3 hr prior to endotoxin injection (25 mg/kg each time in the back leg, intramuscularly). A 3 day regimen of corticosteroid had been shown in another study to
Table II. Perivascular limbal-cell population

<table>
<thead>
<tr>
<th></th>
<th>Lymphocytes and plasma cells</th>
<th>Mononuclear phagocytes</th>
<th>Granulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rabbits (7) 4 hr after endotoxin</td>
<td>8 ± 4</td>
<td>55 ± 10</td>
<td>37 ± 11</td>
</tr>
<tr>
<td>Triamcinolone-treated rabbits (6) 4 hr after endotoxin</td>
<td>4 ± 3</td>
<td>21 ± 3</td>
<td>75 ± 5</td>
</tr>
<tr>
<td>Normal rabbits (4) 8 hr after endotoxin</td>
<td>7 ± 5</td>
<td>77 ± 8</td>
<td>16 ± 13</td>
</tr>
<tr>
<td>Normal rabbits (5) 20 hr after endotoxin, no steroids</td>
<td>5 ± 2</td>
<td>74 ± 6</td>
<td>21 ± 6</td>
</tr>
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</table>

*Data expressed as mean percent ± S.D.; 100+ cells counted.

inhibit limbal vascular permeability after the injection of specific antigen in hyperimmunized rabbits. Either regimen was uniformly effective in abrogating the corneal response 24 hr after the injection of endotoxin.

To determine the types of cells present around limbal blood vessels, over 100 cells were counted in each specimen at 4 and 20 hr after endotoxin injection as well as in a separate group at 8 hr, in which quantitative protein determinations were not made. These cells were grouped as (1) rabbit granulocytes, (2) lymphocytes and plasma cells, and (3) cells of the mononuclear phagocyte series. For cells to be included as the latter cell type, the nucleus had to be oval or indented with a delicately distributed chromatin and the cytoplasm had to be abundant. Electron microscopy of some of these cells confirmed their resemblance to cells of the mononuclear phagocyte line. It is acknowledged that at least some of the cells with these characteristics by light microscopy could be activated lymphocytes ("lymphoblasts").

Results

Data for the extravasation of $^{131}$I-albumin and $^{125}$I-fibrinogen at 4 and 20 hr in untreated and corticosteroid-treated rabbits are given in Table I, and the assessment of the percent of types of inflammatory cells around limbal blood vessels is shown in Table II.

Four hours after the injection of endotoxin in the cornea, approximately 2½ times more isotope-labeled protein was found in the right cornea than the quantity found in the left saline-injected cornea (group A, Table I). This was true for both albumin and fibrinogen. Twenty hours after endotoxin injection and 16 hr after injection of labeled proteins, approximately 6 times more albumin and 10 times more fibrinogen had accumulated in the right cornea than the quantities found in the left ocular tissues (group E, Table I).

In corticosteroid-treated rabbits at 4 hr after endotoxin injection there was a decrease in the quantity of labeled proteins in rabbits pretreated over a 3 day period and also 3 hr before endotoxin injection (groups B and C, Table I), as compared with protein quantities in untreated controls. The results were similar in treated and untreated animals whether average counts per minute or ratios of endotoxin to saline-injected eyes were compared. Although a decrease in protein extravasation was noted, it was not prevented ($p$ values were between 0.10 and 0.05 in all groups compared). The results of a second group of untreated animals at 4 hr (group D, Table I) are given for comparison and to indicate that although the counts per minute values were low, the observations were reproducible.

At 20 hr after endotoxin injection in triamcinolone-treated rabbits, the quantity of extravasated protein was markedly reduced compared with that in untreated animals (group F, Table I). At both 4 and 20 hr the extravasation was reduced in the saline-injected corneas as well. The values for cpm/0.10 gm of tissue 20 hr after saline injection were similar to those for eyes not injected at all (data not shown), suggesting that corticosteroids reduce protein extravasation in the normal animal.

The histopathologic changes at the limbus at 4 hr showed many mononuclear cells as well as granulocytes (Fig. 1). At 20 hr mononuclear cells were more pronounced, and at both times a majority of cells appeared to be of the monocyte-macrophage series (Fig. 1). The ultrastructural appearance of the latter cells confirmed the impression that many of these were mononuclear phagocytes (Fig. 2). Semiquantitative assessment of cells involved...
showed that 75% of cells were mononuclear and 25% granulocytes at 4 hr, and at 20 hr over 80% were mononuclear (Table II).

By contrast, in corticosteroid-treated rabbits at 4 hr the numbers of inflammatory cells were reduced and the majority of cells were heterophils (Fig. 3 and Table II). Evidence of edema and inflammatory cells in saline-injected eyes at 4 hr could usually be found but was minimal. By 20 hr, if inflammation was present, the cells were again predominantly heterophils. The number of cells was usually markedly reduced at this time. Perivascular cells at the limbus in treated rabbits at 4 hr were 75% granulocytes (Table II).

Discussion

The inflammatory response in the corneal and limbal tissues during the first 20 hr after the intracorneal injection of endotoxin is
characterized by a protein extravasation that is measurable at 4 hr and maintained during a 4 to 20 hr period after injection. A characteristic of this early response is the presence of many mononuclear cells around limbal blood vessels, predominantly cells with morphologic characteristics of mononuclear phagocytes. These cells compose a majority of the cell population in this location at 4, 8, and 20 hr. Pretreatment with adrenal corticosteroids decreases but does not prevent the protein extravasation over the first 4 hr, nor does it prevent an extravasation of granulocytes. By contrast, the percent of limbal perivascular mononuclear cells is markedly reduced in treated animals. These findings suggest that the presence of a mononuclear cell population, in particular mononuclear phagocytes, may play a role in establishing a prolonged acute inflammation in this experimental model.

In untreated rabbits 20 hr after endotoxin injection, the accumulation of labeled fibrinogen is greater than that of albumin, suggesting a different rate of accumulation. In studies of delayed hypersensitivity reactions in the skin, a similar differential accumulation of these two proteins has been noted. The accumulation of circulating proteins in tissue was believed to result from the amount initially injected, the time of circulation, the extent of vascular permeability alteration, and the rate of removal.

Corticosteroids given either over a 3 day period or immediately before injection of endotoxin prevented the development of a progressively more severe inflammation. Without such pretreatment, endotoxin in the quantities used produced a corneal vascularization and scarring 2 to 3 weeks after injection. These findings are in contrast to those observed within the eye after intravenous administration of endotoxin. Corticosteroids given for 3 days prior to intravenous
endotoxin injection increased the severity of the ocular response, whereas immediate pre-treatment prevented the response. The differences in results in this and the previous study are probably because of different sites of actions of corticosteroids and the avascular nature of the cornea.

The effect of corticosteroids on limbal vascular permeability within the first 20 hr in this experimental model was not as pronounced as that described in hyperimmunized rabbits injected in the cornea with specific antigen; the reaction was completely prevented in those experiments. In the latter study bovine serum albumin–fluorescein was injected in the cornea and corneal immune rings did not form in steroid-treated rabbits. Thus both the experimental model and the means of measurement of protein extravasation were different.

Of interest in the present study was the apparent decrease in protein extravasation over the 20 hr period in uninjected as well as saline-injected eyes of treated rabbits. At
least a part of this decrease was probably caused by a decreased level of circulating labeled proteins available for extravasation at 20 hr in treated rabbits, since plasma volume increases in animals treated with corticosteroids. In the present study, the average quantity of circulating proteins was 25% less in treated rabbits than that in untreated rabbits, accounting in part for the differences measured in these groups. There remains the possibility that corticosteroids may have an effect on plasma protein turnover in the extravascular space in normal animals.

Interactions of mononuclear cells and endotoxin may have an importance in other in vivo responses. Preparation for the localized Shwartzman reaction may be due in part to mononuclear phagocyte accumulation 24 hr after endotoxin injection in the skin. Procoagulant material derived from intravascular endotoxin–monocyte interactions may be important in the generalized Shwartzman reaction. In small quantities (<0.10 μg), endotoxins are capable of producing some of the features of a delayed hypersensitivity reaction in the skin of man. Conceivably, quantities of endotoxin approaching this amount may reach the limbal tissues during the initial injection of endotoxin in the cornea in this model.

In studies in leukopenic rats, lymphocytes (mononuclear phagocytes?) were found to contribute to a nonspecific acute inflammatory response and to the vascular response to vasoactive amines, a property inhibited by corticosteroids. Supernatants of lymphocytes in tissue culture medium had similar effects, suggesting a lymphokine-like substance might be involved (proinflammatory factor).

In vitro studies have shown that LPS interacts with both mononuclear phagocytes and lymphocytes. Mononuclear phagocytes can be activated to secrete plasminogen, collagenase, and lysozymes as well as to increase other biologic activities. The interaction of LPS and B lymphocytes is well known and indicates both a mitogenic and synthetic activity. In turn, the interaction of LPS with mononuclear phagocytes may be amplified by both T and B lymphocytes. Recent in vitro studies of cultures of human peripheral blood monocytes indicate that many complement components, and in particular alternate complement components, can be synthesized by these cells. Conceivably this may be one of the amplifying mechanisms of mononuclear phagocytes in the present study.

REFERENCES
13. Bechara GH, Sudo L, Ribeiro dos Santos R, and


