In vitro demonstration of cytotoxic antibodies and their possible role in corneal graft rejections. **Perry S. Binder,** *J. W. Chandler,** and **H. E. Kaufman.***

Rabbit corneas were exposed to heterologous guinea pig anti-rabbit antibodies in the presence of complement and cytotoxic damage to the endothelium was demonstrated using para nitroblue tetrazolium. The possible role of such antibodies as "blocking antibodies" and as cytotoxic antibodies in the corneal graft rejection process are discussed.

The mechanism by which the donor corneal endothelial cell is destroyed by invading host lymphocytes has been demonstrated in rabbits, in humans, and the clinical presentation of the homograft rejection in humans in now well known. It is accepted that the host lymphocyte is primarily responsible for the destruction of the donor endothelial cell but the role of antibodies has not been determined.

Manski, Ehrlich, and Polack1 bathed normal rabbit endothelium with heterologous duck anti-rabbit cornea serum and could not demonstrate a deleterious effect on normal endothelium, but they were able to demonstrate damage to regenerating endothelium.

Chandler, Gebhardt and Kaufman2 soaked whole rabbit corneas in heterologous antilymphocyte serum (ALS) and globulin (ALG), and succinylated antilymphocyte globulin (S-ALG), with and without complement. They were able to demonstrate that complement was necessary to produce cytologic damage to the endothelial cells and that antibodies that had the complement-fixing piece (Fc) chemically modified (S-ALG) were not cytotoxic in the presence of complement.

The purpose of this report is to demonstrate the presence of antibodies cytotoxic for rabbit corneal endothelial cells and to discuss the possible role of such antibodies in the corneal graft rejection process.

**Materials and methods.** Adult 2 to 3 kilogram New Zealand white rabbits were sacrificed by an overdose of intravenous pentobarbitol and the eyes were immediately enucleated. The cornea with a scleral rim was carefully removed and placed endothelial side up in a 10 c.c. beaker. The test reagents and tissue culture media were carefully injected down the side of each beaker to minimize trauma to the endothelium. The preparation of the guinea pig anti-rabbit lymphocyte serum has been described elsewhere.3,4 A total of 1.5 c.c. of fluid was necessary to completely cover each cornea. Twelve control corneas were separately incubated with either tissue culture medium (Minimum Eagles medium, Microbiological Associates), guinea pig complement (Microbiological Associates) undiluted and diluted, or antilymphocyte serum with a lymphocytotoxicity titer of 1:1024 (Table 1). In preliminary experiments we found the complement to be cytotoxic if it were diluted with normal saline or water, but that dilution with tissue culture fluid eliminated the cytotoxicity. Nine corneas were treated with equal volumes of undiluted complement and serum and two were treated with undiluted complement and ALS diluted 1:2 (Table 1).

The corneas were incubated for 30 minutes at 37° C., the test fluids were aspirated and replaced with tissue culture fluid and incubated at 37° C. for 30 minutes. Each cornea was then stained (paranitroblue tetrazolium).5 fixed in 10 per cent formalin for 24 hours, and mounted on glass slides as whole preparations.

Coded slides were evaluated by light microscopy. Staining involving more than 10 per cent of the cells was considered as evidence of cytotoxicity.

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**Table I. Effect of antilymphocyte serum and complement on corneal endothelial viability**

<table>
<thead>
<tr>
<th>Cornea No.</th>
<th>Incubating medium</th>
<th>Tissue culture fluid</th>
<th>Complement ALS</th>
<th>Viable endothelial cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>3, 4</td>
<td>+</td>
<td>1:2</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>1:5</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>6-12</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>13-21</td>
<td>+</td>
<td>Undiluted</td>
<td>+</td>
<td>5-20</td>
</tr>
<tr>
<td>22, 23</td>
<td>+</td>
<td>Undiluted 1:2</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
Fig. 1. One hundred per cent viable rabbit corneal endothelial cells following incubation with undiluted guinea pig anti-rabbit lymphocyte serum (nitroblue tetrazolium ×160).

Fig. 2. Rabbit corneal endothelium following incubation with guinea pig complement and undiluted guinea anti-rabbit lymphocyte serum. Note missing endothelial cells and presence of staining cells (nitroblue tetrazolium ×100).
Fig. 3. Normal rabbit corneal endothelium following incubation in diluted guinea pig complement (1:2) and undiluted guinea pig anti-rabbit lymphocyte serum (nitroblue tetrazolium x160).

**Results.** Table I summarizes the results of the nitroblue tetrazolium staining. When cytotoxicity occurred, at least 75 per cent of the cells stained. The undiluted complement, and the undiluted serum, did not produce a cytotoxic effect (Fig. 1). However, the combination of undiluted complement plus serum was cytotoxic as evidenced by severe endothelial cell staining and loss of endothelial cells (Fig. 2). When complement, diluted 1:2 or 1:5, or ALS diluted 1:2 was used, no cytotoxic effect was seen (Fig. 3).

**Discussion.** Previous work using rabbit corneas soaked in ALS prior to transplantation demonstrated an increase in graft survival as compared to unsoaked corneas. More recent work has demonstrated that heterologous antibody can specifically bind to rabbit endothelial cells, and can protect rabbit grafts from rejection. Manski, Ehrlich, and Polack failed to demonstrate a cytotoxic effect on rabbit endothelium using duck anti-rabbit corneal antibodies which are now known not to fix complement. In contrast, Ehrlich and Halbert found that when dispersed corneal endothelial cells were exposed to duck anti-rabbit serum a definite cytotoxic effect was seen. The addition of guinea pig complement diluted 1:10 did not increase the amount of cytotoxicity.

Recently, Manski and Whiteside have demonstrated endothelial cytotoxicity with duck anti-rabbit corneal antibodies only in dispersed actively metabolizing cells. They further demonstrated that certain antigens were only found on the metabolically active cells, but were “hidden” within cell membrane in resting cells (i.e., whole mounts of corneas or undamaged in vivo endothelial cells). While examining the endothelium from two rabbit corneas which had undergone rejection, Polack found cells which were edematous and contained cytoplasmic vacuoles. These changes were similar to those found in cells treated in tissue culture with heterologous antibody. Interestingly, the examined cells were not in an area of active lymphocyte infiltration or fibroblast activity which are common concomitants of the homograft rejection.

It is known that heterologous anticorneal antibodies can fix directly to rabbit endothelial cells and then protect from graft rejection episodes. It is possible that an early step in the graft rejection process is the production of antibodies to the foreign endothelium which fix to the cells thereby rendering the surfaces more easily “accessible” to the incoming lymphocytes. The reason these complement-dependent antibodies are not cytotoxic in themselves may be due to the low levels of com-
plement in the anterior chamber. The mechanism of "blocking" antibody would then be to block the afferent arc of the immunologic reflex by covering surface antigens.

The results of this study clearly demonstrate a direct cytotoxic effect of heterologous complement-activated antibodies on rabbit corneal endothelial cells in vitro when serum in high titer or undiluted complement are used. The mechanism of cell damage is most probably associated with an increased cell wall membrane permeability because the intracellular enzymes are accessible to the nitroblue tetrazolium. To date, no one has been able to demonstrate by transmission electron microscopy any "holes" in the cell walls of rejected endothelium. Specific fixation of these cytotoxic antibodies can be identified by electron microscopy and their role in the corneal graft rejection process elucidated.

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REFERENCES


The glycosaminoglycans of isolated rabbit corneal stroma, clamped between two lucite plates at near normal hydration, were digested with testicular hyaluronidase in saline solution. After equilibration with 0.9 per cent saline solution alone the sodium and chloride content of the stroma was determined. Chloride was in equilibrium with both normal and hyaluronidase-treated stroma, allowing use of the Donnan calculation for excess or bound sodium to be made. Normal stromas contained 200 mEq. bound sodium per kilogram of dry weight calculated from the Donnan calculation; hyaluronidase-treated stromas contained 110 mEq. bound sodium per kilogram of dry weight. The data show that about half of the bound sodium in the corneal stroma is on nonsaccharide binding sites. Quantitative verification of the loss of glycosaminoglycans was performed.

It has been demonstrated that the polyanionic glycosaminoglycan structure of the stroma is responsible for a significant amount of the total tissue cation binding capacity.1,2 A detailed mathematical analysis of the ion binding data3 has indicated, however, that about one-half of the sodium which is bound by the stroma is on nonsaccharide sites, a point of importance in relation to stromal physiology.

Glycosaminoglycans can be degraded by various methods and recent examples of such procedures, relevant to the present study, are those which have been used on the arterial wall to examine the relationship of ion binding to glycosaminoglycan content in this tissue.4,5 In the present study a digestive procedure was used in an at-