Immunologic protection of rabbit corneal allografts: preparation and in vitro testing of heterologous “blocking” antibody

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Corneal allografts, like other foreign tissues, are subject to immunologic attack and rejection. Efforts to prolong graft survival have included the use of antilymphocyte serum and blocking antibodies. In this study antilymphocyte serum was tested as a potential corneal allograft-blocking reagent. It has been demonstrated that guinea pig anti-rabbit lymphocyte serum and the gamma globulin fraction of this serum bind to rabbit lymphocytes and corneal cells. In the presence of serum complement the antiserum and globulin are cytotoxic for cells of the cornea. Chemical modification of the anti-lymphocyte globulin renders it incapable of complement fixation, creating a reagent which retains the capacity to bind to cells and, in addition, to protect them from the cytotoxic effects of unaltered antibody and serum complement. The benign and protective nature of modified antilymphocyte globulin suggests that it is an excellent candidate for use in the prevention of corneal graft rejection.

Effective procedures to prevent immunologic destruction of corneal allografts are desperately needed. Topical or systemically administered reagents of many types have been tested with relatively little success. One of the more promising biologic reagents effective in prolonging allo- geneic tumor, skin, and kidney grafts is blocking antibody, which can inhibit the graft rejection response. The mechanism of action of blocking antibody is unknown. However, soaking an organ in antibody reactive against antigenic determinants on the graft affords protection against rejection. The protection is presumably monitored directly at the site or in the regional lymph nodes.

Intrigued by the possibilities offered by blocking antibody, we decided to derive nontoxic antibodies from serum antibodies of proved cytotoxic potency and to test their capacity for prolonging corneal allograft survival. In this report it is demonstrated that heterologous antilymphocyte serum can be modified chemically so that it is not only incapable of comple-
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Fig. 1. Comparison of ALS-mediated cytotoxicity in the presence of C to that of the anti-serum or normal guinea pig serum without C.

ment-dependent lysis of cells but also retains its ability to bind to and coat corneal and lymphoid cells, thus affording them protection from other cytotoxic antibodies.

Methods*

Experimental animals. Young (1 to 4-week-old) New Zealand white (NZW) male rabbits were used as thymocyte donors. Corneas were obtained from adult (2 to 3 kilogram) NZW male rabbits. Adult (200 to 300 gram) male Hartley strain guinea pigs were used for the production of anti-rabbit lymphocyte serum (ALS) and anti-sheep red blood cell (SRBC) antibody.

Production of ALS. Young rabbits were exsanguinated via cardiac puncture, and the thymus was removed aseptically. The tissue was minced on sterile wire mesh, and the cell clumps were reduced to a single cell suspension by passing them through increasingly smaller syringe needles. The cells were washed, counted in a hemocytometer, and adjusted to a concentration of 10⁶ cells per 0.5 ml. in 0.15 M NaCl. The suspension was emulsified in an equal volume of Freund's complete adjuvant, and a total of 1 ml. (0.1 ml. in each footpad and the remainder in the extremities near the trunk) was injected into several guinea pigs. Three weeks later each animal was given an intraperitoneal (i.p.) injection of 10⁶ cells in 0.15 M NaCl. One week after this injection the animals were bled out, and the pooled serum was separated, heated at 56° C. for 30 minutes, and stored frozen. Normal guinea pig serum collected in a similar manner served as a control sample.

Chemical modification of ALS. Anti-lymphocyte globulin (ALG) was prepared by ammonium sulfate precipitation of pooled ALS. Following precipitation and washing, the ALG was exhaustively dialyzed against 0.15 M NaCl. Disruption of the complement-binding region of the guinea pig immunoglobulin was achieved by adopting the succinic anhydride procedure of Lenard and Singer. The ALG, at a concentration of 10 mg. per milliliter in saline, was maintained at 4° C. in an ice bath, and the pH of the solution was adjusted to 8.0. Succinic anhydride (7 mg. per 20 mg. protein ALG) was added and allowed to dissolve while maintaining a pH of 8.0 with 5 M potassium hydroxide. The reaction was allowed to proceed for 40 minutes, at which time the succinylated ALG was exhaustively dialyzed against phosphate-buffered saline. Normal guinea pig globulin was subjected to identical modification as a control substance.

Preparation of anti-sheep erythrocyte antibody. Sheep erythrocytes were washed three times in 0.15 M NaCl. A 10 per cent suspension was then injected i.p. into guinea pigs at a concentration of 0.1 ml. per 100 grams of body weight. Ten days later the animals were given a second i.p. injection. After 21 days they were bled out by cardiac puncture. The serum was heat-inactivated and stored frozen. The hemolytic antibody titer of the immune and control sera was determined using a standard microriter assay system.

Determination of guinea pig anti-rabbit lymphocyte antibody titer. Lymphocytotoxicity titers were determined by a modification of the micro-
method of Kaliss. Serial twofold dilutions of test sera were made in microtiter plates after adding 0.025 ml. RPMI-1640 culture medium to each well. Subsequently, 0.025 ml. 95 + per cent viable rabbit thymus cells (5 x 10⁶ per milliliter) and 0.025 ml. of a 1:5 dilution of either rabbit or guinea pig complement (C') were also added to each well. The plates were incubated at 37° C. for 45 minutes. The per cent of cells killed was determined for each well by uptake of the vital dye trypan blue.

In one series of experiments 3 x 10⁶ rabbit thymus cells were preincubated for 30 minutes at 37° C. in succinylated normal guinea pig globulin or succinylated ALG (3.6 mg protein per milliliter). These thymocytes were then washed twice in culture medium and used in the above-described cytotoxicity assay. This latter approach was adopted as a means of determining whether succinylated ALG would bind to and protect thymus cells from the cytotoxic effects of ALS and C'.

Determination of antibody-binding to corneal tissue. Fresh 8 mm. corneal trephine buttons were placed in 1 ml. test sera for 30 minutes at 37° C. The antibody concentration of the absorbed serum was then compared with that of unabsorbed serum using either the cytotoxicity or anti-SRBC microtiter methods outlined above. As a control, serum was preincubated with 10⁸ thymocytes and then tested for reduction in lymphocytoxicity titer.

Determination of the effects of incubation in serum on corneal viability. Eight-millimeter rabbit corneal buttons were soaked in varying concentrations of test sera for 30 minutes. The viability of the corneal endothelium was tested immediately after treatment and 2 hours after incubation in antibody. The enzyme content of the cells was determined using para-nitroblue tetrazolium, as previously described by Kaufman, Capella, and Robbins.

Results

Characteristics of ALS. Using the above techniques a potent guinea pig anti-rabbit ALS was produced. Fig. 1 shows a typical cytotoxicity curve obtained with the ALS in the presence of rabbit C'. At a dilution of 1:1,024 the ALS remained potent and killed 50 per cent of the thymocytes. Identical results were obtained using guinea pig C'. A prozone effect is seen with the undiluted ALS, but the cytotoxic titer rises rapidly as the serum is diluted. This phenomenon was observed repeatedly. It is also evident that in this in vitro assay exogenous C' is necessary for cell death. Normal guinea pig serum did not kill significant numbers of rabbit thymocytes in the presence or absence of C'. Incubation of the cells in C' alone caused fewer than 10 per cent of the test cells to die.

Characteristics of ALG and modified ALG. The guinea pig anti-rabbit ALG obtained by ammonium sulfate fractionation had cytotoxic potency equal to the ALS when used at a concentration of 3.6 mg per milliliter of protein (Fig. 2).
addition, the prozone phenomenon observed with the undiluted ALS was lost when only the globulin portion of the serum was used. The ALG, like ALS, remained capable of killing 50 per cent of thymus cells at a dilution of 1:1,024. Normal guinea pig globulin lacked cytotoxicity.

Neither succinylated normal guinea pig globulin nor succinylated ALG (Fig. 2) was significantly lymphocytotoxic in the presence of C'. The apparent explanation for the loss of cytotoxicity by the succinylated ALG is that acylation disrupts the conformation of the antibody molecule to the extent that complement fixation is no longer possible.

However, evidence that the succinylated ALG did, in fact, bind to the thymus cells was obtained by performing an "indirect" lymphocytotoxicity test (Fig. 3). Thymus cells preincubated in succinylated ALG were protected from lysis by either ALS or ALG in the presence of C'. Succinylated normal guinea pig globulin failed to provide such protection.

**Antibody binding to corneal tissue.** Evidence for the binding of ALS to corneal cells was obtained by soaking 8 mm. corneal buttons in serum and then using the absorbed serum in standard lymphocytotoxicity tests. Progressive decreases in the potency of the ALS were produced by increasing the number of corneas used to absorb the serum. Fig. 4 is an example of one such experiment. Absorption with one or two corneas produced intermediate reductions in cell death. Similarly, preincubating the ALS with 10⁶ thymocytes markedly reduced the cytotoxic capabilities of ALS. To demonstrate that the binding of the ALS was not due to nonspecific absorption of antibody, similar experiments were carried out using corneas soaked in guinea pig anti-SRBC sera (Table I). No reductions in either serum hemagglutinin or hemolysin antibody titers were produced by contact with from one to three corneas.

**Effects of sera on corneal cell viability.**
Table II demonstrates that incubation of corneal endothelium in guinea pig anti-rabbit ALS, ALG, or succinylated ALG in the absence of C' is not injurious. The cells retain their intracellular enzymes at 2 hours postincubation in tissue culture media. In contrast, incubation of corneal
Fig. 4. Evidence that the heterologous ALS has specificity for antigens present on the rabbit cornea as well as the rabbit lymphocyte. Absorption of aliquots of ALS with three intact corneas reduces the potency of ALS in terms of its ability to kill lymphocytes.

### Table I. Lack of nonspecific absorption of anti-SRBC serum to cornea

<table>
<thead>
<tr>
<th>Serum tested</th>
<th>Hemagglutinin titer</th>
<th>Hemolysin titer</th>
</tr>
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<tbody>
<tr>
<td>NGPS*</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>Anti-SRBC</td>
<td>1:32</td>
<td>1:1,024</td>
</tr>
<tr>
<td>Abs. Anti-SRBC</td>
<td>1:16</td>
<td>1:1,024</td>
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</tbody>
</table>

*NGPS = normal guinea pig serum.
Anti-SRBC = guinea pig anti-sheep red blood cell serum.
Abs. Anti-SRBC = serum was preincubated for 30 minutes with rabbit corneas.

Discussion

The concept of using blocking antibody to protect tissue from graft rejection derives from a laboratory phenomenon known as immunologic enhancement of tumor growth or immunologic blockade of tissue rejection. In the early 1900's, Flexner and Jobling noted that rats injected with non-viable Jensen sarcoma cells several days before the injection of viable tumor cells developed larger tumors than unsensitized animals. Therefore, instead of the tumor regressing, the animals were usually overwhelmed by it. Several years later it was proved that immunologic enhancement was related to histocompatibility antigens and mediated by antibodies. Since the term immunologic enhancement has become associated with tumor immunology, the term immunologic blockade has generally been applied to the wider spectrum of this immunologic phenomenon, including its application to transplantation biology.

The use of homologous blocking antibody serum to produce prolonged survival of normal tissue grafts has been successful in the transplantation of diverse organs and tissues, including skin, kidney, endocrine organs, and lymphoid cells. Burde, Waltman, and Berrios reported that soaking donor corneal tissue in heterologous ALS prolonged graft survival; this may have been due to the protective effect of heterologous blocking antibody. The prolongation of graft life by blocking antibody...
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Fig. 5. Nitroblue tetrazolium staining of corneal endothelium. A. Complete destruction of cells incubated in ALS and C' as evidenced by loss of intracellular enzymes. B. Corneal endothelial cells soaked in succinylated ALG and C' remain viable and retain intracellular enzymes.

appears to be more successful when weaker histocompatibility differences are involved; it may, therefore, be of greater practical promise in corneal transplants than in the overwhelming rejection seen in mismatched skin or kidney transplants.

The mode of action of the ALS in lengthening graft survival time is not understood, but several hypotheses may be critically examined. It seems unlikely that the relatively small quantity of antibody present on an ALS-pretreated corneal graft could cause central suppression of the immune response. Furthermore, studies by Zimmerman and Feldman using rat skin allografts and enhancing antibodies strongly suggest that immunosuppression takes place at the graft site or in the peripheral draining lymph nodes.

Inhibition of graft rejection by ALS present on the graft could be caused by either of two mechanisms—the antibodies could act to inhibit sensitization by blocking directly, or the antibodies could bind to and mask graft antigens, preventing lymphocyte contact, recognition, and stimulation. Over a period of hours or days, the ALS-coated corneal cells may undergo antigenic modulation so that the graft becomes less immunogenic to the host.

The present studies were designed to evaluate the use of heterologous anti-lymphocyte serum, or its modified components, as a protective agent for corneal allografts. The selection of thymocytes as

Table II. Effect of incubation in sera on corneal endothelial viability

<table>
<thead>
<tr>
<th>Sera</th>
<th>C'</th>
<th>Per cent of viable endothelial cells</th>
</tr>
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<tbody>
<tr>
<td>ALS*</td>
<td>-</td>
<td>95+</td>
</tr>
<tr>
<td>ALG†</td>
<td>-</td>
<td>95+</td>
</tr>
<tr>
<td>S-ALG§</td>
<td>-</td>
<td>95+</td>
</tr>
<tr>
<td>ALS</td>
<td>+</td>
<td>&lt;10</td>
</tr>
<tr>
<td>ALG</td>
<td>+</td>
<td>&lt;10</td>
</tr>
<tr>
<td>S-ALG§</td>
<td>+</td>
<td>95+</td>
</tr>
</tbody>
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*Guinea pig anti-rabbit lymphocyte serum.
†Guinea pig anti-rabbit lymphocyte globulin.
§Succinylated anti-lymphocyte globulin.
antigen was based on their ready availability in relatively pure form and on the ease with which the immunizing dose could be quantified. Likewise, the ease of performing lymphocytotoxicity tests provided a well-controlled and quantifiable method for evaluating the potency of the blocking antibody.

In vivo ALS is thought to exert a suppressive effect on immunocompetent cells. This is the rationale for its systemic administration in graft recipients. However, it has been shown that antilymphocyte serum also possesses the ability to bind to nonlymphoid cells by attaching to antigen shared by lymphocytes and other cells. This is accomplished by antibodies directed against histocompatibility antigens and forms the basis for immunologic blockade. The present studies give evidence that anti-rabbit ALS can also bind to rabbit corneal cells, while anti-SRBC antibody is not absorbed nonspecifically.

Previous investigations have demonstrated that antibodies which mediate graft and tumor enhancement are of the 7S gamma-G class. It is also known that antibody fragments devoid of complement-binding sites are capable of prolonging the survival of primate renal allografts. The present studies give evidence of the binding of noncompement-binding antibody to thymus cells and the subsequent protection of cells from the lethal effects of ALS and complement. It is also demonstrated that soaking corneal buttons in ALS in the absence of complement is not injurious to corneal cells.

The exact mechanism by which blocking antibodies protect grafts and prolong their survival remains unclear. The preparations used in these in vitro studies are now being used to treat rabbit corneal allografts. It is believed that these studies will elucidate the mechanism by which blocking antibodies prevent corneal graft rejection.

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