Effect of Topically Applied Anti-CD4 Monoclonal Antibodies on Orthotopic Corneal Allografts in a Rat Model

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Purpose. Monoclonal antibodies (mAb) have generated interest as therapeutic agents. Limited data are available on the treatment of corneal graft rejection. The purpose of this study was to assess the use of topically applied mAb on experimental corneal grafts.

Methods. W 3/25, an IgG 1 mouse antirat mAb that recognizes a CD4+ cell subset, was used to treat Lewis recipient rats that received orthotopic corneal grafts of Wistar–Furth donors. Recipients were randomly assigned to receive topically applied drops of liposome-incorporated anti-CD4 mAb (LIP-anti-CD4 mAb), an equivalent amount of free anti-CD4 mAb, an isotype-matched control mAb encapsulated in liposomes (LIP-control mAb), or empty liposomes (emp-LIP) 5 times daily for 10 days. To investigate the immunologic effect of mAb treatment, flow cytometry of the targeted cells and cytotoxic activity of lymphocytes were analyzed.

Results. Application of LIP-anti-CD4 mAb was effective in reducing the rejection rate (P < .05) and in prolonging the mean survival time of corneal grafts that underwent rejection (P < .05). In contrast, no significant effect on graft outcome was observed after the application of control agents. Flow cytometry analysis did not reveal systemic depletion of the targeted lymphocyte subset in any anti-CD4 mAb treated animals. Rejected grafts elicited a cellular cytotoxic immune response in a cell-mediated lymphocytotoxic assay independent of the treatment given.

Conclusion. The results suggest that treatment with topically applied LIP-anti-CD4 mAb prolongs graft survival in orthotopic corneal grafts in a rat model. The beneficial effect of LIP-anti-CD4 mAb, probably due to enhanced intraocular delivery, was achieved by using relatively low doses of mAb. Invest Ophthalmol Vis Sci. 1996;35:52-61.

Allograft rejection remains the most frequent cause of corneal graft failure. Despite the routine use of topical immunosuppressive agents, 10% to 30% of corneal transplants undergo allograft rejection.1-3 The precise mechanisms by which corneal grafts are rejected remain a subject of investigation, but the central role of T lymphocytes is unquestioned. Advances in hybridoma technology have produced monoclonal antibodies (mAb) directed against T-cell surface molecules that allow regulation of the immune response.4 In animal models, the application of mAb not only provided insight on the role of T-cell subsets in immunity, it also offered promise as a therapeutic agent. Recently, anti-CD4 mAb generated interest as a potential immunosuppressant in organ transplantation. T cells expressing the CD4 marker play a key role in mediating allograft rejection. Anti-CD4 mAb markedly prolonged acceptance of skin, cardiac, and renal transplants in several species, including subhuman primates.5-8 Systemically applied anti-CD4 mAb significantly prolonged corneal graft survival in a mouse model.9 However, the systemic use of mAb may be complicated by side effects caused by general immunosuppression and adverse reactions to foreign protein.9,10 Therefore, topical application of mAb would
be desirable in a condition that is not life threatening, such as in corneal transplantation.

We previously demonstrated that mAb can be topically delivered to the eye when it is encapsulated in liposomes. In that study, liposome-incorporated mAb reached measurable levels in cornea, aqueous humor, and vitreous after topical application. In contrast, nonincorporated mAb applied topically was not detectable intraocularly. In this study, we investigated the role of anti-CD4 mAb in prolonging the survival of experimental corneal grafts. Free mAb and LIP-mAb was administered as eye drops in rat corneal transplants. The results suggest a role for anti-CD4 mAb in the prevention of experimental corneal graft rejection, providing further insight into the immunobiology of keratoplasty.

MATERIALS AND METHODS

Animals

In all experiments, inbred female rats (Harlan Sprague–Dawley, Indianapolis, IN), each weighing 200 to 250 g, were used. Lewis (LEW, RT 1') rats served as recipients of Wistar–Furth (W/F) grafts. These strains differ at the entire major histocompatibility complex (MHC) and are not congeneric. All animals were housed in wire-bottomed cages with controlled light–dark cycles, fed a standard laboratory diet, and given free access to tap water. Only the left eye of the recipient underwent corneal transplantation so that no animal was blinded. Animals were handled in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Animal Model

Orthotopic corneal transplantations were performed using a modified method of that reported by Williams and Coster. Donor animals were anesthetized and sacrificed by an overdose of inhalation anesthesia using methoxyflurane. Before surgery, recipient eyes were dilated by 1% atropine (Elkins–Sinn, Cherry Hill, NJ) and 1:1000 epinephrine (Parke–Davis, Morris Plains, NJ) was used to maintain maximal dilation of the iris.

Surgical Procedure

Anesthesia in recipient animals was induced by inhalation of methoxyflurane, followed by the injection of a combination of 100 mg/kg ketamine (Parke–Davis) and 33 mg/kg of promazine-hydrochloride (Schein, Port Washington, NY). In brief, donor eyes were harvested with forceps. A 3.5-mm-diameter trephine was applied to the central cornea and was twisted until the anterior chamber was entered. To complete the dissection, corneal scissors were used. Both donor corneal buttons were temporarily stored in RPMI 1640 (Gibco, Grand Island, NY) media. A 3.0-mm trephine was used to enter the anterior chamber of the recipient eye. On entering the anterior chamber, viscoelastic substance (Healon, Pharmacia, Uppsala, Sweden) was instilled to reform the anterior chamber and to avoid lens or iris damage. The donor button was secured with eight interrupted 11-0 monofilament nylon sutures (Alcon Surgical, Ft. Worth, TX). Before tying the last suture, the viscoelastic material was removed by irrigation with balanced salt solution (Alcon), and a small air bubble was placed in the anterior chamber to elevate the cornea away from the iris. Half the sutures were removed within the first week of surgery, whereas the remaining were removed the following week. Gentamicin ointment (Altana, Melville, NY) was applied immediately after grafting and weekly thereafter.

Monoclonal Antibodies

The murine mAb W3/25 is an IgG1 immunoglobulin directed against CD4+ cells. The mAb-producing hybridoma was grown in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics. When cell cultures reached confluence, they were resuspended in serum-free medium and incubated for 48 hours, at which time supernatants containing the mAb were collected. Protein concentrations were measured with the standard Bio-Rad ( Hercules, CA) assays. Supernatants were concentrated using ultrafiltration with YM 10 filters (Amicon, Danvers, MA), dialyzed against phosphate-buffered saline (PBS) using membranes of a molecular weight cut-off of 12 to 14 kd ( Spectrum, Los Angeles, CA), sterilized by passage through 0.22-μm filters (WVR Scientific, Cerritos, CA) and stored at −70°C until use. The concentration of the mAb was adjusted to 1.4 mg/ml and was diluted with normal sterile saline to a concentration of 0.6 mg/ml. This concentration corresponds to the concentration obtained in the LIP-mAb preparation.

Liposome Preparation

Encapsulation of mAb. Large unilamellar vesicles of uniform size were prepared by fast, controlled dialysis of mixed detergent–lipid micelles. Phosphatidylycho-

line and phosphatidylserine (Sigma, St. Louis, MO) in a 7:3 molar ratio were mixed together with n-octyl-β-D-glucopyranoside in methanol. Solvent was removed completely under reduced pressure. The dry lipid–detergent mixture was dissolved in PBS containing the mAb to yield a final lipid concentration of 17 mmol/l and a lipid detergent ratio of 0.2 mol/mol. The clear solution was dialyzed for 24 hours against a continuous flow of buffer and a dialysis membrane with a cutoff of 10,000 daltons. For dialysis, a commercially available dialysis device (Lipoprep, Diachema AG, Langnau, Switzerland) and a highly permeable dialysis membrane were used. Liposomes containing an isotype matched mouse-antirat mAb (LIP-control mAb) directed against IgG (Jackson Immuno Research, West Grove, PA) also were prepared by fast, controlled dialysis. This antibody does not react with CD4+ cells, and it was adjusted to a concentration of 0.6 mg/ml.

Removal of Nonincorporated mAb. Monoclonal antibodies not entrapped in vesicles were separated by ultracentrifugation. Liposomes containing mAb were pelleted by centrifugation (140,000g, 90 minutes, 25°C). The pelleted liposomes were washed twice with PBS, and the resultant pellet was resuspended in PBS to yield the final lipid concentration of 17 mmol/l.

Empty Liposomes. Liposomes containing the same constituents, except mAb, were prepared by fast, controlled dialysis.

Characterization of the Resultant Liposomes. Vesicles were uniform in size, with mean diameters of 145 nm for the LIP-mAb preparations and 130 nm for the emp-LIP preparation, as determined by laser autocorrelation spectrometry (Nanosizer, Coulter, UK), gel filtration (Sepharose CL-2B, Pharmacia/LKB, Bromma, Sweden), and freeze-fracture electronmicroscopy (Fig. 1). For freeze-fracture electronmicroscopy, liposome samples were placed between two copper plates, cryofixed, plunged into liquid ethane, etched under high vacuum, and vapor-coated with platinum. Even though the preparations contain identical phospholipids, the presence of mAb accounts for the larger size of these liposomes. Trapping efficiency was calculated as the ratio of the amount of mAb in liposomes to mAb in the supernatant after lysis of liposomes with Tween 80. The mAb concentration was determined by ELISA, and the protein level was determined by the method of Lowry. Trapping efficiency was determined to be 45%, resulting in a liposome solution containing 0.6 mg/ml LIP-mAb.

Treatment Protocol

One hundred eleven animals underwent orthotopic corneal transplantation. Grafted animals were divided into the following groups. Allogeneic grafts in 40 LEW rats that underwent with W/F transplant without postoperative treatment served as controls. Syngeneic grafts in 15 LEW recipients were used as controls for technical failure. Allogeneic grafts in 56 LEW rats that received W/F corneal grafts were randomly assigned to receive topically applied free mAb, LIP-anti-CD4 mAb, LIP-control mAb, or emp-LIP. Treatment was applied as 15-μl drops 5 times daily for 10 days. All treatments began on the day of surgery when corneal transplantation was complete. After drop application, animals were held for about 1 minute and wrapped in a towel to prevent ingestion of the agent and to minimize potential systemic absorption.

Graft Assessment

Grafts were examined under the operating microscope on days 2, 4, and 7 after transplantation, and then 3 times weekly for a minimum of 4 weeks. Transplants surviving longer than this were examined weekly. The methodology for clinical evaluation of corneal grafts in rats has been described previously. Briefly, grafts were scored on a scale of 0 to 4 for clarity, edema, and neovascularization as absent, minimal, moderate, or severe. If the parameters became moderate or severe after grafting beyond the first week, the transplant was recorded as rejected. Complications such as infection, loss of anterior chamber, or cataract formation were recorded. Animals with any of these complications were excluded from further analysis.

Histopathology

Representative recipient animals were sacrificed for histopathology. Enucleated globes were fixed with 4% buffered formalin and processed for paraffin embedding. Four-micron-thick sections were stained with hematoxylin and eosin.

Cytotoxicity Assay

The cytotoxic activity of lymphocytes from experimental animals was measured in a standard 51Cr release assay.
mAb in Corneal Transplantation

assay. Single-cell suspensions of target W/F lymphocytes were obtained from cervical lymph nodes. Lymph nodes were excised, minced in tissue culture media consisting of RPMI 1640 supplemented with 5% fetal calf serum (Gibco), gentamicin 0.05% (Elkins-Sinn), and 25 mM HEPES buffer (Sigma, St. Louis, MO), and were then passed through a tissue sieve. Target cells were suspended in culture medium at a concentration of 2 × 10⁶ and cultured at 37°C in a humidified atmosphere of 5% CO₂/95% air for 2 days in 75 cm² tissue flask (Costar, Cambridge, MA). These cells were stimulated on day 3 with 10 μg/ml concanavalin A (Sigma, St. Louis, MO). On day 3, concanavalin A-stimulated cells were harvested and centrifuged at 800g for 10 minutes. To the pellet, 100 μl of radioactive ⁵¹CrO₄⁻ was added. These cells were incubated for 1 hour, washed twice, and then adjusted to 10⁶ cells/ml. On the day of the assay, effector cells from LEW recipients were harvested like the target cells described above and were plated with 1 × 10⁴ target cells in triplicate at ratios of 25:1, 50:1, and 100:1, respectively. Spontaneous release was determined from ⁵¹Cr release in the absence of effector cells, whereas the maximum release was determined by adding 0.5% Tween 20 to three wells. The percent specific cytotoxicity at each dilution was calculated using the formula:

\[
\text{Flow Cytometric Evaluation} \quad \frac{\text{experimenatal release mean} - \text{spontaneous release mean}}{\text{maximal release mean} - \text{spontaneous release mean}} \times 100
\]

Flow Cytometric Evaluation

To detect a systemic effect of mAb treatment on W3/25 cells, flow cytometry was performed. Lymphocytes of three animals in each group treated with the previously mentioned regimens were analyzed. Single-cell suspensions were washed twice with PBS supplemented with 2% serum and 0.1% sodium azide and then incubated with anti-CD4 mouse antirat IgG (Zymed Laboratories, San Francisco, CA) for 15 minutes at room temperature protected from light. The cells were washed, resuspended in 1% paraformaldehyde, and analyzed using the fluorescence-activated cell sorter (Becton Dickinson, Mountain View, CA). Gates were set at 1% of total cells based on staining by secondary antibody alone.

Statistical Analysis

Graft survival times were compared using a Mantel-Haenszel survival analysis. Animals that died prematurely or developed one of the complications noted above were eliminated from the study on the day the complication was recorded. In addition, mean survival times for rat transplants that underwent allograft rejection were compared using a one-way analysis of variance with post hoc Student’s t-test. Statistical significance was accepted at P < .05. Statistical analysis of flow cytometry was performed using the Mann–Whitney rank sum test. P < .05 was considered statistically significant.

RESULTS

Of the 111 animals used, 15 were excluded because of technical failure, mature cataract, premature death, or infection (Table 1). Rats receiving syngeneic corneas retained clear grafts beyond 100 days after transplantation. After transplantation, grafts had minimal edema that cleared by day 7. At the end of the first week after transplantation, the corneas became slightly vascularized with vessel ingrowth directed toward the sutures. Vessels did not progress far beyond the stitches and regressed when sutures were removed.

Conal grafts in a W/F and LEW strain combination, without any immunosuppressive treatment, resulted in a rejection rate of 62.9%. No rejections were noted after 35 days. Using only those grafts that were rejected gave a mean survival time of 14 ± 4 days. Grafts that remained clear had a clinical course similar to transplants in the syngeneic combination. Initially, neovascularization developed, but this was related to sutures because regression occurred after suture removal. Conal graft rejection in allogeneic rats was observed as increasing transplant opacity accompanied by edema (Fig. 2). Severe neovascularization accompanied or followed opacification of the graft and often progressed to the center of the cornea.

When W/F corneas were grafted to LEW rats and were treated with emp-LIP given as frequent drops, the rejection rate was 63.6% with a survival time of 14 ± 5 days for grafts that were rejected. Grafts in this group of animals, used as controls for drop treatment, had a clinical course similar to grafts in untreated animals. When W/F corneas were grafted to LEW rats and were treated with topical application of anti-CD4 mAb drops, 58.3% of the grafts were rejected. The rejection rate did not differ significantly compared to animals treated with emp-LIP or to untreated animals. The mean survival time of grafts that were rejected was 16 ± 6 days, which was not significantly different from animals that received emp-LIP or untreated animals. When W/F corneas were grafted to LEW rats and were treated with LIP-control mAb, the rejection rate was 63.6% with a survival time of 13 ± 5 days for grafts that were rejected. Grafts in this group of animals, used as controls for a nonspecific LIP-mAb effect, had
TABLE 1. Number of Postoperative Complications in Experimental Corneal Grafts That Led to Exclusion From the Study

<table>
<thead>
<tr>
<th>Group</th>
<th>Anterior Chamber Loss</th>
<th>Cataract</th>
<th>Infection</th>
<th>Died</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allogeneic (35) untreated</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Emp-LIP (11)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Free anti-CD4 mab (12)</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LIP-control mab (11)</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>LIP-anti-CD4 mab (12)</td>
<td>1</td>
<td></td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

Emp-LIP = empty liposomes; Free anti-CD4 mab = nonincorporated anti-CD4 monoclonal antibody; LIP-control mab = liposome incorporated IgG monoclonal antibody; LIP-anti-CD4 mab = liposome incorporated anti-CD4 monoclonal antibody.

a clinical course similar to grafts in nontreated animals.

When W/F corneas were grafted to LEW rats and were treated with topically applied LIP-anti-CD4 mAb drops, 25% of the grafts were rejected (Fig. 3). The rejection rate was significantly lower compared to animals treated with emp-LIP, LIP-control mAb, or free mAb. The mean survival time of grafts that were rejected was 26 ± 7 days, which was significantly different from animals that received emp-LIP drops or free mAb. The incidence of graft rejection in the different experimental groups is summarized in Table 2.

Histopathology

When histologic sections of rejected allogenic grafts were examined, these grafts showed edema of the donor stroma infiltrated with neutrophils, macrophages, and lymphocytes (Fig. 4). The donor epithelium and endothelium were swollen, and in some sections endothelial cell loss was seen. There was neovascularization of the recipient corneal bed with vessels that extended into the donor stroma. Allografts that underwent rejection showed similar features that were independent of treatment.

When histologic sections of syngeneic grafts and nonrejected allogenic grafts were examined, they showed normal morphology with normal thickness and minimal stromal infiltrate consisting of neutrophils in some sections (Fig. 5). Epithelium and endothelium appeared normal. The wound margins were approximated without signs of inflammation and were minimally vascularized in some animals.

Cytotoxic T Lymphocytes Assay

To examine the immunosuppressive effect of the agents, grafted animals were examined for the presence of antigen-specific cytotoxic T lymphocytes (CTL). The results are summarized in Figure 6. They demonstrate that any animal that rejected the orthotopic corneal graft developed an antigen-specific CTL response. However, no significant difference in CTL activity was observed in animals that rejected

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**FIGURE 2.** Clinical appearance of rejected Wistar–Furth corneal graft in Lewis recipient rats 9 days after transplantation. Half the sutures are still in place. No treatment was applied after surgery. Original magnification, ×4.

**FIGURE 3.** Clinical appearance of nonrejected Wistar–Furth corneal graft in Lewis recipient 28 days after transplantation. Recipient received topical liposome-incorporated anti-CD4 monoclonal antibody 5 times daily for 10 days beginning on the day of transplantation. Graft remained clear during the entire observation period. Original magnification, ×4.
TABLE 2. Incidence of Graft Rejection and Mean Survival Time (±SD) in Orthotopic Corneal Transplants

<table>
<thead>
<tr>
<th>Group</th>
<th>(N)</th>
<th>Incidence of Rejection (%)</th>
<th>Mean Survival Time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Syngeneic</td>
<td>15</td>
<td>0</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2. Allogeneic untreated</td>
<td>35</td>
<td>62.9</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>3. Emp-LIP</td>
<td>11</td>
<td>63.6</td>
<td>14 ± 5</td>
</tr>
<tr>
<td>4. Free W3/25 mab</td>
<td>12</td>
<td>58.3</td>
<td>16 ± 6</td>
</tr>
<tr>
<td>5. LIP-control mab</td>
<td>11</td>
<td>63.6</td>
<td>12 ± 5</td>
</tr>
<tr>
<td>6. LIP-anti-CD4 mab</td>
<td>12</td>
<td>25.0</td>
<td>26 ± 7</td>
</tr>
</tbody>
</table>

Groups 1 and 2 did not receive any treatment postoperatively. Groups 3 to 6 received topically applied drop treatment five times daily for 10 days of the indicated agents. Emp = empty; LIP = liposome; mab = monoclonal antibody.

grants relative to the type of treatment. In contrast, animals in which syngeneic grafts were performed and animals that accepted grafts did not develop allospecific cell-mediated cytotoxicity even at high effector:target cell ratios.

Flow Cytometry Analysis
In untreated LEW rats, CD4+ cells constitute approximately 72% of total lymphocytes. There were no significant differences in CD4+ cells between untreated LEW rats and animals receiving topical treatment (Fig. 7).

DISCUSSION
Corneal grafts in experimental animals have been performed to characterize in more detail the immune response involved in graft rejection and to evaluate the effect of immunosuppressive agents. Orthotopic transplants in rabbits provided valuable information about the immune process in graft rejection. Khodadoust demonstrated that corneal endothelium could be destroyed by placing sensitized lymphocytes into the anterior chamber, indicating the prominent role of these cells in graft rejection. However, further characterization of the immune response in rabbits is limited by the lack of understanding of that animal’s immune system. The orthotopic corneal graft model in rats used in this study was introduced by Williams and Coster and overcomes these problems. The advantages include a well-characterized immune system in inbred animal strains and detailed knowledge about the MHC antigen system. In addition, mAb directed against specific cell surface markers allows study of the importance of cell subpopulations. By using this corneal graft model, the cell subsets infiltrating the rejecting corneal graft have been described. Using immunohistochemical staining, the presence of MHC class II+ cells and CD4+ cells have been reported. A different experimental approach to investigate cell-
mediated immunity in the rat cornea was used by Gebhardt. The injection of purified CD4+ cells, together with class II antigen-positive macrophages into allogeneic rat corneas, resulted in "reverse corneal allograft reaction." Injection of macrophages alone or in combination with CD8+ cells failed to produce significant corneal disease. It was concluded from these observations that CD4+ cells and macrophages are the major components that cause cytotoxicity to allogeneic corneal cells.

This report on the use of topically applied anti-CD4 mAb stresses the importance of CD4+ cells as potential targets for immunomodulation in corneal transplants. Thus, treatment with liposome-encapsulated anti-CD4 mAb significantly prolonged survival of orthotopic corneal transplants in rats compared to
control animals receiving empty liposomes and animals treated with free mAb. In addition, using specific mAb encapsulated in liposomes did not reduce the rejection rate or prolong graft survival. A specific effect of Fc-binding seems, therefore, to be unlikely.

The mechanism by which anti-CD4 mAb permits immunomodulation is not completely understood. Depletion of target cells has been suggested as a main effect of specific mAb treatment approaches. More recent investigations demonstrated that systemic target cell depletion does not seem to be an essential requirement of unresponsiveness. In addition, previous studies demonstrated that W3/25 did not significantly deplete CD4+ cells. It must be noted, however, that this mAb inhibited assays of T-cell activation in vitro and that, in autoimmune disease models, experimental allergic encephalomyelitis and experimental autoimmune uveitis W3/25 were effective at preventing the disease.

Because CD4 is an important component in T-cell activation, the effect of CD4 mAb may be attributed to interference with T-cell activation. No consensus has been reached on how this process takes place. Several possibilities have been proposed. The CD4 molecule may serve as an accessory molecule in which direct interaction with the constant region of MHC class II molecule increases adhesion between CD4+ cells and antigen-presenting cells. Consequently, anti-CD4 mAb may inhibit the rejection process by inhibiting cell adhesion with antigen-presenting cells. Alternatively, it has been suggested that anti-CD4 mAb may also transmit a negative signal to T cells. In addition, some macrophages express the CD4 molecule on their surfaces, and the treatment effect may, to some degree, be related to inhibition of host inflammatory macrophages. Another alternative, though rare in corneal grafts, donor passenger cells may have been affected by the LIP-anti-CD4 mAb treatment.

To study further the immunologic status of recipients, a standard CTL assay was performed. This assay assesses the ability of lymphocytes to respond to MHC disparities in vitro by generation of cytotoxic cells. Our observations are in line with previous reports that orthotopic corneal allografts undergoing rejection consistently generate antigen-specific cytotoxic lymphocytes. On the other hand, corneal grafts across a full MHC barrier that did not undergo rejection were unable to sensitize the recipient, as measured by the low levels of cytotoxic lymphocytes. Interestingly, in a previous study, anti-CD8 mAb eliminated in vitro CTL activity, but this treatment did not promote corneal allograft survival. It was suggested that either residual CTL activity remained in vivo or that the remaining CD4 cells mediated cytolysis. This may have occurred as direct cytolytic activity of CD4+ cells or as cell-activating, second-level effector cells (e.g., macrophages).

Also of note, a specific CTL response in our study was observed in animals that rejected their grafts regardless of what treatment was given. It has been suggested that when in vitro CTL activity was eliminated by anti-CD8 treatment, the remaining CD4+ cells were able to mediate cytotoxicity. Therefore, anti-CD4 treatment may not only interfere with the antigen recognition, it may also have an effect on cytotoxic CD4+ cells. Whatever the mechanism involved, it is clear from this and previous studies that interference with the CD4 molecule by anti-CD4 mAb provided an immunosuppressive effect in the experimental setting of corneal allografts.

As with other immunosuppressive agents used in keratoplasty, mAb can be applied systemically, locally, or topically. Systemic application of the chimeric IL-2PE 40 mAb significantly reduced corneal graft rejection in rat corneal allografts. Daily intraperitoneal injection of this mAb for 10 days prevented graft rejection in 50% of the transplants, whereas graft rejection occurred in all control animals within 2 weeks of surgery. Using the rat corneal graft model, intraperitoneal administration of anti-CD4 (W3/25) at a total dosage of 1 mg/kg was also effective and significantly prolonged graft survival.

Local application of antibodies in animal models of corneal transplantation was less effective. Subconjunctival injections of L11/135, a murine mAb that identifies primarily rabbit T cells in addition to neutrophils and macrophages, were used in a rabbit corneal graft model. No significant beneficial effect on graft survival was found after either subconjunctival injection or intravenous application of the mAb. The authors concluded that the poor results may have been due to a subtherapeutic dosage or lack of specificity of the mAb used. L11/135 was also used in a different approach by Williams. In a high-risk model of rabbit corneal grafts, mAb was injected directly into the anterior chamber when graft rejection occurred. Reversal of an ongoing immune reaction was observed in some of the animals. According to these studies, injections of mAb were well tolerated and intraocular access of mAb was suggested to be an important requirement for therapeutic efficacy.

Because in the present study equivalent dosages of mAb were used for animals that received either LIP-anti-CD4 mAb, control LIP-mAb, or free mAb, incorporation of anti-CD4 mAb in liposomes was probably responsible for the beneficial effect in the anti-CD4 mAb group. Liposomes, membrane-like vesicles consisting of one or more concentric bilayers alternating with aqueous compartments, have been studied as a modality to enhance ocular drug absorption.
are a promising drug delivery device because of their ability to protect the incorporated substance from metabolic enzymes in tears and corneal epithelium and to increase intracorneal drug delivery. Liposomes can be prepared easily from nontoxic materials, they lack antigenicity, they are not irritating to the ocular surface, and they do not obscure vision.\(^\text{37}\) We previously reported the improved intracorneal access of liposome-incorporated immunomodulatory agents, as well as its improved immunosuppressive effect in experimental corneal grafts.\(^\text{38-40}\)

In this study, therapy with an anti-CD4 mAb instituted in experimental corneal grafts was tested for its ability to prevent corneal graft rejection across the full MHC barrier. Although it is difficult to envision direct clinical applicability of our experimental approach, the observations stress the potential role of CD4+ cells as targets for immunomodulation. Liposome-encapsulated mAb directed against specific target cells warrants further attention as a potent immunomodulator.

**Key Words**
graft rejection, liposomes, monoclonal antibodies, penetrating keratoplasty, transplantation

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