Prevention of Corneal Allograft Rejection in Rats Treated With Subconjunctival Injections of Liposomes Containing Dichloromethylene Diphosphonate

Gerard Van Der Veen,* Lidy Broersma,* Christien D. Dijkstra,† Nico Van Rooijen,† Gabriel Van Rij,‡ and Ruth Van Der Gaag*  

Purpose. The drug dichloromethylene diphosphonate (CL2MDP) encapsulated in liposomes depletes macrophages but not other immunocompetent cells. The authors investigated whether subconjunctival injection of CL2MDP containing liposomes (CL2MDP-LIP) could prolong survival of corneal allografts in rats.

Methods. Male Fisher rats received orthotopic corneal grafts of Dark Agouti origin. Rats were treated postoperatively with subconjunctival injections of 0.1 ml CL2MDP-LIP at the time of transplantation and on days 2, 4, 6, and 8 after transplantation. Control groups received either liposomes containing phosphate-buffered saline subconjunctivally at the same time points or no additional treatment. Corneal grafts were evaluated every other day and were scored for neovascularization, opacity, and edema. Immunohistologic evaluation was performed 12 and 19 days after surgery.

Results. Corneal grafts in both control groups were rejected within 17 days. In the CL2MDP-LIP treated rats, grafts were not rejected during the maximum follow-up of 100 days. Cellular infiltration in these grafts was clearly reduced. There was also a strong reduction in neovascularization of the cornea.

cells. After phagocytosis, the phospholipid bilayers are disrupted and the drug is released, causing death of the macrophage due to its activity as a chelator of calcium, metal ions, or both. This treatment has proved to be effective in depleting macrophages from specific organs and tissues. The effect depends on the route of administration and the dose given. The influence of treatment with C12MDP-LIP on macrophage infiltration in corneal grafts has not yet been reported. We conducted experiments using subconjunctival injections of C12MDP-LIP to study the influence on corneal graft rejection and on macrophage infiltration in rats receiving an allogeneic corneal graft.

Our results clearly demonstrate that clinical signs of corneal graft rejection can be prevented and immunohistologic signs reduced by repeated subconjunctival injections of C12MDP-LIP.

**MATERIALS AND METHODS**

**Experimental Animals**

Inbred male Fisher (F344; RT1<sup>1+</sup>) and Dark Agouti (DA; RT1<sup>-</sup>) rats<sup>5</sup> were obtained from HSD/CBP (Harlan Sprague Dawley, Central Institute for the Breeding of Laboratory Animals, Zeist, The Netherlands), housed at our institute under standard conditions and given food and water ad libitum. In all experiments, F344 rats were used as recipients of DA corneal grafts. At the time of transplantation, F344 rats were between 12 and 18 weeks of age. Donor DA rats ranged in age from 10 to 20 weeks. Treatment of the animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Pretreatment of Recipient Eyes**

In this study, we used a model of corneal transplant rejection in rats that we previously developed.<sup>14</sup> Using a modification of the antigen-induced keratitis model<sup>15</sup> for the pretreatment of recipient eyes, we were able to obtain a high percentage of rejection within a circumscribed number of days. Heat-inactivated normal rabbit serum (5 to 15 μl/eye; Dr. Van Haeringen Laboratory, Wageningen, The Netherlands) was injected intracorneally in the recipient eye to induce keratitis. One week after injection, the corneal stroma became edematous and slightly vascularized. This reaction reached its maximum in the second week, after which the corneal edema gradually subsided and neovascularization was arrested. Four to five weeks after injection, the cornea was clear and blood vessels were no longer seen. At this time, an orthotopic corneal transplantation was performed. The acceptor cornea still contained macrophages and a few T lymphocytes. All recipient eyes were pretreated in this way.

**Corneal Transplantation**

The surgical technique used was a modification of the technique described by Williams and Coster.<sup>16</sup> Recipients were anesthetized with an intramuscular injection of a mixture of fluanison and phentanyl citrate (0.5 ml/kg; Hypnorm, Janssen Pharmaceutica, Tilburg, The Netherlands) and an intraperitoneal injection of 0.25 ml diazepam solution (5 mg/ml). Maximal dilatation of the iris was obtained by an intramuscular injection of 0.25 ml/kg atropine sulphate (0.5 mg/ml).

Before transplantation, a single drop of oxybuprocaine hydrochloride (0.2%) was administered to the eye. Donor rats were killed with an intracardiac injection of 0.1 ml sodium pentobarbital (200 mg/ml). A 3-mm trephine was used to score the central areas of donor and acceptor corneas, and the buttons were removed using curved corneal scissors. The donor button was then sewn into the recipient cornea using 8 to 10 stitches of a continuous 10/0 monofilament nylon suture placed intrstromally. The sutures were left in place for the duration of the experiment (day 42) but were subsequently removed in the remaining rats (days 60 and 100). Recipients were operated on one eye only. No attempt was made to reform the anterior chamber at the end of the procedure.

**Clinical Evaluation**

Grafted animals were examined under a slit lamp and operating microscope on the first postoperative day and then at least three times a week for a minimum of 3 weeks or until rejection occurred. Long-term surviving grafts and rejected grafts were examined at least once a week. Opacity of the graft, edema of the graft, and neovascularization of graft and recipient corneas were graded from 0 to 4 as described previously.<sup>5</sup>

Neovascularization was scored separately for every quadrant of the cornea, leading to a maximum possible score of 16 (Fig. 1). Corneal graft rejection was defined as a sudden increase in graft opacity.

**Liposome-Encapsulated Dichloromethylene Diphosphonate**

Multilamellar liposomes were prepared as described earlier.<sup>8</sup> Briefly, 75 mg phosphatidylcholine and 11 mg cholesterol (Sigma Chemical, St Louis, MO) were dissolved in chloroform in a round-bottomed flask. After low vacuum rotary evaporation at 37°C, a thin film was formed on the interior of the flask. This film was then dispersed by gentle rotation for 10 minutes in 10 ml phosphate-buffered saline (PBS [0.15 M NaCl/10 mm phosphate buffer, pH 7.4]) for the preparation of PBS-containing liposomes (PBS-LIP). For C12MDP-LIP, 2.5 g C12MDP (Clodronate; Boehringer
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FIGURE 1. Neovascularization of the cornea was scored as follows: 1 = blood vessels reaching the sutures; 2 = blood vessels reaching the corneal wound; 3 = vascularization of the peripheral graft; 4 = vascularization reaching the center of the graft.

Mannheim, Mannheim, Germany) was added to 10 ml PBS; more C12MDP could not be dissolved in the buffer. The liposomes were washed twice by centrifugation in PBS at 100,000g for 30 minutes to remove free C12MDP and then were resuspended in 4 ml PBS.

Experimental Design

Intracorneal injection of normal rabbit serum followed by corneal transplantation as described above was carried out in three groups of Fisher 344 rats. Grafts were monitored weekly for technical failures. From a total of 49 rats, six showed one of the following postoperative complications in the first postoperative week and were excluded from the study: wound dehiscence, hyphema, or synechiae between the iris and the graft. In one rat, a buphthalmus developed that became evident in the fourth postoperative week, and it was also excluded from the study. Successful grafts were treated as follows.

Untreated Group. This group received no additional postoperative treatment (n = 20). Nine rats were sacrificed for immunohistologic evaluation on postoperative days 12, 19, and 60.

PBS-LIP Treated Group. Repeated subconjunctival injections of 0.1 ml PBS-LIP were given at the time of transplantation and on days 2, 4, 6, and 8 (n = 10). Six rats in this group were scored to establish rejection time. Immunohistologic evaluation was performed on days 12 and 19.

C12MDP-LIP Treated Group. Repeated subconjunctival injections of 0.1 ml C12MDP containing liposomes were given at the same time points as above (n = 12). Animals in this group were sacrificed for immunohistology on postoperative days 12, 19, and 60. The mean corneal graft survival time was determined by clinical evaluation of the nine grafts with a minimum follow-up of 19 days.

Subconjunctival Injections

Subconjunctival injection of liposomes resulted in the formation of a bleb restricted to an area around the injection site. A more equal distribution of liposomes around the limbus could be obtained by injecting at three to five different places near the limbus. This method resulted in a circular subconjunctival bleb (Fig. 2). Because of leakage at the injection sites, a small amount of the liposome solution was lost each time. Apart from general anesthesia, a single drop of oxybuprocaine hydrochloride (0.2%) was administered to the eye before the subconjunctival injections.

FIGURE 2. Clinical appearance of a recipient eye 8 days after corneal transplantation in the untreated group (A) and in the C12MDP-LIP-treated group directly after the fifth subconjunctival injection of C12MDP-LIP. Note the moderate cloudiness of the graft. The injection sites are indicated by arrowheads (B).
FIGURE 3. Schematic representation of the anterior segment of the eye showing the different sites where cell numbers were counted: 1 = around the limbal blood vessels; 2 = the subconjunctival space; 3 = the iris; 4 = the peripheral part of the recipient cornea; 5 = the wound area in the recipient cornea; 6 = the wound area in the graft; 7 = the center of the graft.

**Immunohistologic Evaluation**

A minimum of three animals was killed for immunohistologic evaluation at various intervals after transplantation in each group. Eyes were removed and embedded in gelatin capsules containing OCT (Optimal Cutting Tissue compound 4585; Tissue-Tek; Miles, Elkhart, IN) and frozen in liquid nitrogen. Immunoperoxidase staining, as described by Verhagen et al., was carried out on serial cryostat sections, 8-μm thick, cut across the central area of the graft along the optical axis. Mouse anti-rat antibodies (supplied by CDD) were used as primary antibodies and consisted of ED1 reacting with a cytoplasmic antigen in macrophages, monocytes, and dendritic cells; OX4 reacting against rat Ia antigen; OX19 as a surface marker of T lymphocytes (both from Serotec, Oxford, UK); OX8 as a surface marker of rat T cytotoxic-suppressor cells and of a subset of natural killer cells (Sera-Lab, Sussex, UK). Peroxidase-labeled goat anti-mouse IgG antibody (Dakopatts, Glostrup, Denmark) was used as the second antibody. Antibodies were used in dilutions varying from 1:400 to 1:3000 in PBS containing 1% human serum albumin (HSA). Immunopositive cells infiltrating the tissues were quantified using an ocular graticule magnified ×400 (sample area per frame 0.0036 mm²) by an independent observer (LB) who was unaware of the treatment given. The number of positively stained cells was counted in seven different areas: the center of the graft, the wound area in the graft, the wound area in the recipient cornea, the limbal part of the recipient cornea, around the limbal blood vessels, subconjunctivally, and in the iris as shown schematically in Figure 3. The data were expressed as mean cell numbers per 0.01 mm².

**Statistical Analysis**

Graft survival times were compared with the nonparametric Mann-Whitney test. Corneal graft opacity scores and corneal neovascularization scores were compared between groups with the nonparametric Kruskall-Wallis test using the Bonferroni method to correct for multiple comparisons. Cell density scores between the three groups were compared using the Student-Newman-Keuls test and were thus corrected for cumulation of type 1 errors.

**RESULTS**

**Corneal Graft Survival**

All grafts scored to determine the rejection time in the untreated group (n = 20) and in the group treated with PBS-LIP (n = 6) were rejected within 17 days; rejections started at day 7. In the untreated group, the mean rejection time was 12.4 days (SD 2.6 days), and rats treated with PBS-LIP rejected their grafts in 12.1 days (SD 2.6 days). This difference was not significant. In the group treated with C12MDP-LIP, no rejections were seen. Of the nine grafts scored in this group, four had a follow-up of 19 days, three of 60 days, and two of 100 days (Fig. 4).

**Corneal Graft Opacity.** In the first week after transplantation, there was slight clouding of the grafts in the untreated group. In the second week, this progressed rapidly until day 17, after which the grafts cleared slightly. The grafts in the PBS-LIP treated group were, on average, more opaque than in the untreated group, especially in the first weeks after transplantation. The difference was not statistically significant. Opacity in the C12MDP-LIP treated group first increased until day 8 (the day of the last subconjunctival injections) and then decreased. These grafts were significantly more opacified on days 6 and 8 than were the untreated group, and they were significantly less opacified from day 14 on than were the untreated and the PBS-LIP treated groups (Figs. 5, 6).

**Corneal Graft Edema.** The score for corneal graft...
**FIGURE 4.** Survival of corneal grafts in the three experimental groups: the C12MDP-LIP treated group (—); the PBS-LIP treated group (-----), and the untreated group (······).

**FIGURE 5.** Corneal graft opacity scores compared between the three experimental groups: the C12MDP-LIP treated group (——); the PBS-LIP treated group (△△△), and the untreated group (○○○). Values are presented as mean ± SD in each group. Comparisons among the three groups were made with the Kruskall–Wallis test using the Bonferroni method to correct for multiple comparisons. *P < 0.01; †P < 0.001 (from postoperative day 16 on).
edema paralleled the score for corneal graft opacity, although differences between groups were less pronounced. The difference in corneal graft edema between the C12MDP-LIP group and the other groups became statistically significant from day 16 on (data not shown).

**Corneal Neovascularization.** Corneal neovascularization was reduced strongly by treatment with C12MDP-LIP. The difference in neovascularization score was significant between the C12MDP-LIP treated group and the other two groups from day 6 on. There was no difference in neovascularization between the untreated group and the PBS-LIP treated group (Fig. 7).

**Immunohistology**

Immunohistologic evaluation of corneal grafts of PBS-LIP-treated recipients at the mean rejection time (postoperative day 12) and 1 week later showed a cellular infiltrate and MHC class II expression in the center and in the wound area of the graft comparable to the untreated group and consistent with corneal graft rejection (Figs. 8, 9A, 9B).

There were, however, differences between these two groups in the number of infiltrating cells at other sites. Near the limbal blood vessels in the recipient cornea and in the iris, the number of macrophages, T cells, and Tc/s cells was lower in the PBS-LIP treated group than in the untreated group (Table 1, Fig. 9C). Expression of MHC class II antigens in the recipient cornea was also lower in the PBS-LIP treated group at these time points.

Subconjunctival injections of C12MDP-LIP resulted in a clear inhibitory effect on macrophage infiltration in the graft on postoperative days 12 and 19 compared to the untreated and the PBS-LIP-treated groups (Figs. 9A, 9B). There was hardly any difference in macrophage infiltration between the C12MDP-LIP treated group and the PBS-LIP-treated group at other sites (Table 1, Fig. 9C).

MHC class II expression in the C12MDP-LIP-treated group follows the same pattern as that for macrophage infiltration. It is comparable to the expression in the PBS-LIP-treated group in most tissues examined. A clear reduction in expression can be seen only within the graft (Figs. 9A, 9B). In the untreated group at postoperative day 19, the number of MHC class II-positive cells is lower than the number of macrophages (Figs 9B, 9C). This indicates that not all of the macrophages present express class II antigens.

Subconjunctival injections with C12MDP-LIP also influence infiltration of T lymphocytes. In contrast to macrophages, however, their presence in the limbal area is mainly restricted to the limbal blood vessels; few are found in the subconjunctival space (Table 1). In relation to the number of T lymphocytes in the untreated group, injections with C12MDP-LIP, as well as injections with PBS-LIP, result in diminished T cell infiltration on postoperative days 12 and 19 around the limbal blood vessels, in the iris, and in the wound area in the recipient cornea (Table 1, Fig. 9C). The number of T lymphocytes in the center of the graft is relatively small in all three groups (Fig. 9A).

The number of Tc/s cells at postoperative days 12 and 19 is clearly reduced as a result of treatment with C12MDP-LIP in the graft and in the wound area in the recipient cornea (Fig. 9A).

In the wound area of the acceptor cornea in the untreated group and in the center of the graft in the untreated and PBS-LIP-treated groups, there are more OX8+ cells than OX19+ cells, possibly indicating the presence of natural killer cells.

Sixty days after surgery, the number of T lymphocytes in the graft in the C12MDP-LIP-treated group was significantly higher than in the untreated group (Table 2). Because few Tc/s cells appeared to be pres-
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FIGURE 7. Comparison of corneal neovascularization scores between the three groups: the C12MDP-LIP-treated group (—■—); the PBS-LIP-treated group (—▲—); and the untreated group (—●—). Values are presented as mean ± SD in each group. Comparisons between the three groups were made with the Kruskall–Wallis test using the Bonferroni method to correct for multiple comparisons. *P < 0.01; †P < 0.001 (from postoperative day 8 on).

ent, these cells were probably mostly Th/i cells. Their presence did not result in an increase in infiltration of other cell types nor in the increased expression of MHC class II on corneal graft cells. There were also no clinical signs of corneal graft rejection. Their presence alone was obviously not enough to induce corneal graft rejection. These cells disappeared by the 100-day follow-up (data not shown).

DISCUSSION

Repeated subconjunctival injections of C12MDP-LIP clearly prevent clinical signs and reduce immunohistologic signs of corneal graft rejection. C12MDP-LIP selectively kills phagocytizing cells and does not affect proliferation and functions of T- and B-cell clones in vitro. Suppression of corneal graft rejection is, therefore, due to direct or indirect effects of local macrophage elimination.

Immunohistologic evaluation revealed a considerable reduction of the number of macrophages infiltrating the graft, acceptor cornea, and conjunctiva as a result of subconjunctival injections of C12MDP-LIP. Although injections of PBS-LIP also lead to reduced macrophage infiltration in most tissues examined, corneal graft rejection was not inhibited.

MHC class II expression by the ED1-positive cells was not consistent. In the untreated group, some macrophages were MHC class II negative. The presence of ED1-positive, MHC class II-negative cells has also been demonstrated in rat corneas at the site of immune precipitates. This variability in MHC class II expression may be a reflection of the presence of subtypes of macrophages, different stages of maturation, and age, or it may be due to changes in the microenvironment.

The number of T lymphocytes infiltrating the central part of the graft and the wound area in C12MDP-LIP-treated animals 12 and 19 days after surgery is much lower than that in untreated recipients, all of which rejected their corneal graft during this period. In the model of autoimmune encephalomyelitis in rats, a reduction of clinical manifestation due to intravenous injection of mannosylated C12MDP-LIP was not associated with a reduction in the number of T lymphocytes in the central nervous system compared to controls.

The presence of natural killer cells in rejecting corneal allografts is consistent with the findings in kidney transplants. Although their presence is associated with a high cytolytic natural killer activity in situ, depletion experiments have failed to demonstrate a prolongation of allograft survival. In corneal graft rejection, a possible role of natural killer cell activity directed against corneal endothelium has been suggested.
FIGURE 8. Comparison of corneal allografts stained for macrophages-monocytes and dendritic cells (MoAb ED1) 19 days after surgery. The C12MDP-LIP-treated group (A), the PBS-LIP-treated group (B), and the untreated group (C). There is heavy infiltration of ED1-positive cells and substantial edema in the untreated and the PBS-LIP-treated groups. The appearance of corneal grafts in the C12MDP-LIP-treated group is in accordance with that of syngeneic corneal grafts 19 days after surgery (data not shown). Examples of positively stained cells are indicated by arrows. Bar = 0.1 mm.

Another effect of subconjunctival injections of C12MDP-LIP is the reduction of angiogenesis of the acceptor cornea. In 1977 it was shown that macrophages could induce angiogenesis in vivo. Hunt et al showed that macrophages derived from wounds were angiogenic in the rat cornea. The central role played by macrophages in the induction of neovascularization has since been confirmed by many studies. Angiogenic activity is only found in activated macrophages. Because macrophages in wound areas can be expected to be active, macrophages in the wound area of a corneal graft will normally provide angiogenic stimulation. The inhibitory effect of treatment with C12MDP-LIP on corneal angiogenesis is already significant at postoperative day 6. Therefore, a more extensive analysis of the immunohistologic characteristics of C12MDP-LIP-treated corneal graft recipients in the first 2 weeks after transplantation is in progress, using immunoenzyme histochemistry and antibodies against macrophage differentiation antigens.

There may be several reasons for the inhibitory effect of C12MDP-LIP on corneal graft rejection. Macrophages can function as effector cells in graft rejection and/or can play an important role as antigen-presenting cells. They appear to be the most efficient cells in antigen processing when intracellular degradation of particulate antigens is required. It is not clear if corneal graft tolerance in our experiments is the result of a failure in the capacity of macrophages for processing and presentation of antigens to T lymphocytes and/or effector functions. In future experiments, the ability of C12MDP-LIP-treated corneal graft recipients to induce cytotoxic T lymphocytes will be tested.

A possible influence of C12MDP-LIP treatment on dendritic cells has not been evaluated. However, because these cells only have a limited phagocytic activity, they are not expected to take up any appreciable amount of liposomes and are probably not directly influenced by C12MDP-LIP.

The inhibition of blood vessel formation possibly also contributes to acceptance of the graft. In the absence of blood vessels, the movement of the various immune-competent cells in and out of the cornea is
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Figure 9. Comparison among the three experimental groups of the numbers of cells staining positively with the MoAb's EDI, OX4, OX19, or OX8 in the center of the graft (A), the wound area of the graft (B), and the wound area of the recipient cornea (C) at 12 (top) and 19 (bottom) days after surgery. Cellular infiltration in the graft in the C12MDP-LIP-treated group is somewhat higher than in syngeneic corneal grafts 12 and 19 days after surgery (manuscript in preparation). Values are presented as mean ± SD. Cell numbers between groups were compared using ANOVA followed by the Student-Neuman-Keuls test. *P < 0.05 compared with the C12MDP-LIP-treated group; †P < 0.05 compared with the PBS-LIP-treated group; ‡P < 0.05 compared with the untreated group.

Probably restricted. An interesting observation is that the two rats treated with C12MDP-LIP in which postoperative synechiae developed in the wound area (they were then excluded from the study) rejected their grafts in a normal time course (postoperative days 12 and 15). Although corneal angiogenesis in these rats was inhibited, blood vessels from the iris entered the graft and were clearly not affected by the treatment with C12MDP-LIP.

The importance of macrophages in corneal graft rejection has been suggested by several authors. Additionally, Gebhardt found in his model of the reverse corneal allograft reaction in the rat that injection of different cell types from an immunized rat into the corneal stroma of the immunizing strain only caused persistent central opaque zones when helper T cells were injected in combination with MHC class II-positive macrophages. Sensitized T cells alone or macrophages alone had no effect. Our results support these findings and indicate that macrophages are indeed essential for the pathogenesis of corneal graft rejection.

Since the development of the "macrophage suicide" technique, a growing number of reports have elaborated on the role of macrophages in the initiation of immune reactions in a variety of tissues and in the etiology of several diseases. In the lung and in the peritoneal cavity, macrophages were found to have a suppressive influence on the humoral immune response. The development of disease could successfully be prevented by macrophage depletion in experimental allergic encephalomyelitis. Elimination of

Table 1. Mean Cell Numbers in the Graft 60 Days After Corneal Transplantation in the C12MDP-LIP Treated Group and in the Untreated Group

<table>
<thead>
<tr>
<th>Graft: Wound Area</th>
<th>Graft: Center</th>
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</thead>
<tbody>
<tr>
<td><strong>C12MDP-LIP</strong></td>
<td></td>
</tr>
<tr>
<td>EDI</td>
<td>2.9 ± 1.6</td>
</tr>
<tr>
<td>OX4</td>
<td>0.2 ± 0.1*</td>
</tr>
<tr>
<td>OX19</td>
<td>1.7 ± 0.8</td>
</tr>
<tr>
<td>OX8</td>
<td>0.4 ± 0</td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
</tr>
<tr>
<td>EDI</td>
<td>1.7 ± 1</td>
</tr>
<tr>
<td>OX4</td>
<td>4.6 ± 1</td>
</tr>
<tr>
<td>OX19</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>OX8</td>
<td>0.9 ± 0.1</td>
</tr>
</tbody>
</table>

Values are cell number/0.01 mm² (mean ± SD). Cell numbers were compared between groups using ANOVA followed by the Student-Neuman-Keuls test.

* P < 0.05 compared with the untreated group.
TABLE 2. Influence of Subconjunctival Injection of C12MDP-LIP and PBS-LIP on Numbers of Immune Competent Cells 12 and 19 Days After Corneal Transplantation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>12 Days Postoperatively</th>
<th>19 Days Postoperatively</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EDI</td>
<td>OX4</td>
</tr>
<tr>
<td>Iris</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C12MDP-LIP</td>
<td>3.9 ± 0.8</td>
<td>7.2 ± 1.9</td>
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<tr>
<td>PBS-LIP</td>
<td>7.8 ± 0.8</td>
<td>15.2 ± 2.8</td>
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<tr>
<td>Untreated</td>
<td>30.2 ± 10.0*</td>
<td>63.0 ± 13.6</td>
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<td>Subconjunctival space</td>
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<tr>
<td>C12MDP-LIP</td>
<td>1.9 ± 1.1</td>
<td>4.7 ± 1.7</td>
</tr>
<tr>
<td>PBS-LIP</td>
<td>3.6 ± 1.4</td>
<td>5.3 ± 2.8</td>
</tr>
<tr>
<td>Untreated</td>
<td>6.1 ± 0.6</td>
<td>9.4 ± 2.5</td>
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<tr>
<td>Limbal blood vessels</td>
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<tr>
<td>C12MDP-LIP</td>
<td>4.2 ± 1.7</td>
<td>11.9 ± 0.6</td>
</tr>
<tr>
<td>PBS-LIP</td>
<td>10.8 ± 5.6</td>
<td>33.0 ± 13.0*</td>
</tr>
<tr>
<td>Untreated</td>
<td>31.9 ± 5.5*</td>
<td>48.5 ± 12.7*</td>
</tr>
<tr>
<td>Peripheral part of the recipient cornea</td>
<td></td>
<td></td>
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<tr>
<td>C12MDP-LIP</td>
<td>2.8 ± 0.6</td>
<td>5.5 ± 3.9</td>
</tr>
<tr>
<td>PBS-LIP</td>
<td>3.3 ± 1.4</td>
<td>11.6 ± 4.7</td>
</tr>
<tr>
<td>Untreated</td>
<td>11.4 ± 1.7</td>
<td>27.7 ± 7.2</td>
</tr>
</tbody>
</table>

Values are cell number/0.01 mm² (mean ± SD). Cell numbers were compared between groups using ANOVA followed by the Student-Neuman-Keuls test.
* P < 0.05 compared with the PBS-LIP treated group.
† P < 0.05 compared with the C12MDP-LIP treated group.

macrophages delayed the onset of clinical signs of experimental autoimmune uveitis. We now have shown that corneal graft rejection can also be prevented by repeated subconjunctival injections of C12MDP-LIP without, however, achieving total elimination of macrophages locally. Intracorneal injection of C12MDP-LIP does not seem to be an option. A preliminary experiment showed that injection of C12MDP-LIP into the intact corneal stroma resulted in clouding, the formation of bullae, and infiltration with inflammatory cells.

The treatment described in this report could be useful for future studies investigating the role of macrophages in the pathogenesis of corneal disease. It also holds promise for clinical applications in those patients in whom temporary inhibition of local macrophage functions may be beneficial.

**Key Words**

corneal transplantation, graft rejection, dichloromethylene diphosphonate, liposomes, macrophage

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**References.**


