Augmentation of Suppression in Cyclophosphamide-Treated Rats Bearing Allogeneic Skin Implants in the Anterior Chamber of the Eye

James D. Grogan, D. S. V. Subba Rao, and Susan E. Henry

This study shows that alloantigen presentation via the anterior chamber (AC) of the eye coupled with a single high dose cyclophosphamide (CP) (100 mg/kg) treatment effectively suppresses the skin graft rejection reaction of the recipient. Lewis (Le) rats bearing allogeneic Brown Norway (BN) skin implants in the AC of the eye demonstrate a modest increase in the survival time of orthotopic BN skin grafts. A slight prolongation of the survival of orthotopic BN skin grafts was also demonstrated in nonimplant or syngeneic implant-bearing Le recipients which received a single injection of a large dose of CP. Augmentation of suppression was evident in rats which were treated with a single dose of 75 mg/kg CP but not 25 mg/kg. The augmentation of suppression was evident when CP treatment and skin grafting of the recipient occurred on either 0, 7, or 14 days postimplantation. Recipient splenectomy did not interfere with the augmentation of suppression. Invest Ophthalmol Vis Sci 26: 1230-1235, 1985

Antigen pretreatment as an immunosuppressive regimen in transplantation has been studied for several years with varying success. However, recent reports that blood transfusions prior to renal transplantation increase the survival time of human kidney transplants have produced renewed interest in methods of eliciting active immunosuppression. Although the precise mechanism by which pretreatment of the recipient with blood or other antigens increases the efficacy of immunosuppressive drugs has not been identified, there is good evidence that such treatment is effective. Terasaki proposed recently that antigen pretreatment immunizes the recipient, resulting in a strong anamnestic response soon after transplantation. High doses of immunosuppressive drugs given during this period kill or inactivate clones of reactive cells, thus potentiating immunosuppression.

Whereas antigen presentation to the host for immunosuppression has generally been via the intravenous route, in the present study antigen presentation via the anterior chamber (AC) of the eye was employed. Studies from this laboratory and others indicate that antigen presentation via the AC of the eye can alter the host's immune response. Since the implant within the eye remains viable for several weeks, it presumably exerts a continuous antigen challenge to the host immune system. If the immunosuppressive drug cyclophosphamide (CP) were added to the treatment regimen, would immunosuppression be enhanced? This study extends a previous study from this laboratory and indicates that CP augments the survival of orthotopic allogeneic skin grafts in rats bearing allogeneic skin implants of donor origin in the AC of the eye.

Material and Methods

Experimental Animals

Inbred female rats weighing 150–200 g were purchased from a commercial supplier (M. A. Lab Animals; Walkersville, MD). Brown Norway (BN, RT1\textsuperscript{b}) and Fischer (Fi, RT1\textsuperscript{lv}) rats were used as donors and Lewis (Le, RT1\textsuperscript{a}) rats served as recipients. All rats were housed in plastic cages with continuous water and fed without restriction. Control animals and those used for implantation were the same age.

Skin Grafting Procedure

Orthotopic skin grafts were transplanted according to the standard procedure described by Billingham with modification reported by Grogan et al. A circular graft bed of 2 cm diameter was made on the upper back region of recipient rats, and to this a full-thickness graft from donor skin was fitted and held
in place with tissue adhesive (Eastman 910, Eastman Kodak; Rochester, NY). Le rats were doubly grafted in this manner with the skin of BN and Fi donors. The grafts were dressed with vaseline-impregnated gauze and the bandages were removed 7 days after grafting. Grafts were inspected visually every day and rejection was recorded when complete destruction of graft epithelium occurred. The mean survival times of skin grafts were compared using the Student t-test.

AC Implantation

Implantation was performed according to the procedure described by Medawar\(^\text{14}\) and Woodruff\(^\text{15}\) with modification by Raju et al.\(^\text{16}\) Tissue implants (0.5 mm\(^3\)) were prepared from the ear skin of BN rats. Ketalar\(^\text{®}\) (25 mg/rat, Parke Davis; Morris Plains, NJ) anesthesia was employed. The grafts were inserted into the AC of the eyes of Le rats through a 2.0–3.0 mm lateral incision made directly above the limbus.

CP Administration

CP (Cytoxan\(^\text{®}\), Mead Johnson; Evansville, IN) was injected intraperitoneally in doses of 25, 75, and 100 mg/kg average weight.

Use of Animals

The animal studies in this report conform to the ARVO Resolution on the Use of Animals in Research.

Statistics

The Student’s t-test for unpaired data was employed in this study to determine significance.

### Results

#### Survival Time of Orthotopic BN Skin Grafts on Le Rats Either Bearing BN Skin Implants in the AC or Treated with a Single Dose of 100 mg/kg CP

In an earlier study, we showed that the survival time of orthotopic BN skin grafts was prolonged in Le rats that harbored BN skin implants in the AC of the eye,\(^\text{17}\) suggesting that the presence of the implant actively immunosuppressed the host. In Table 1, the immunosuppressive effect of allogeneic skin implants in the AC is compared to that of a single dose of 100 mg/kg CP given intraperitoneally. When compared to nonimplanted, control rats significant skin graft prolongation was noted in the rats bearing the implants for 14–28 days (\(P < 0.001\)). Skin grafts performed on rats harboring the implant for 42 days were rejected in accelerated fashion. CP treatment alone produced modest immunosuppression at the three time intervals studied. Thus, the host’s ability to reject orthotopic skin grafts was modified to a similar extent by either a single treatment of CP (100 mg/kg) or a BN skin implant in the AC for 14–28 days.

#### Immunosuppression of Implant-bearing Recipients Treated With CP

Studies were initiated to determine whether greater immunosuppression could be obtained if the implant-bearing host were treated with CP. In Table 2 is shown the survival of orthotopic BN skin grafts on Le rats bearing BN skin implants in the AC of the
Table 2. Survival of Brown Norway (BN) skin grafts on cyclophosphamide-treated Lewis (Le) rats with and without BN anterior chamber implants

<table>
<thead>
<tr>
<th>Group</th>
<th>Day of treatment postimplantation</th>
<th>Mean survival time (days) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without implant</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>12.1 ± 1.3 (18)</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>11.2 ± 1.6 (23)</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>11.0 ± 1.6 (26)</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>12.1 ± 1.3 (18)</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>11.2 ± 1.6 (23)</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>11.0 ± 1.6 (26)</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>12.1 ± 1.3 (18)</td>
</tr>
<tr>
<td>8</td>
<td>14</td>
<td>11.0 ± 1.3 (26)</td>
</tr>
<tr>
<td>9</td>
<td>14</td>
<td>11.2 ± 1.6 (18)</td>
</tr>
<tr>
<td>10</td>
<td>28</td>
<td>11.0 ± 1.3 (26)</td>
</tr>
<tr>
<td>11</td>
<td>28</td>
<td>11.2 ± 1.6 (23)</td>
</tr>
</tbody>
</table>

NS: not significant.
* Cyclophosphamide 100 mg/kg ip.
† All implants performed on day 0.
‡ Number of animals.

Table 3. Relationship between the time of cyclophosphamide (CP) treatment and skin grafting and the survival of Brown Norway skin grafts on Lewis rats bearing skin implants in the anterior chamber of the eye

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Mean survival time ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No implant</td>
</tr>
<tr>
<td>I</td>
<td>CP* and skin grafting 0–14 days postimplantation</td>
<td>11.6 ± 1.6 (67)†</td>
</tr>
<tr>
<td>II</td>
<td>CP and skin grafting 28–42 days postimplantation</td>
<td>11.4 ± 1.3 (27)</td>
</tr>
<tr>
<td>III</td>
<td>CP given 7–14 days before implantation and skin grafting</td>
<td>11.8 ± 1.5 (41)</td>
</tr>
</tbody>
</table>

NS: not significant.
* 100 mg/kg.
† Number of rats skin grafted.
of immunosuppression was evident by the presence of the implant in the AC.

Specificity of the Suppressive Effect of CP-treated Le Hosts Bearing BN Skin Implants in the AC

To determine whether the suppression was antigen specific, third party skin grafts were placed on CP-treated Le rats bearing BN skin implants in the AC. As shown in Table 4, the survival of the third party orthotopic Fi skin grafts was not altered by the presence of the BN implant in the Le recipient’s eye. It appears that the host suppression allowing prolonged skin graft survival is antigen specific.

Suppression of Graft Rejection in CP-treated Recipients Bearing Syngeneic Skin Implants in the AC

To determine whether trauma from the implantation procedure was responsible for the increased survival time, BN skin grafts were placed on Le rats treated with CP, both unmodified CP-treated Le recipients and recipients bearing syngeneic skin implants in the AC. The results are shown in Table 5. It is clear that the survival time of BN skin grafts was not altered by the presence of syngeneic skin implants in the CP-treated Le rats.

Effect of CP Dosage on the Suppression of Implant-bearing Le Hosts

Table 6 shows the effect of varying the dose of CP on the survival of BN skin grafts on Le rats bearing skin implants in the AC of the eye. The skin graft survival time was modestly increased as the dose of CP was increased in the nonimplant-bearing recipients. In comparison, rats with an allogeneic skin implant in the AC exhibited a highly significant increase in the survival time of test BN skin grafts at both the 75 and 100 mg/kg dosages. Higher dosages (150–200 mg/kg) of CP were attempted but the drug toxicity precluded valid results. Reducing the dose to 25 mg/kg failed to augment suppression.

Effect of Splenectomy on the Suppression of CP-treated Implant-bearing Hosts

Since the spleen appears to be vital for host immune suppression after antigen presentation via the AC,18,19 studies were performed to determine whether splenectomy would affect the immunosuppression detected in implanted hosts treated with CP (Table 7).

The survival time of orthotopic BN skin grafts on either nonimplanted or implant-bearing Le hosts was not affected by splenectomy. However, the presence of an allogeneic BN implant in the AC of the CP-treated rats produced an increased survival time of the test skin grafts on the splenectomized as well as on the nonsplenectomized hosts.

Discussion

It has been known for some time that effective immunosuppression may be obtained by antigen pretreatment alone,9 but this modality is usually amplified by adjunctive treatment with immunosuppressive drugs.8,20–22 In the rat, the pregrafting antigenic stimulation has been provided by whole cells,3 tissue extract,9 and blood transfusions.8 Prolongation of mouse skin allografts20 and rat heart allografts8 has been reported after CP and donor antigen treatment. The results presented herein support our previous report11 and indicate that skin allografts can also be prolonged in CP-treated rats that received antigen via the AC of the eye. The survival of orthotopic BN skin grafts in Le recipients harboring BN skin implants in the AC of the eye was augmented by the administration of a single dose of either 75 or 100 mg/kg of CP. A reduced dose of 25 mg/kg did not increase the survival time presumably because suppressor T lymphocytes are preferentially affected at this dosage.23
Table 6. Comparison of different doses of cyclophosphamide on Brown Norway (BN) skin graft survival in Lewis rats bearing BN anterior chamber implants

<table>
<thead>
<tr>
<th>Implant</th>
<th>Day of treatment</th>
<th>Mean survival time (days) ± SD for various doses of cyclophosphamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>CP</td>
<td>25 mg/kg</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>10.2 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>10.6 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>NS</td>
</tr>
</tbody>
</table>

CP: cyclophosphamide; NS: not significant.

The enhanced immunosuppression was antigen specific inasmuch as third party Fi skin grafts placed on the side opposite the BN grafts on the Le recipients were not prolonged. Rats receiving 100 mg/kg demonstrated an increased survival of orthotopic skin grafts if the recipient rats were treated with CP and skin grafted on either the day of implantation or 7 to 14 days postimplantation. Rats implanted on day 0 but not treated with CP and skin grafted until 28 to 42 days postimplantation showed no prolongation of survival, nor did rats that received the CP treatment 7 to 14 days before implantation. These results support those of other investigators who found that enhanced suppression as measured by increased allograft survival times was achieved when CP was administered within a short time of antigen exposure.8,24,25

The suppression produced by tumor cells and skin implants placed into the AC of the eye is abolished by splenectomy.19 However, splenectomy did not abolish the augmented suppression noted in CP-treated implant-bearing rats. This would be expected if the enhanced suppression was evoked by the drug's elimination of antigen reactive cells stimulated in the host by antigenic presentation via the AC of the eye.3

Immunization via the AC of the eye induces antigen reactive cells in the recipient, but the end product of the immune response varies according to the antigen dose and form. For instance, some investigators have injected lymphoid or tumor cells into the AC, inducing a strong humoral antibody response by the host while cellular immunity was suppressed. This phenomenon is termed anterior chamber associated immune deviation (ACAID).25 However, solid skin implants do not induce this form of ACAID since very weak or no cytotoxic or hemagglutinating antibody responses occur in these recipients (manuscript in preparation). On the other hand, spleen cells from rats bearing skin implants in the AC demonstrate reduced activity in several in vivo and in vitro assays,10,28,29 indicating antigen recognition by the host which in turn results in down regulation of the immune response. Treatment of the recipients with low dose CP (20 mg/kg) reverses the inhibition noted in the MLR assay,23 suggesting that suppressor cells are induced in the recipient by the presence of implant within the AC. High dose CP treatment would, of course, eliminate the suppressor cells along with reducing other lymphoid cells within the host,30 therefore, the augmentation of suppression noted in this study in CP-treated recipients 0–14 days postimplantation may result from either partial clonal deletion of antigen reactive cells by CP5 or production of suppressor cells,23 or a combination of both.

CP given preimplantation resulted in no augmentation of suppression, particularly if the CP was given 14 days prior to implantation and skin grafting, because the recipient would have recovered immunologically by the time of transplantation.23 Thus, only the suppression created by the presence of the skin implant in the eye would be noted. Since suppressor cells have been reportedly induced by CP treatment,31 suppression exhibited in rats that received
CP near the time of implantation may be a result of suppressor cells induced by the implant in the AC of the eye of the recipient during recovery from the high CP dosage. Regardless of the mechanism, it is clear that a single properly spaced CP treatment can augment the survival of BN orthotopic skin grafts on Le recipients bearing donor skin implants in the AC of the eye.

**Key words:** skin implant, anterior chamber, cyclophosphamide, skin graft, splenectomy

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**References**