Promotion of Corneal Allograft Survival With Leflunomide

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Purpose. The efficacy of the antirejection drug leflunomide was evaluated in a rat model of penetrating keratoplasty.

Methods. Corneal grafts from inbred Lewis rats were transplanted orthotopically to inbred Wistar-Furth (WF) recipients. WF rats received either Leflunomide (HWA 486), the active metabolite of leflunomide (A77-1726A), or cyclosporin A, administered orally beginning 2 days before transplantation and continuing for 30 days thereafter. Graft survival was assessed clinically three times per week, and mean survival times were determined.

Results. Oral administration of either leflunomide or the salt of its active metabolite resulted in a significant prolongation of graft survival time. Moreover, almost one third of the grafts survived for an additional 3 weeks, even after drug treatment was discontinued.


In spite of the high success rate of keratoplasty, immunologic rejection remains the leading cause of corneal graft failure. Immunosuppressive drugs, such as corticosteroids and cyclosporin A (CsA), have been used effectively in reducing the risk of immunologic rejection. However, the prolonged use or high dose of either immunosuppressive agent can produce serious side effects, including glaucoma, cataract, hypertension, nephrotoxicity, and hepatotoxicity.1 Therefore, alternative immunosuppressive agents are needed for patients at high risk who are undergoing corneal transplantation.

Leflunomide, an isoxasol derivative, has been shown to be highly effective in preventing or ameliorating several autoimmune diseases in animal models.2,3 More important, this agent was as effective as cyclosporin A in preventing the rejection of skin and kidney allografts in rats.4 The present study evaluates the efficacy of leflunomide in preventing corneal allograft rejection.

MATERIALS AND METHODS. Rats. Female Lewis and Wistar–Furth inbred rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). These two strains differ at the entire major histocompatibility complex. All animals used were older than 8 weeks of age. In all experiments, latex bead-treated corneas from Wistar–Furth donors were grafted onto Lewis recipients (see below). Animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Grafting. Full-thickness 3-mm penetrating corneal grafts were performed as described.4 Donor corneas were pretreated with sterile latex beads to induce centripetal migration of peripheral Langerhans cells into the body of the cornea to be used for transplantation.4 Corneal graft rejection was assessed according to the severity of graft opacity, edema, and neovascularization.4,5 Clinical criteria were scored as minimal, moderate, or severe by two independent investigators in a masked fashion. If all three parameters became moderate or severe more than 7 days after grafting, the graft was recorded as rejected on that day.4,5

Administration of Immunosuppressive Agents. Leflunomide, HWA 486 (N-(4-trifluoromethylphenyl)-5-methylisoxazol-4-carboamide; Hoechst AG, Wiesbaden, Germany), and cyclosporin A (Sandimmune; Sanzo Pharmaceutical, East Hanover, NJ) were dissolved in olive oil and administered at a dosage of 10 mg/kg per day. The triethanolamine salt of the active metabolite of leflunomide, A77-1726A, (Hoechst AG) was prepared as an aqueous solution and administered at a dosage of 6.5 mg/kg per day. All three compounds, as well as an olive oil vehicle control, were administered daily by gavage 2 days before transplantation and for 30 days thereafter.

Statistical Analysis. Graft survival times were compared between two different ways. Mean survival time was calculated and compared using a one-way analysis of variance. A second method used a Mantel Haenszel survival analysis program. This analysis involved the calculation of chi-square values at each time point during the 60-day observation period. A probability (P) value less than 0.05 was considered significant.

RESULTS. The mean survival time for grafts transplanted to untreated rats was 11 days (Table 1). Vascularization of the graft bed up to the graft margin...
sig}naled the onset of graft rejection. Blood vessels became increasingly congested and eventually invaded the body of the graft. Histopathologically, the rejected corneal grafts contained a moderate to severe mixed inflammatory infiltrate comprised of mononuclear cells and occasional neutrophils.

Neither cyclosporin A (10 mg/kg per day) nor its olive oil carrier prolonged corneal allograft survival (mean survival time = 14.6 and 14.7 days respectively; \( P > 0.05 \)). The clinical and histopathologic features of these two experimental groups differed little from the untreated control group.

Orally administered leflunomide (10 mg/kg per day) produced an impressive prolongation of corneal allograft survival (Table 1). Of the 10 grafts treated with leflunomide, five demonstrated survival times that were at least twice those of the untreated controls. Moreover, three grafts survived at least 3 weeks after drug treatment was discontinued. Two grafts were terminal at days 60 and 61 with no signs of rejection.

Treatment with the salt of the active metabolite of leflunomide (A771726A; 6.5 mg/kg per day) produced a similar prolongation of corneal allograft survival (Table 1). Half the experimental grafts survived at least three weeks after cessation of drug treatment. As in the leflunomide-treated group, the mean survival time of this group was almost triple that of the untreated controls (Table 1).

**DISCUSSION.** The results reported here indicate that orally administered leflunomide or its active metabolite produced impressive prolongation of corneal allograft survival in a rat model of penetrating keratoplasty. Graft survival time was almost tripled in both treatment groups compared to untreated controls. Moreover, almost one third of the grafts in the leflunomide-treated group survived even after drug treatment was discontinued.

The inability of orally administered CsA to promote corneal allograft survival was surprising. Others have reported that topical application of CsA prevented corneal allograft rejection in the rat and rabbit. In the present study, we wanted to compare the efficacy of systemically administered leflunomide with a known immunosuppressive agent, CsA. Therefore, it was necessary to administer CsA in the same manner as leflunomide. The common protocol for use of CsA in preventing solid organ rejection uses a CsA dosage of 10 to 12.5 mg/kg per day during the first 6 months, with prednisone added as a second drug. Although the dosage used in the present study was within this range, it is possible that higher dosages of CsA (for example, 25 mg/kg per day) are needed to inhibit corneal allograft rejection. However, at higher doses, nephrotoxic and possible hepatotoxic effects are known to occur. It is conceivable that higher systemic doses of CsA would have produced similar prolongation of corneal allograft survival in this model, but at the risk of toxic side effects.

In vitro studies have demonstrated that leflunomide suppresses proliferation of human lymphocytes stimulated by one-way mixed lymphocyte reactions, anti-CD3 antibody plus phorbol myristate acetate, and anti-CD28 plus phorbol myristate acetate. Although leflunomide partially inhibits IL-2 production, its mode of action appears to be through inhibition of T cell responsiveness to IL-2 because addition of exogenous IL-2 fails to restore the proliferative responses of T cells treated with leflunomide. In contrast, the fungal metabolites, CsA and FK506, block T cell activation by preventing the production of IL-2 at the transcriptional level. Leflunomide is chemically unrelated to CSA and FK506, and it exerts its immunosuppressive effects by inhibiting T cell responsiveness to IL-2.

The present findings add to a growing body of evidence indicating the potential efficacy of leflunomide as an immunomodulating agent for use in autoimmune diseases and in organ transplantation. In animal studies, leflunomide has been shown to inhibit the autoimmune sequelae in experimental autoimmune encephalitis and experimental autoimmune uveitis. Systemic administration of leflunomide also prevents skin and kidney allograft rejection in rats. Moreover, leflunomide can reverse

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**TABLE 1. Effect of Leflunomide of Corneal Graft Survival**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MST (days)</th>
<th>( P^* )</th>
<th>Number of Grafts Surviving &gt;22 Days†</th>
<th>Number of Grafts Surviving After Drug Removal‡</th>
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<tr>
<td>None</td>
<td>11.9</td>
<td>—</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CsA§</td>
<td>14.6</td>
<td>0.06</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Olive oil</td>
<td>14.7</td>
<td>0.18</td>
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<td>1</td>
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<tr>
<td>Leflunomide</td>
<td>29.7</td>
<td>0.002</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Leflunomide (A771726A)§</td>
<td>32.4</td>
<td>0.005</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

* Probability (\( P \)) value determination by Mantel Haenszel survival analysis.
† Graft survival 22 days or longer represents a doubling of the mean survival time (MST).
‡ Grafts surviving at least three weeks after cessation of drug treatment.
§ Salt of the active metabolite of leflunomide.
ongoing rejection of heterotopic cardiac allografts in rats. In patients with rheumatoid arthritis, leflunomide produced dramatic clinical improvements without signs of toxicity, even at the highest dose levels. Thus, leflunomide holds considerable promise as an anti-rejection drug for use in patients in whom steroids and CsA are contraindicated.

**Key Words**
corneal graft, keratoplasty, leflunomide, HWA 486, rat

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**References**