Effects of the Immunosuppressant FK506 on a Penetrating Keratoplasty Rejection Model in the Rat

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Purpose. The immunosuppressive effects of FK506 on allogeneic corneal transplantation were tested in a rat model.

Methods. Inbred-strain Lewis rats were used as recipients, and Fisher rats were used as donors. Intraperitoneal injection of FK506 (0.3, 1.0, and 3.0 mg/kg per day) was administered for 2 weeks, and the grafts were inspected by clinical evaluation. Mixed lymphocyte culture assay, using lymphocytes from recipients of penetrating keratoplasty as responder cells and irradiated splenocytes from naive Fisher or Brown Norway as stimulator cells, was used to identify allogeneic stimulation. The rejection process was studied by histology and immunohistochemistry.

Results. The rat strain combination developed 100% graft rejection in about 2 weeks after the penetrating keratoplasty. FK506 prolonged the graft survival in a dose-dependent manner, as observed by clinical evaluation. In mixed lymphocyte culture assay, Lewis rats that had been primed to allogeneic stimulation at the time of cornea transplantation presented significant proliferation to Fisher stimulator splenocytes. FK506 suppressed this primed lymphocyte proliferation. Immunohistochemical and histologic studies confirmed the clinical evaluations. Untreated rat corneas, at the second postoperative week, presented a large number of helper/inducer T cells, macrophages, IL-2 receptor-expressing cells, and Ia-antigen-expressing cells. In the same period, FK506-treated rats appeared normal and had no cellular infiltration. Corneas rejected after FK506 cessation had less intense cell infiltration than the control corneas.

Conclusions. These data indicate that FK506 prolonged the corneal graft survival and can be a potentially useful drug in the immunotherapeutic arsenal to suppress corneal graft rejection.


Corneal graft rejection is a major problem in corneal transplantation. Patients with corneal neovascularization, a history of previous graft rejection, or both, have a greater than 50% rate of graft failure from rejection, despite conventional treatment. Therefore, more effective immunosuppressive drugs are needed for the future successful management of such high-risk corneal grafts.

FK506 is a macrolide isolated from the fermentation broth of a strain of soil fungus, Streptomyces tsukubaensis, found in the Tsukuba area in Japan. FK506 was the product of a deliberate drug discovery program to identify new fungal metabolites that would inhibit interleukin 2 (IL-2) production. In vitro studies have demonstrated that FK506 has a powerful immunosuppressive effect, with concentration levels 40- to 200-fold lower than CsA. Some authors have claimed that FK506 is a po-
tent drug for the prevention of allograft rejection. Kobayashi et al. have used FK506 to treat corneal graft rejection in a rabbit model. But the corneal rejection model used was not totally appropriate. Twenty-five percent of nontreated rabbits did not spontaneously reject the grafts. Also, rejection emerged at a disparate time because a well-defined inbred strain is not available for rabbit. In such a noncontrolled environment, the evaluation of an immunosuppressant is less than ideal because nonrejection can be the consequence of a model variation.

In the present study, we tested the effect of FK506 in an orthotopic-penetrating keratoplasty model in allogenic inbred rats. This model was reliably shown to have a 100% corneal rejection rate. The effect of FK506 was investigated by clinical evaluation, by mixed lymphocyte culture using lymphocytes from rats submitted to penetrating keratoplasty to identify allogeneic stimulation, and by immunohistochemical and histologic examination of the grafts.

MATERIALS AND METHODS

Animals

Inbred strains of Lewis rats (Rt1b) and Fisher rats (Rt1a) were purchased from Charles River Japan Co (Atsugi, Kanagawa, Japan); Brown Norway rats (Rt1a) were obtained from Seiwa Experimental (Fukuoka, Japan). The animals were male and weighed 200–250 g (8–12 weeks old). Lewis rats were used as recipients and Fisher rats were used as donors of the corneal grafts. Fisher and Lewis rats differ only in their medial and minor histocompatibility antigens. This strain combination produces only weak rejection of visceral organs. However, rejection in skin graft is as strong as when combinations of rats with major histocompatibility antigen discrepancies are used. Our experiments adhered to the ARVO guidelines on the use of animals in research.

Surgical Technique

The orthotopic penetrating keratoplasty technique described by Herbst et al. was employed in the present study. Mydriasis in both donor and recipient eyes was obtained with eye drops of atropine sulfate 1% (Nitten, Nagoya, Japan), tropicamide 0.5% (Santen Pharmaceutical, Osaka, Japan), phenylephrine hydrochloride 5% (Kowa, Nagoya, Japan), and indomethacin 1% (Senju Pharmaceutical, Osaka, Japan). They were administered 3 times at 10-minute intervals just before the operative procedure. The rats were anesthetized by intramuscular injection of ketamine hydrochloride (Parke-Davis, Tokyo, Japan) 250 mg/kg of body weight, and xylasine (Bayer Japan, Tokyo, Japan) 80 mg/kg of body weight. Full-thickness donor corneas from Fisher rats were excised eccentrically using a 3.0-mm trephine (Onishi type, Inami, Tokyo, Japan). Two grafts were obtained from one donor animal; they were kept in SM-2 corneal preservation medium (Senju Pharmaceutical, Osaka, Japan) until used for grafting. The grafts were transplanted onto 2.5-mm recipient beds of Lewis rats. Eight sutures were placed using a 10-0 monofilament nylon (CU-5 needle, Alcon, Fort Worth, TX). The anterior chamber was reformed by air at the end of the procedure. The whole procedure was done under strict asepsis. Blepharorraphy was performed with a 5-0 black silk suture (Keisei Medical, Tokyo, Japan) and left for 3 days. Animals were kept in a laminar flow cabinet. Postoperative care consisted of daily application of atropine 1% ointment (Nitten, Nagoya, Japan) and gentamicin eye drops (Schering, Kenilworth, NJ) once daily for 7 days, together with intramuscular gentamicin (Shering) 5 mg/kg per day for 4 days. The corneal sutures with 10-0 monofilament nylon were left in place to stimulate neovessel formation and to reproduce the clinical situation of a vascularized cornea.

Treatment Schedules

FK506 (a gift from Fujisawa Pharmaceutical, Osaka, Japan) was suspended in physiologic saline. The treated group of rats received FK506 intraperitoneally for 15 days, initiated on the day of surgery. Allogeneic grafts were treated with FK506 and divided in 3 groups with different doses: 0.3, 1.0, or 3.0 mg/kg of body weight per day, with 6 animals in each group. One rat from the group that received FK506 at 1.0 mg/kg per day was eliminated from the evaluation because of wound opening observed on the third postoperative day. Twelve rats with allogeneic grafts did not receive any treatment and formed the control group.

Clinical Evaluation

All rats underwent weekly clinical examinations for 8 weeks. Each animal was submitted to a masked examination under an operating microscope (model OME-G, Olympus, Tokyo, Japan). A scoring system was used to evaluate graft opacity, edema, and vascularization. Graft opacity was scored from 0 to 4: 0 = no opacity; 1 = faint opacity with iris details clearly visible; 2 = some details of the iris seen with difficulty caused by corneal opacity; 3 = extensive opacity, but pupil still could be seen; 4 = total opacification. Graft edema was scored from 0 to 2: 0 = absence of edema; 1 = edema existed but was mild; and 2 = very important edema when graft was clearly elevated, more easily observed at its edge. Graft neovessels were scored 0 if no vessel was present in the graft, and from 1 to 3 depending on whether vessels reached only to the periphery (grade 1), the intermediary zone (grade 2), or the central part.
of the graft (grade 3). After the first week, if the total score was higher than 3, grafts were considered to be rejected.

**Mixed Lymphocyte Culture**

**Preparation of cells.** Nonadherent lymph node cells obtained from corneal transplant recipient (Lewis) rats were used as responder cells. Irradiated spleen cells from naive Lewis, Fisher, or Brown Norway rats were used as stimulator cells. The responder cells and stimulator cells were prepared as follows. Spleen and cervical lymph nodes were excised and teased in RPMI 1640 medium (Nikken Bio-Medical Lab, Kyoto, Japan). Spleen cells were isolated from red blood cells by centrifugation with a Ficoll-Hyphaque (Pharmacia, Uppsala, Sweden) gradient and irradiated for 2500 R (MBR-1520 R-B, Hitachi, Tokyo, Japan). Responders cells were plated in a 100 × 15 mm petri dish (Fisher Scientific, Ottawa, Ontario, Canada) for 1 hour to allow monocytes to adhere to the dish surface. Thereafter, the nonadherent cells were harvested by gentle pipetting. Responder cells were prepared from 4 different groups—naive Lewis rats, rats submitted to syngeneic corneal transplantation (Lewis to Lewis), allogenic (Fisher to Lewis) corneal transplantation without treatment, and the allogenic corneal transplantation followed by FK506 (1 mg/kg per day) treatment for 15 days. Experiments were repeated 4 times. The animals submitted to penetrating keratoplasty were killed during the third postoperative week. Besides this experiment, other triplicated lymphocyte cultures from three rats treated with FK506 were submitted to concanavalin A 2 μg/ml to find out if proliferation capability was preserved.

**Culture condition.** RPMI 1640 medium was supplemented with glutamine (GIBCO, Grand Island, NY) (2 mM), penicillin (100 U/ml), and streptomycin (0.1 mg/ml) (GIBCO), 10% fetal bovine serum (GIBCO), and 5 × 10⁻⁵ M/ml 2-mercaptoethanol (Nakarai Chemicals, Kyoto, Japan). Responder and stimulator cell concentrations were adjusted to 5 × 10⁵ cells/well, and cells were incubated in flat bottom 96-well plates (Costar, Cambridge, MA), in a triplicate manner. The cultures were incubated for 4 days at 37 °C in a 5% CO₂ humidified air incubator. Pulsing with ³H-thymidine 0.5 μCi/20 μl/well (Amersham Japan, Tokyo, Japan) was carried out for the last 16 hours of the culture. Harvesting on a glass fiber filter followed, using a cell harvester (PHD, Cambridge Technology, Watertown, MA).

**Scintillation counter.** The filter pads were placed in vials with 3 ml toluene scintillator (Cambreria Packard, Zurich, Switzerland) and counted in a liquid scintillation counter (Beckman LS 6800, Rutherford, NJ). The mean of the triplicate cultures in counts per minute was calculated for each set of replicate cultures. The stimulation index was derived by dividing the mean for each allogeneic culture by the mean of syngeneic cultures.

**Immunohistochemistry and Histology**

Immunohistochemical and histologic studies of the grafts were performed for the control group and the FK506 1.0 mg/kg per day group. Grafts from 32 recipients were used.

**Periods of study.** After surgery, animals were killed and examined immunohistochemically at four different stages: prerejection stage, rejection onset stage, 1 week after rejection onset, and 2 weeks after rejection onset. Because the onset of the acute rejection in allograft usually occurs on postoperative days 12–14, the control rats were killed on day 10, day 14, day 21, or day 28 after surgery. For the treated groups, the prerejection time was chosen as day 14 after surgery because though grafts were still clear, control corneas were usually rejected. This clinical finding was well confirmed immunohistochemically. The treated rats were observed under an operating microscope every other day, starting from postoperative day 14, to determine the rejection time. The corneal parameters were based on clinical evaluation already described. Rejection was diagnosed when there was important deterioration in all three parameters. After the observation, rats were killed at the time of rejection onset, 1 week after rejection onset, or 2 weeks after rejection onset. The eyeballs were enucleated, embedded in OCT compound (Miles Lab, Naperville, IL), and snap-frozen by immersion in n-hexane previously cooled with dry ice-acetone. They were subsequently stored at −70°C until use. There were four grafts for each period of sacrifice and group of treatment.

**Immunohistochemistry.** The frozen tissues were cut at 4-μm thickness, fixed with 4% paraformaldehyde, and stained immunohistochemically by avidin-biotin-peroxidase complex method previously described. The primary monoclonal antibodies used consisted of OX6 reacting specifically against rat 1A antigen; W3/25 as a surface marker of rat T helper/inducer lymphocytes (Th/i cells); OX8 as surface marker of rat T suppressor/cytotoxic lymphocytes (Ts/cells, SeraLab, Sussex, England); OX42 as surface marker of rat macrophage (Serotec, Kidington Oxford, England); ART18 as interleukin-2 receptor (IL-2R) (provided by Dr. T. Diamantstein, Immunology Research Unit, Berlin, Germany). The secondary antibody (biotin-labeled horse anti-mouse IgG) and reagents for the third phase reaction (avidin-biotin-peroxidase complex) were purchased from Vector Laboratories (Burlingame, CA). For the staining substrate of peroxidase, 3,3′-diaminobenzidine was used. After the staining procedures were completed, the positive cells in the central part of the cornea were counted using a grid...
mounted in a contact lens in the microscope (0.015 mm²), at 100X magnification.

**Histology.** The same samples used for the immunohistochemical study were submitted to microscopic examination and stained with hematoxylin and eosin.

**Statistical Analysis**

Rejection onset time was compared between FK506-treated and untreated groups using Wilcoxon's signed rank test. The clinical scores between the treated and untreated groups for each period of evaluation were compared by Wilcoxon's rank sum test. The number of positively stained cells by immunohistochemistry for each first antibody was compared between control and FK506-treated groups using Wilcoxon's signed rank test. The stimulation index between different mixed lymphocyte culture conditions and the weight gain rates between the control and the FK506-treated groups were analyzed using the unpaired Student's t-test.

**RESULTS**

**Clinical Evaluation**

Figure 1 summarizes the effect of FK506 on the survival rates of allograft-penetrating keratoplasties compared to the untreated control group grafts. All untreated allografts were rejected within 3 weeks. The majority of the group (83%) was rejected within 2 weeks after surgery. In contrast, all FK506-treated groups showed high graft survival rates on postoperative day 14. At this time, all treated groups had significantly lower clinical scores than the control group (P < 0.05). In relation to the rejection onset time, the statistical analysis showed a significant difference between the control group and all FK506-treated groups: 0.3 mg/kg per day (P < 0.05), 1.0 mg/kg per day (P < 0.05), and 3.0 mg/kg per day (P < 0.01), respectively, on Wilcoxon's signed rank test.

† p < 0.05
‡ p < 0.01

(These p values were determined by Wilcoxon's rank sum test for comparison of clinical scores at each period of evaluation.)

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933174/) *Survival rates of grafted eyes from untreated allogeneic grafts (In1) and FK506-treated rats divided into three groups with different doses: 3.0 mg/kg per day group (In2), 1.0 mg/kg per day group (In3), and 0.3 mg/kg per day group (In4). Although all FK506 groups had high survival rates, 85% of control grafts were rejected the second week after transplantation. There was a significant difference in rejection onset time between the control group and all FK506-treated groups: 0.3 mg/kg per day (P < 0.05), 1.0 mg/kg per day (P < 0.05), and 3.0 mg/kg per day (P < 0.01), respectively, on Wilcoxon's signed rank test."

**FIGURE 2.** Clinical appearance of orthotopic corneal heterografts on postoperative day 14. (A) Untreated group graft with sign of rejection (18X). (B) FK506-treated group (1 mg/kg per day) graft with clear graft and no clinical signs of rejection (14X).
2B shows a clear graft of the same period from an FK506-treated rat (1 mg/kg per day); no sign of rejection is observed.

**Mixed Lymphocyte Culture**

Table 1 summarizes the results of the mixed lymphocyte cultures. Responder cells from naive Lewis rats did not proliferate when mixed with Fisher rat stimulator cells. However, proliferation occurred when responder cells from naive Lewis rats were challenged by Brown Norway stimulator cells, representing the primary mixed lymphocyte culture usually seen in combinations of completely disparate strains. The penetrating keratoplasty itself did not cause more proliferation because Lewis rats that received syngeneic grafts still had a low stimulation index. On the other hand, the untreated allograft (Fisher to Lewis) group had a high stimulation index compared to naive groups ($P < 0.05$) in which Fisher rat cells were used as stimulator cells. This proliferation was as high as observed for the Brown Norway stimulator cell. After challenge by Fisher stimulator cells, the Lewis lymphocyte proliferation represented the reaction of lymphocytes primed to allogenic stimulation at the time of cornea transplantation. This stimulation was suppressed in the FK506-treated group ($P < 0.05$). All four heterografts treated with FK506 (1 mg/kg per day), whose cells were used as responders at the mixed lymphocyte reaction (MLR), were not rejected by the time of clinical evaluation. Cultures with Brown Norway stimulator cells were also suppressed by FK506. FK506 suppressed only allogeneic stimulation because the addition of concavalin A to the culture provoked extensive proliferation; three experiments using lymphocytes from rats submitted to allogeneic cornea transplantation followed by FK506 (1 mg/kg per day) treatment for 15 days, stimulation index equal to 204, 245, and 251.

**Histology and Immunohistochemistry**

The allografts of the control rats at the time of rejection (postoperative day 14) had edema and a heavy infiltration of mononuclear cells (Fig. 3A). The edema was recognized by the augmentation in the thickness of the total cornea and vacuoles, especially at the basal cell layer of the epithelium. Mononuclear cell infiltration was observed in all layers of the cornea. Cells infiltrated the deep layer of the epithelium, significantly in the stroma, and around the endothelium. Some cells were attached to the surface of the corneal endothelium. Allografts from rats treated with FK506 (1 mg/kg per day) obtained on day 14 did not have such mononuclear cell infiltration (Fig. 3B). Some endothelial cells were lost, but that could result from surgical trauma.

Figures 4A and 4B show the dynamic change of different types of infiltrating cells in the rejecting corneas of untreated and FK506-treated groups. In the control group, OX42- and Ia-antigen-expressing cells

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**TABLE 1. Effect of PKP and FK506 Treatment on the Responsiveness of Mixed Lymphocyte Culture**

<table>
<thead>
<tr>
<th>Responder Cells*</th>
<th>Stimulator Cells</th>
<th>Fisher (Rat)</th>
<th>Brown Norway (Rat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penetrating Keratoplasty</td>
<td>FK506 (1 mg/kg per day)</td>
<td>1.0 ± 0.1†</td>
<td>3.8 ± 0.9</td>
</tr>
<tr>
<td>Lewis → Lewis</td>
<td>(−)</td>
<td>0.8 ± 0.2</td>
<td>5.6 ± 1.7</td>
</tr>
<tr>
<td>Fisher → Lewis</td>
<td>(−)</td>
<td>5.6 ± 1.5</td>
<td>5.6 ± 1.3</td>
</tr>
<tr>
<td>Fisher → Lewis</td>
<td>(+)</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.2</td>
</tr>
</tbody>
</table>

*Responder cells were nonadherent lymph node cells from naive or corneal transplant recipient Lewis rats. Stimulator cells were irradiated splenocytes.
†Stimulation index (mean ± SEM from four experiments) was derived by dividing each mean of allogeneic culture by the mean of syngeneic culture (Lewis as stimulator cells).
‡“Donor — > recipient” in the penetrating keratoplasty.
were in the grafts even before clinical rejection became evident. W3/25-, OX42-, Ia-antigen-, and IL-2R-bearing cells were maximal at the time of rejection.

Figure 5 shows some examples of immunohistochemical staining of grafts from control and FK506-treated rats during the second postoperative week. Fig. 5A exhibits marked staining for Ia antigen, a major histocompatibility complex class II antigen. Many round cells also stained for Ia antigen, possibly representing activated T cells and macrophages. Some elongated cells also stained for Ia antigen and may have been dendritic type cells or Ia-expressing keratocytes and neovascular endothelial cells. Figure 5B clearly shows cells that bear IL-2 receptors, confirming the acute immunologic activation of cells infiltrating the graft. Figures 5C and 5D show grafts from animals treated with FK506 (1 mg/kg per day) for 15 days. No cells bearing Ia antigen or interleukin-2 receptor were observed in these grafts.

The dynamic change of cell infiltration of Ts/c cells was different from other cells tested because their highest expressions were observed 1 week after the rejection began (Fig. 4A).

The effect of FK506 was also noticed in the rejected grafts of treated groups, which showed less infiltration of the immune cells (Fig. 5B) than the control grafts. The immunosuppressive effects of the drugs were most remarkable at the time when cell infiltration became highest in the control group. Comparing each marker tested, the FK506-treated graft had significantly lower infiltration for OX8-staining cells \((P < 0.05)\), OX42-staining cells \((P < 0.05)\), Ia-antigen-bearing cells \((P < 0.01)\), and IL-2 receptor-expressing cells \((P < 0.05)\).

**FK506 Toxicity**

Some rats had diarrhea during the first week of FK506 administration. Lower weight gains were detected in all FK506-treated groups than in the control group; however, the difference was significant only for the high-dose group (3 mg/kg per day) \((P < 0.01)\). During the second postoperative week, when the drug was still being administered, no diarrhea was observed, and the level of weight gain was the same as in the control group.

**DISCUSSION**

The leading cause of late corneal graft failure is allograft rejection. In a series of 600 transplants, Gibbs et al. reported an overall rejection rate of 44%, half of which were irreversible. The situation is even worse in patients with dense vascularization of recipient corneas or with a history of previous rejection. These patients are known to be at high risk for rejection that runs a more fulminant course, leading to graft failure in more than 50% of the cases. Hence, the development of more effective treatment for corneal rejection is needed. Recently, Hill reported the use of CsA in the management of high-risk keratoplasties and noted a significant improvement when compared with corticosteroid treatment alone. Nevertheless, there were still patients who experienced rejection, even with combined CsA and corticosteroid treatment. FK506...
FK506 on PKP in the Rat

FIGURE 5. Allograft stained by the avidin-biotin-peroxidase complex method during the second week after surgery. (A) Antibody OX6 was used for specific binding to the Ia antigen (MHC class II) in an untreated group graft. (B) Antibody ART18 was used for specific binding to the IL-2 receptor antigen in an untreated group graft. (C) FK506-treated grafts were stained by immunohistochemistry with antibody OX6. (D) A treated graft stained with antibody ART18 (295X).

has been shown to be a more potent immunosuppressant than is CsA, both in vitro\(^9\) and in a variety of experimental organ transplantation models.\(^{5,17,18}\) Fung et al\(^7\) have recently reported the use of FK506 as a “salvage” therapy for liver, kidney, and pancreas transplantation in patients who had intractable rejection while under treatment with CsA. Chronic rejection could be controlled in about half of these patients. To test the effect of FK506 on corneal graft rejection, we used a penetrating keratoplasty model in rats. The advantage of using rats is that we can obtain a reproducible corneal graft behavior by using inbred strains that have a constant histocompatibility discrepancy. Our previous study showed 100% corneal rejection in this rat model, with graft rejection occurring usually by the second postoperative week.\(^9\) FK506 was administered for 2 weeks at 3 different doses (0.3, 1.0, or 3.0 mg/kg per day). At the end of the second postoperative week (treatment period), a clear effect was noted. A high corneal survival rate was observed for all treated groups (67% for the lowest dose and even higher rates for others), whereas only 17% of grafts survived in the control animals (Fig. 1). After cessation of FK506 treatment, rejection invariably occurred at different intervals depending on the dose. The highest dose of FK506 (3 mg/kg per day) produced the longest graft survival, with one rat presenting a clear cornea at 7 weeks after transplantation. With lower doses
of FK506, earlier onset of graft rejection occurred. As shown in other rejection models, FK506 did not prevent rejection after the drug was stopped. In our previous study, rats treated with CsA also had high rates of corneal graft survival as long as the treatment was given, but rejection developed after interruption of drug administration. Comparing the survival rates of treated groups in the present study with those in the previous work, CsA at 10 mg/kg per day and FK506 at 1 mg/kg per day seemed to be equipotent.

A Fisher-to-Lewis strain of rats was chosen for our experiments so we could use the MLR to confirm the effect of FK506 in vitro. Differing only in the medial and minor histocompatibility antigens, this strain combination does not elicit a primary MLR, as seen in Table 1. On the other hand, Lewis lymphocytes primed to Fisher antigens after penetrating keratoplasty had significant proliferation when mixed with Fisher splenocytes. This stimulation resulted from allograft sensitization because proliferation did not occur in Lewis-to-Lewis syngeneic grafts. Lymphocytes from FK506-treated rats were not able to react to Fisher splenocytes. This immunosuppression caused by FK506 administration in vivo was not specific; it also inhibited the primary MLR with Brown Norway splenocytes, a third-part stimulator cell. The immunosuppressive effect of FK506 was still present at the time of the mixed lymphocyte test, even though it was performed 1 week after drug cessation. Because MLR is an in vitro analog system for the afferent arm of the immunologic process, the present data suggest that FK506 causes inhibition in the early phase of lymphocyte stimulation. It is also possible that proliferation of the responder cells from FK506-treated animals may start to take place in a later interval than that tested in the present study. If this happens, the result would be analogous to what we have observed clinically, which is that rejection onset was delayed by FK506 treatment.

Another advantage of the rat model is that it allows use of monoclonal antibodies for an immunohistochemical study of rejection. The present immunohistochemical evaluation of the grafts demonstrated a marked infiltration of T lymphocyte subsets, macrophages, and Ia-antigen and IL-2R-presenting cells at the time of the acute, clinically observed rejection. The following observations suggest that, in the present rat model, graft rejection is caused, at least in part, by a delayed type of hypersensitivity. IL-2 R-positive cells were scarce, but there was a high proportion of macrophages at the time of acute rejection. The high proportion of OX8-positive cells (Ts/c cells) at 1 week into rejection, when clinical rejection was quieting down, probably indicates downregulation of the immune reaction. The clear corneas of treated rats during the second postoperative week, in contrast to those in the control animals, presented normal features by microscopic examination of hematoxylin-eosin-stained sections and no infiltration by specific immune cells in the immunohistochemical study. The immunosuppressive effect of FK506 persisted even after cessation of drug administration because cell infiltration of rejected corneas was less intense than in the controls. This suggests that even though FK506 administration did not result in permanent survival of the graft, the rejection process was less destructive.

FK506 was the product of a drug discovery program to screen new fungal metabolites that would inhibit IL-2 production and, consequently, would limit the activation of T lymphocytes. Suzuki et al have demonstrated that FK506 could inhibit B-cell activation that is dependent on T-cell stimulation. Besides its effect on immunosuppression of cell-mediated phenomena, FK506 can inhibit corneal rejection if the humoral factor represents an important mechanism in the rejection process. The rat model of penetrating keratoplasty is not the best model to study the role of alloantibody in the rejection process of corneal transplantation. Attempts at allograft rejection of different organs in rodents by antibody-mediated mechanisms have proved unsuccessful possibly because the lytic capacity of the rodent complement is less than that of the human complement. Paradoxically, the administration of alloantibody to rodents at the time of other organ grafting often leads to improved graft survival, and it is generally accepted that antibody per se does not mediate allograft rejection in rodents.

Previous studies involving experimental transplantation have demonstrated that FK506 is a much more potent immunosuppressant than is CsA. Comparing the survival rate data of FK506 with our previous results using CsA shows that FK506 was 10 times more potent than CsA on a weight-to-weight basis, yet it did not seem to be more toxic. Diarrhea and weight loss developed in some FK506-treated rats (3 mg/kg per day) during the first week of drug administration, but normal weight gain returned during the second week of treatment. These findings are in accord with the toxicologic study in the rat performed by Nalesnik et al, which showed that weight loss was limited to the first week when a high-dose treatment regimen (4 mg/kg per day) was used; rats treated with FK506 at 2 mg/kg per day or less did not have any weight loss. Their findings also described histologic changes in the pancreas, corresponding to an increased pancreatic acinar cell turnover. Another 13-week toxicologic evaluation of living rats given oral FK506, performed by Ohara et al, revealed lens opacities, as seen by ophthalmoscopic examination, in 2 of 24 animals. Unfortunately, the authors did not mention if these opacities were present before treatment. Although the side effects seen in rats could be
considered "acceptable," there seems to be a considerable interspecies difference in toxicity.\textsuperscript{31} Assessment in humans is in progress. Some initial studies on human transplant patients who were treated with FK506 have shown that nephrotoxicity, neurotoxicity, and possibly diabetes are some of the side effects.\textsuperscript{7,8,35} The better therapeutic index found for FK506 is particularly desirable in treating corneal allograft rejection because this is not a life-threatening condition. It is hoped that FK506 can be administered by a topical route (eye drops or ointment) to avoid systemic toxicity. Topical use of FK506 is under investigation. However, like CsA, FK506 is liposoluble, and an appropriate vehicle has to be found. FK506 immunosuppression might be used both preventively, in high-risk patients, and for the treatment of acute corneal rejection. Reports in the literature that FK506 is effective in treating ongoing rejection\textsuperscript{17,20,34} could be significant because corneal rejection is the main cause of graft failure.\textsuperscript{4} The future use of FK506 might affect rejection prevention in high-risk patients and become an effective treatment for acute corneal rejection. Other ophthalmologic diseases that are potential candidates for FK506 treatment are those thought to be immune mediated, among them Mooren’s ulcer, pemphigoid, and Behçet’s disease. Until more information is available regarding its ocular side effects, such as cataracts,\textsuperscript{30} FK506 should be used cautiously.

In summary, our clinical, immunohistochemical, and immunologic data show that FK506 effectively prevented corneal graft rejection for as long as it was administered but did not sustain graft survival after cessation of treatment. The mixed lymphocyte test was in vitro evidence that the minor and medial histocompatibility complex mismatched rat combination did not induce allografts. FK506 in inhibiting the afferent arm of the rejection process. Immunohistochemical analysis confirmed the immunosuppressive effect of FK506 and added to our understanding of the physiopathology of corneal rejection.

**Key Words**
FK506, penetrating keratoplasty, rat, graft rejection, immunohistochemistry

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**References**


