Use of Topical FK506 in a Corneal Graft Rejection Model in Lewis Rats

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Purpose. To evaluate the immunosuppressive effect of topical FK506 on allograft corneal rejection in rats.

Methods. Lewis rats were used as recipients and Fisher rats as corneal graft donors. In Experiment 1, all rats received intraperitoneally FK506 (0.3 mg/kg per day) for 7 days to ensure equal baseline parameters. The rats then were assigned randomly to treatment with topical 0.3% FK506 or vehicle alone. In another set of experiments, rats were treated only with topical treatment. The grafts were inspected by clinical evaluation. Corneas obtained at the time of maximum rejection were used for histology and immunohistochemistry.

Results. The selected combination of rat strains caused 100% graft rejection in untreated animals within 2 weeks after the penetrating keratoplasty. In the treated animals, rejection was delayed until the end of topical therapy. One third of corneal grafts remained clear until day 30. Histologic and immunohistochemical studies confirmed the clinical evaluations. Untreated rat corneas had a large number of infiltrating helper-inducer T cells, macrophages, interleukin-2 receptor-expressing cells, and Ia-antigen-expressing cells. At the same timepoint, topically treated corneas showed a limited inflammatory response characterized by a 2/3 reduction in the number of infiltrating helper and cytotoxic cells, and a five-fold decrease in the expression of class I and class II major histocompatibility antigens.


FK506 is a neutral macrolide isolated from the fermentation broth of Streptomyces tukubaensis.4-6 It suppresses T-cell-mediated lymphokines,7 interleukin-2 receptor expression, and the generation of cytotoxic T cells.8-10 These immunomodulatory properties of FK506 are 10 to 100 times more potent than those of cyclosporine, which has been shown to prevent graft rejection when given continuously either topically or systemically.7,11-13

Previous studies of systemic FK506 in experimental animals have shown that it causes systemic adverse side effects, such as diarrhea, weight loss, and liver dysfunction.14,15 In a clinical trial in refractory uveitis, oral FK506 caused nephrotoxicity, neurotoxicity, and hyperglycemia.16 Topical use of FK506 might be desirable in avoiding its systemic side effects and managing the immune-mediated disorders in the ocular surface tissues, such as allograft corneal rejection. A major concern in the topical use of FK506 is whether it penetrates well into the eye. Our previous study, however,
showed that the topical FK506 sufficiently suppressed endotoxin-induced uveitis in rats, suggesting considerable penetration of the agent in the eye.\textsuperscript{15} In the current study, we investigated the immunosuppressive effects of topical FK506 preparation on an allogeneic corneal graft rejection model in inbred rats (Lewis and Fisher rats). The strains differed from each other only in minor histocompatibility antigens. In this model, corneal graft rejection occurs in 100% of untreated animals within 2 weeks after graft surgery,\textsuperscript{18,19} making it a good model for evaluating the immunosuppressive effect of a novel agent on corneal graft rejection.

\textbf{MATERIALS AND METHODS}

\textbf{Animals}

Two inbred rat strains, Lewis (RT1-1\textsuperscript{b}) and Fisher (RT1-1 \textit{vl}), differing only in minor histocompatibility antigens were used in this study.\textsuperscript{20} All animals were obtained from National Institutes of Health-approved sources and housed in environmentally controlled rooms with a 12-hour light and dark cycle. The study was approved by the Institutional Animal Care Committee and complies with the Public Health Service Policy on the Humane Care and Use of Laboratory Animals, and all procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All rats were females weighing between 200 and 230 g (10 to 12 weeks old). Fisher rats were used as corneal graft donors and Lewis rats were used as recipients.

\textbf{Surgery}

Before surgery, all animals were given the following drops to achieve maximum pupil dilation: atropine sulfate 1% (AKORN, Abita Springs, LA), tropicamide 1% (AKORN, Abita Springs, LA), phenylephrine hydrochloride 2.5% (Winthrop, New York, NY), cyclopentolate hydrochloride 2% (ALCON, Fort Worth, TX), and flurbiprofen sodium 0.03% (Allergan America, Hormigueros, Puerto Rico). Each drop was given three times at 5-minute intervals. Surgery was performed with the animals under general anesthesia after an intramuscular injection of ketamine hydrochloride (Fort Dodge Laboratory, Fort Dodge, LA) 250 mg/kg of body weight and xylazine (Mobay Corporation Animal Health Division, Shawnee, KS) 80 mg/kg of body weight. Full-thickness donor corneas were excised eccentrically using a 3.0-mm trephine (ALKON, Fort Worth, TX). Before excision, a 10–0 nylon suture was placed through the edges of the graft 180° apart, to ensure that the proper orientation of the epithelium and the endothelium could be maintained. Two grafts were obtained from each donor animal. They were kept in McCoy–Kauffman medium (Tissue Banks International, Baltimore, MD) until used. The grafts were sutured into a full-thickness 2.5-mm central corneal bed of one eye of recipient rats. Eight 10–0 monofilament nylon sutures (CU-5 needle; ALCON, Fort Worth, TX) were placed without burying the knots. These were left in place to stimulate neovascularization, because it mimics the high-risk situation in humans more closely. The anterior chamber was reformed with air. The entire surgical procedure was done with the animal under strict asepsis. At the end of the procedure, a tarsorrhaphy was performed with 4–0 silk suture (Ethicon, Somerville, NJ) and left in place for 3 days.

\textbf{Postoperative Treatment Schedule}

\textbf{Experiment 1.} Postoperative care consisted of daily applications of atropine sulfate 1% ointment (Pharmafair, Hauppage, NY) and gentamycin 0.3% ointment (Schering, Kenilworth, NJ) twice a day for 2 weeks starting on the third postoperative day. Immediately after surgery, the rats were given intramuscular injections of gentamycin (4 mg/kg per day) for 4 days and intraperitoneal injections of FK506 (0.3 mg/kg per day) for 7 days. Systemic FK506 was given for 1 week to ensure that all the grafts that were used in the study equally were successful. On the seventh day after surgery, all animals were examined with an operating microscope to exclude any animals in whom a surgical complication had occurred, such as anterior chamber hemorrhage, shallow anterior chamber, anterior synechia, or infection, thus ensuring that the animals in both experimental groups had the same baseline characteristics. The selected animals, 36 in all, were assigned randomly to 1 of 2 groups. The treated group received 0.3% FK506 eye drops, whereas the control group was given the vehicle alone (FK506 eye drops is isotonic ophthalmic suspension and pH 5.25). FK506 was provided by Fujisawa Pharmaceutical, Osaka, Japan. The eye drops were given every 4 hours, six times a day, for 2 weeks. Drops were applied using a micropipette with each drop containing 7 μl. All animals were given two drops at each timepoint. Immunohistopathologic changes were evaluated on the corneal grafts from these experiments.

\textbf{Experiment 2.} In another set of experiments, systemic FK506 was not given after the penetrating keratoplasty; 0.3% FK506 eye drops or vehicle alone were given for 2 weeks starting on the third postoperative day. This experiment was aimed at examining whether the systemic FK506 used in the experiment 1 to ensure the equal baseline had a significant effect on the graft survival rate in experiment 1. Clinical evaluation but no immunohistochemical evaluations were carried out.
Eyes from both control animals and treated animals were harvested at the same time. Six millimeter serial frozen sections of each frozen eye were prepared for immunohistologic study by the avidin–biotin–peroxidase complex method.\textsuperscript{5,22} Adjacent frozen tissue sections were stained with hematoxylin and eosin. The following primary mouse monoclonal antibodies were used: W3/25 for T helper–inducer cell (CD4), OX8 for T suppressor–cytotoxic cell (CD8), OX42 for macrophage (CD11c/CD18), OX33 for B cell (CD19), OX39 for interleukin-2 receptor (CD25), antiintercellular cell adhesion molecule-1 (ICAM-1) antibody (CD54), antilymphocyte function-related antigen-1 (LFA-1) antibody (CD11a/CD18), OX27 for major histocompatibility (MHC) class I (RT1-A), OX6 for MHC class II (RT1-B), and OX17 for MHC class II (RT1-D). All monoclonal antibodies were purchased from Accurate Chemical and Scientific (Westbury, NY), except for anti–ICAM-1 antibody and anti-LFA-1 antibody, which were provided by Dr. Miyasaka, Metropolitan Institute (Tokyo, Japan). Mouse ascites fluid containing 1 to 2 µg of nonspecific protein per milliliter were used as a control. The secondary antibody was biotin-conjugated goat antimouse immunoglobulin-G (American Qualtex, CA), which was depleted of cross-reacting antirat immunoglobulin-G.

**Evaluation of Immunopathology**

Using the staining protocol described above, positive cells were stained with a bluish–black color. To quantify the degree of graft infiltration by each cell type, the following grading scheme described previously was used.\textsuperscript{25} Disease intensity was scored on a scale of 0 to 4+ as follows: 0.5+ ≤ 10 cells per 40X high power field (HPF); 1+ 11 to 49 cells per 40X HPF; 2+ ≥ 50 to 100 cells per 40X HPF; 3+ 101 to 150 cells per 40X HPF; 4+ ≥ 151 cells per 40X HPF. To ensure consistency in having the slides read, cells were counted and recorded by a masked examiner from the same anatomic area in each section. Counting and grading the stained slides were limited to the central zone of each cornea to avoid the inflammatory response surrounding sutures and areas of poor staining.

**Statistical Analysis**

Clinical scores of the treated and untreated groups for each period of evaluation were compared using the Mann–Whitney test. The number of cells stained positively by immunohistochemistry for each first antibody was compared between the control and FK506-treated groups using the same nonparametric test.

**RESULTS**

**Clinical Evaluation**

Eighteen rats (8 rats in the control group and 10 rats in the treated group) in experiment 1, and 10 rats (4 rats in the control group and 6 rats in 0.3% FK506 group) in experiment 2 were used to evaluate the clinical course. All rats were examined every other day for 4 weeks (experiment 1) and up to 7 weeks (experiment 2) by at least two independent examiners using an operating microscope (OPM-1; Zeiss, Oberkochen, Germany). The clinical appearance of each graft was scored using the following three parameters: opacity, edema, and vessels. The exact scoring scheme used is listed in Table 1. To assess the effect of surgical trauma on the scoring system, orthotopic Lewis-to-Lewis corneal grafts also were performed on a group of seven animals (Figs. 1A, 1B, and 1C). The maximum score for each clinical parameter in the syngeneic grafts was as follows: opacity 1.3 ± 0.3, edema 0.8 ± 0.4, neovascularization 1.8 ± 0.2, and total score 3.7 ± 0.4. To take into account the possible effect of surgical trauma alone, a corneal graft was considered as being rejected only when the graft met at least one of the following criteria: an opacity score of 2.0 or more or a total score above 4.5 (2 standard deviation points above the maximum mean of syngeneic grafts).

**Immunohistochemistry and Histology**

The grafts were studied using standard histologic and immunohistochemical analysis (18 rats were used, 8 untreated and 10 treated). Animals were killed when rejection was well established in the control grafts. Eyes from both control animals and treated animals were harvested at the same time. Six millimeter serial frozen sections of each frozen eye were prepared for immunohistologic study by the avidin–biotin–peroxidase complex method.\textsuperscript{5,22} Adjacent frozen tissue sections were stained with hematoxylin and eosin. The following primary mouse monoclonal antibodies were used: W3/25 for T helper–inducer cell (CD4), OX8 for T suppressor–cytotoxic cell (CD8), OX42 for macrophage (CD11c/CD18), OX33 for B cell (CD19), OX39 for interleukin-2 receptor (CD25), antiintercellular cell adhesion molecule-1 (ICAM-1) antibody (CD54), antilymphocyte function-related antigen-1 (LFA-1) antibody (CD11a/CD18), OX27 for major histocompatibility (MHC) class I (RT1-A), OX6 for MHC class II (RT1-B), and OX17 for MHC class II (RT1-D). All monoclonal antibodies were purchased from Accurate Chemical and Scientific (Westbury, NY), except for anti–ICAM-1 antibody and anti-LFA-1 antibody, which were provided by Dr. Miyasaka, Metropolitan Institute (Tokyo, Japan). Mouse ascites fluid containing 1 to 2 µg of nonspecific protein per milliliter were used as a control. The secondary antibody was biotin-conjugated goat antimouse immunoglobulin-G (American Qualtex, CA), which was depleted of cross-reacting antirat immunoglobulin-G.

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**RESULTS**

**Clinical Evaluation**

Experiment 1. After the corneal graft, opacification began to appear in both the 0.3% FK506 and vehicle-
treated animals as early as 7 days after surgery (1 day after stopping systemic FK506) (Fig. 1A). In the vehicle-treated group, opacification peaked on day 15. This intense opacification lasted for another 7 days, after which the grafts began to clear with most grafts becoming clear by the fourth week after surgery. In the 0.3% FK506-treated group, the intensity of opacification was similar to the control group until day 10 (day 3 of topical FK506 treatment). Then, the level of opacity began to decrease when compared to that of vehicle-treated animals (Fig. 1A). All grafts remained clear while the animals were on topical 0.3% FK506, but 3 days after the eye drops were stopped, some grafts began to opacify. The temporal profile for graft edema nearly is identical to that of graft opacity (Fig. 1B). Both parameters showed a statistically significant difference between treated and untreated grafts on day 15 ($P < 0.001$). In both groups, neovascularization appeared at the edge of the graft around the seventh day, progressing until day 17 (Fig. 1C). There was no statistically significant difference between the two groups in relation to the severity of neovascularization.

When graft survival was examined, a significant difference was noted between treated and untreated grafts (Fig. 2). In control animals, graft rejection occurred around day 13 (6 days after stopping systemic FK506). The graft survival rate was 62.5% on day 12 and only 12.5% on day 13. All untreated grafts were rejected by day 14. In contrast, all grafts treated with 0.3% FK506 eye drops survived until day 22 and 30% of grafts still were clear 4 weeks after surgery. As Figure 3A shows, the graft taken from a vehicle-treated animal on day 14 after surgery shows a totally opacified cornea. Its clinical score was as follows: opacity 4+, edema 3+, neovascularization 3+. A cornea with these findings was considered totally rejected. Figure 3B
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FK506, and 33% of grafts still remained clear 7 weeks after surgery (Fig. 4).

**Histology and Immunohistochemistry**

Allografts from control rats taken at the point of maximum rejection (day 14) had edema and a heavy mononuclear cell infiltration (Fig. 5A). The edema was recognized by an augmentation in the thickness of the total cornea and vacuoles present at the basal epithelial layer. Mononuclear cell infiltration was present in all layers of the cornea, and neovascularization was present in the subepithelial layer as well as in the stroma (Fig. 5A). However, corneas taken on the same day from animals treated with topical 0.3% FK506 had normal corneal thickness, fewer infiltrating cells, and no neovascularization (Fig. 5B). Mononuclear cells mainly were located at the graft margin and in proximity to the sutures. No inflammatory cells were seen adjacent to the endothelium. The corneal epithelium in FK506-treated animals had a normal appearance, suggesting that the FK506 microspheres were not toxic to the corneal epithelium.

Results of the immunohistochemical studies are summarized in Figure 6. In both groups, the inflammatory cell population was composed of CD4+(W3/25), CD8+(OX8), and macrophages (OX42). However, there were significant differences in the number of cells observed. CD4+ and CD8+ cells were reduced by 68% and 67%, respectively, in treated animals. Fewer macrophages were seen in either group, but they also were reduced by 76% in the treated grafts. The B cells (OX33) did not contribute much to the inflammatory process, and no statistical difference was
FIGURE 4. Survival plot of FK506-treated versus vehicle-treated corneal grafts (experiment 2). Topical FK506 prolonged the corneal graft survival rate in a dose dependent manner. During the treatment, all the grafts treated with topical 0.3% FK506 survived and 33% of grafts remained clear until 7 weeks after surgery, while all the grafts treated with vehicle were rejected by 13 days after surgery. There were significant differences in the survival rate between FK506 (0.3%) treated and vehicle treated groups on day 11 after surgery († P < 0.02).

FIGURE 5. Histologic picture of FK506-treated and vehicle-treated corneal grafts on day 14 after surgery (hematoxylin and eosin staining). There was marked corneal thickening, inflammatory cell infiltration, and neovascularization in the cornea treated with vehicle alone (A, x100). In contrast, the 0.3% FK506-treated corneal graft shows normal corneal thickness and almost complete absence of inflammatory cells (B, x200).

DISCUSSION
FK506 has been shown to be a potent immunosuppressive agent in several models of organ transplantation and in clinical practice. Kobayashi et al first reported the usefulness of FK506 in preventing corneal graft rejection using a rabbit model. Their treatment protocol involved the use of subconjunctival injections of FK506, which was given 0.1 mg/kg twice a week for 100 days and 0.01 mg/kg once a week for 100 days. However, as a model for graft rejection, the rabbit is not the best choice because histocompatibility differences between donors and recipients cannot be controlled. Nishi et al showed in an orthotopic graft model in rats that intraperitoneal injection of FK506 could prevent graft rejection in a dose-dependent manner. In the current study, we used the same inbred strains of rats to examine the immunosuppressive effect of topically applied FK506. Fisher and Lewis rats have identical major histocompatibility antigens and differ only in the minor histocompatibility antigens that they express. Nevertheless, all corneal grafts are

noted between treated and control animals. In addition to reducing the number of infiltrating cells, topical FK506 had a dramatic effect on the expression of inflammatory markers. Expression of CD25 (interleukin-2 receptor) was reduced by 59% in treated animals (P < 0.007). Expression of MHC class I and class II antigens also was affected. In the control group, MHC class I antigens heavily were expressed in all layers of the cornea. MHC class II antigens were expressed by both corneal-resident cells and infiltrating cells. In treated animals, the expression of both markers was reduced by 75% or more. FK506 also affected the expression of intercellular adhesion molecule-1 (CD54) and its receptor LFA-1 (CD11a/18). In the treated group, there was a decreased expression of both CD54 and CD11c/18 (71% reduction and 63% reduction, respectively).
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FIGURE 6. Immunohistochemical score of placebo-treated and 0.3% FK506-treated corneal grafts. There were significant differences between the vehicle-treated (closed circle) and 0.3% FK506-treated (open circle) groups in the expression of T-cell markers and major histocompatibility class II antigens. The expression of cell adhesion molecules was reduced strongly in the 0.3% FK506-treated group compared to that of the vehicle-treated group († P < 0.007; †† P < 0.003, — median value).

rejected in approximately 2 weeks if left untreated.18,19 All our animals in experiment 1 received systemic intraperitoneal FK506 injections (0.3 mg/kg per day) for 1 week after surgery to ensure equal baseline parameters. Both the rejection time and clinical course of control animals were consistent with those of previous reports.18,19 To exclude the possibility that a short course of systemic FK506 might influence significantly the outcome of the graft survival rate, another set of experiments (experiment 2) was carried out by treating rats only with topical FK506 eye drops.

In experiment 1, all corneas in the control group were rejected by day 13. In contrast, the grafts treated with topical 0.3% FK506 survived until day 22, with 30% of the grafts still remaining clear 4 weeks after surgery. In experiment 2, topical FK506 prolonged graft survival until day 19, with 35% of grafts remaining clear 7 weeks after surgery. Thus, the graft survival rate in 0.3% FK506 eye drops groups in the two experiments were almost identical. Our results with topical treatment with 0.3% FK506 eye drops are comparable to those obtained with 1.0 mg/kg per day of systemic FK506 given intraperitoneally for 2 weeks after surgery.14 At this dosage, 40% of grafts also were clear at 4 weeks, although some of the animals experienced diarrhea and lowered weight gain when compared to control subjects. Such side effects were not noted in our animals. These results also compare favorably with topical application FK506. In a rat transplant model, Williams et al13 were not able to show any effect on graft rejection when using a 1% cyclosporine solution in chromophore EL-ethanol. In contrast, Foets et al25 were able to prolong allogenic corneal grafts in rabbits by using a 1% cyclosporine preparation in oil. However, the effect might have been mediated by systemic absorption of the drug, which was quite elevated in some cases. Systemic FK506 levels taken on day 20 after grafting in the topically treated animals were 0.8 ng/ml ± 0.3. This low systemic level indicates that systemic absorption also was occurring in this case. However, this blood level was not associated with adequate immunosuppression in either a renal transplantation model or in experimental uveitis.25,27

The immunosuppressive capability of topical FK506 further was confirmed by histology and immunohistochemistry. Comparing the control group to the topical FK506 treatment, there was a significant reduction in the levels of inflammatory cell infiltration of the CD4+ and CD8+ T-cell subsets. The number of infiltrating macrophages (CD11c/CD18+) also was decreased in treated grafts, but as noted in Figure 6, these cells were not present in high numbers in either group. A significant reduction in the expression of CD25+ (IL-2-R), a marker of T-cell activation, also was noted. Thus, there are fewer immunocompetent cells present within the treated grafts, and these cells are not activated to the same degree as those in the non-treated grafts. These results are similar to findings in experimental autoimmune uveitis where systemic FK506 was shown to be an effective immunosuppressive agent.14,27,28 FK506 significantly suppressed the recruitment of inflammatory cells such as CD4+ and CD8+ T-cell subsets, which are major mediators of inflammation in this model.14,29,30

For activation of T cells to occur, processed antigen must be presented by competent antigen-presenting cells expressing an appropriate MHC antigen on its cell surface.31,32 Helper T cells (CD4+) require the presence of MHC class II for activation, whereas cytotoxic T cells (CD8+) require MHC class I antigen. Several studies have looked at the pattern of MHC
class I and II antigens expression in corneas undergoing rejection.33-38 Both MHC class I and II antigens were detected on corneal endothelial cells, stromal cells, and basal epithelial cells in rejected allografts. FK506 appears able to reduce the expression of MHC class I and II antigens on corneal resident cells as well as on the infiltrating immunocompetent cells. Reduced MHC antigen expression probably is mediated through the action of FK506 on the production and secretion of lymphokines by T cells. It has been shown to suppress within T cells the production of mRNA for interleukin-2, interleukin-3, interleukin-4, tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ).39-42 This latter cytokine induces the expression of class II antigens on the cell surface, including corneal endothelial and stromal cells.40-45 FK506 also suppressed the expression of ICAM-1 and LFA-1. Both antigens are involved in the margination of cells into the area of inflammation. Expression of these two cell surface antigens can be induced by a variety of inflammatory mediators. Lipopolysaccharide, IFN-γ, interleukin-1, and TNF-α can all cause a strong induction of ICAM-1 in a variety of tissues and thereby greatly increase binding of lymphocytes and monocytes through their cell surface ligand LFA-1 first to vascular endothelium and then to other cells expressing ICAM-1 in the zone of inflammation.44 Expression of MHC class I and II antigens as well as cell adhesion molecules plays an important role in the induction of graft rejection. By reducing the expression of MHC class I, class II antigens, and cell adhesion molecules, FK506 can inhibit the influx of immunocompetent cells and thereby further reduce the likelihood of rejection. This effect requires the continued application of the agent for more than 2 weeks, because a considerable number of treated grafts (60%) will show signs of rejection beginning 3 days after cessation of the drops.

Topical FK506 thus appears to act through several mechanisms to inhibit graft rejection. By inhibiting adhesion molecule expression, topical FK506 is able to reduce the influx of immunocompetent cells. By decreasing the expression of both class I and II MHC antigens, it reduces the activation of wandering T cells. By directly inhibiting T-cell activation and inhibiting the synthesis of cytokines that can induce the expression of various surface molecules, it prevents the inflammatory cascade from becoming established. All of these methods of action are possible with either systemic or topical therapy.

**Key Words**

FK506, graft rejection, immunohistochemistry, penetrating keratoplasty, topical treatment

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**References**

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