Conditions Affecting Enhanced Corneal Allograft Survival by Oral Immunization

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PURPOSE. To determine the optimal conditions for enhancing corneal allograft survival by oral immunization with donor-specific alloantigens.

METHODS. CB6F1 mice were orally immunized with various doses of C3H/Hej corneal epithelial and endothelial cells before receiving orthotopic C3H/Hej corneal allografts. Paraformaldehyde-fixed corneal cells were compared with viable corneal cells for their capacity to promote corneal allograft survival. The mucosal adjuvant, cholera toxin B (CTB), was examined for its capacity to enhance corneal graft survival when given separately or conjugated to corneal cells used for oral immunization. Oral immunization was also evaluated for its capacity to prevent immunologic rejection in three high-risk settings: preimmunized hosts, hosts with prevascularized graft beds, and grafts that contain donor-specific Langerhans' cells.

RESULTS. Optimal graft survival occurred when 2 × 10⁶ corneal cells were administered orally 10 days before orthotopic corneal transplantation. Paraformaldehyde-fixed corneal cells were as effective as viable cells in preventing corneal graft rejection. Cholera toxin B enhanced the efficacy of oral immunization when conjugated with the orally administered corneal cells but was ineffective when administered separately. Oral immunization with donor corneal cells enhanced corneal graft survival in all three high-risk settings.

CONCLUSIONS. Oral immunization with donor cells is an effective strategy for enhancing corneal graft survival and preventing graft rejection in high-risk settings. Graft enhancement is optimized when the orally administered cells are conjugated with CTB and administered before corneal transplantation. Because fixed cells retain their capacity to enhance corneal graft survival, it may be possible to store donor cells for long-term use in high-risk hosts. (Invest Ophthalmol Vis Sci. 1998;39:1835–1846)

Corneal transplantation is the oldest and most common form of solid tissue transplantation.¹ Corneas have been transplanted successfully to humans for almost a century.² In the United States alone, more than 45,000 corneal transplants are performed each year.³ Although the success rate for first-time corneal transplants is 85% to 90%, approximately 10% of the grafts will fail because of immunologic rejection.³ The rejection rate for corneal transplants soars to 65% to 70% in patients who have previously rejected a corneal transplant.⁴,⁵ Preventing graft rejection in such high-risk hosts requires the use of systemic immunosuppressive drugs. However, many of these corneal allografts will undergo immunologic rejection even in the face of cyclosporin A and similar potent immunosuppressive drugs.¹ Although corticosteroids and cyclosporin A have greatly reduced the rejection rate of corneal allografts, their prolonged use can produce deleterious side effects including glaucoma, cataract formation, nephrotoxicity, hypertension, and hepatotoxicity.⁶,⁷ Thus, less-toxic alternative methods of immunosuppression are still needed for corneal transplantation, especially for use in the high-risk host.

Oral administration of antigens is a unique and effective method for downregulating the immune response to a variety of antigens, including alloantigens.⁸,⁹ This curious phenomenon was largely ignored until the late 1980s when several investigations showed that oral administration of autoantigens mitigated a wide variety of experimental autoimmune diseases including experimental autoimmune encephalomyelitis, rheumatoid arthritis, diabetes, and experimental autoimmune uveoretinitis.⁸,⁹ We have shown that oral administration of alloantigens results in a remarkable reduction in the rejection of orthotopic corneal allografts in mice.¹⁰,¹¹ In the case of fully allogeneic corneal allografts (i.e., major histocompatibility complex and multiple minor histocompatibility mismatches), oral administration of donor cells results in a 50% reduction in graft rejection.¹⁰,¹¹ This graft enhancement can be augmented by conjugating the oral cell inoculum with the nontoxic B subunit of the mucosal adjuvant cholera toxin (CTB).¹¹ Untreated CB6F1 mice consistently reject 100% of their fully allogeneic C3H orthopic corneal allografts.¹¹ However, oral administration of CTB-conjugated C3H corneal cells reduces the rejection rate from 100% in untreated hosts or hosts treated with CTB only, to less than 10% in mice fed CTB-conjugated C3H corneal cells.¹¹

Corneal allograft enhancement can also be achieved by oral immunization with skin epidermal cells (i.e., keratino-
cytes) or spleen cells from the relevant donor mouse strain.\textsuperscript{10,11} Importantly, orally induced graft enhancement is alloantigen specific; third-party corneal allografts are unaffected by oral antigen administration.\textsuperscript{10–12} In a recent study, investigators showed that oral administration of corneal alloantigens induces a profound downregulation of cytotoxic T lymphocyte, delayed-type hypersensitivity, and mixed lymphocyte responses to donor alloantigens, but not to third-party alloantigens.\textsuperscript{12} Thus, alloantigen administration holds considerable promise as a strategy for minimizing the need for immunosuppressive drugs in patients undergoing keratoplasty.

These promising results prompted us to evaluate conditions that might further enhance the beneficial effects of oral immunization on corneal allograft survival. We were particularly interested in determining whether oral immunization would promote corneal allograft survival in high-risk hosts.

\section*{Methods}

\subsection*{Mice}

Female C3H/Hej (H-2\textsuperscript{k}) and CB6F1 (H-2\textsuperscript{b/d}) mice were reared in the Department of Microbiology Animal Colony at the University of Texas Southwestern Medical Center (Dallas) and were used between the ages of 2 and 8 months. The use of animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

\subsection*{Corneal Cell Cultures}

Tissue-cultured C3H/Hej corneal epithelial and endothelial cells were used as alloantigens for the induction of oral tolerance. Cell cultures were established from freshly dissected corneal explants\textsuperscript{13,14} and propagated in minimum essential medium supplemented with 10% fetal calf serum. After the primary cultures were established, the cells were immortalized with human papilloma virus genes E6 and E7 using, the disabled recombinant retroviral vector pLXSN16E6/E7.\textsuperscript{15} These cells proliferate indefinitely while maintaining their original morphologic characteristics. Furthermore, the cells express the same histocompatibility antigens as their nontransformed counterparts.\textsuperscript{10}

\subsection*{Oral Tolerance Induction}

Cultured murine corneal cells were used for inducing oral tolerance. The nontoxic B subunit of cholera toxin was conjugated to C3H/Hej corneal epithelial and endothelial cells before oral administration.\textsuperscript{11} Cholera toxin B subunit (Sigma, St. Louis, MO) was conjugated with cells by incubating 100 \(\mu\)g CTB with a cell suspension containing a mixture of \(5 \times 10^6\) corneal epithelial cells and \(5 \times 10^6\) corneal endothelial cells in 1 ml Hanks' balanced salt solution (HBSS). The cell suspensions were incubated for 2 hours at 37\textdegree C with frequent shaking followed by washing three times in HBSS. The efficiency of the CTB conjugation procedure was confirmed by incubating corneal cell suspensions with 100 \(\mu\)g fluorescein isothiocyanate-labeled CTB, using the same protocol and viewing the conjugated cells by fluorescence microscopy. In most experiments, a mixture of \(1 \times 10^6\) epithelial cells plus \(1 \times 10^6\) endothelial cells was administered directly into the stomach using a gavage tube. In some experiments, CTB-conjugated corneal cells were fixed with 1.0% paraformaldehyde for 10 minutes at room temperature, washed once in HBSS, and resuspended in 5 ml 200 mM L-glutamine, washed three times in HBSS, and used immediately for oral immunization.

\subsection*{Orthotopic Corneal Transplantation}

Full-thickness penetrating C3H (H-2\textsuperscript{k}) corneal grafts (2.5-mm diameter) were transplanted orthotopically in anesthetized CB6F1 (H-2\textsuperscript{b/d}) mice using a procedure described by She et al.\textsuperscript{16} and modified by He et al.\textsuperscript{17} Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (1-2 mg/mouse; Abbott Laboratories, Chicago, IL). Proparacaine was used as a topical anesthetic (Alcon Laboratories, Fort Worth, TX). The donor graft and the recipient graft bed were scored with 2.5-mm and 2.0-mm diameter trephines, respectively (Storz Instruments, St. Louis, MO) before removal of the corneal button using vannas scissors (Storz). The donor graft was sewn into place using 12 interrupted 11-0 nylon sutures and a 50-\(\mu\)m diameter needle (2881G; Ethicon, Somerville, NJ). Sutures were removed 7 days later. No immunosuppressive drugs were used. In some experiments orthotopic corneal grafts were sewn into place with a single running 11-0 nylon suture. The single knot for the running suture was removed on day 7, but the remainder of the running suture was left in place. Occasionally, suture loops would appear and were cut free.

\subsection*{Clinical Observations}

Grafted eyes were examined with a slit lamp biomicroscope at least twice a week throughout the entire study period. Graft opacity, edema, and neovascularization were scored as minimal, moderate, or severe, as previously described.\textsuperscript{17} If all three parameters became moderate or severe more than 7 days after transplantation, the graft was recorded as rejected on that day. Any host in which such complications as cataract, anterior chamber loss, iris synechiae, or infection developed was excluded from the study. Mean survival time was calculated for each group and used for expressing the results in graphic form. Median graft survival times were also calculated and used to determine the statistical significance by the Mann-Whitney test. Differences in the incidence of rejection were evaluated by chi-square analysis.

\subsection*{Induction of Langerhans' Cell Migration}

The central corneal epithelium of the mouse is normally devoid of resident Langerhans' cells (LC), which are usually situated in basal layers at the limbus. However, LCs can be induced to migrate centripetally from the limbus into the central corneal epithelium by the instillation of sterile latex beads (1.0-\(\mu\)m diameter; Sigma) into shallow incisions in the corneal epithelium.\textsuperscript{18,19} Corneal allografts (2.5-mm diameter) were prepared from corneas from C3H/Hej mice treated with sterile latex beads 7 days earlier.

\subsection*{Heterotopic Corneal Transplantation}

Full-thickness C3H/Hej corneal grafts containing peripheral limbus (total 3.0-3.5-mm diameter) were transplanted heterotopically onto vascularized subdermal graft beds on the lateral thoraxes of CB6F1 mice as previously described.\textsuperscript{20} Heterotopic transplantation of such LC-bearing corneal allografts is an effective method for inducing cytotoxic T lymphocyte and delayed-type hypersensitivity alloimmune responses in mice.\textsuperscript{12,18,21}
FIGURE 1. Dose dependency of oral tolerance. CB6F1 mice were immunized orally with 1 dose per day for 10 days of C3H/Hej corneal epithelial and endothelial cells conjugated with cholera toxin B. C3H/Hej orthotopic corneal grafts were transplanted to animals 1 day after the 10th oral inoculum. There were 9 to 11 mice per group. The difference between 1 × 10^6 and 2 × 10^6 groups was significant (P = 0.0008). The difference between 2 × 10^6 and 1 × 10^5 groups was significant (P = 0.0004).

Prevascularization of Corneal Graft Beds
A previously described mouse model of neovascularized corneal graft beds was used in which three interrupted sutures (11-0 nylon) were placed in the central cornea of one eye. Intense corneal neovascularization was present 7 to 10 days later. C3H/Hej corneal allografts were placed in the neovascularized graft beds of CB6F1 mice 10 days after the initial placement of the sutures.

RESULTS

Dose Dependency of Oral Immunization and Corneal Graft Enhancement
Previous studies have demonstrated a remarkable enhancement of corneal allograft survival using 1 dose per day for 10 days of 2 × 10^6 donor-specific corneal cells, spleen cells, or keratinocytes. Because this regimen requires a considerable quantity of donor cells and may create logistic problems in a clinical setting with human subjects, we sought to determine whether corneal allograft enhancement could be achieved with fewer cells in each oral inoculum. Accordingly, CB6F1 mice were immunized with 10 daily doses of 1 × 10^4, 1 × 10^5, 1 × 10^6, or 2 × 10^6 C3H/Hej corneal cells. Mice received full-thickness C3H/Hej orthotopic corneal allografts 1 day after the 10th oral inoculum. As in previous studies, oral immunization with 10 doses of 2 × 10^6 corneal cells resulted in a remarkable enhancement of corneal allograft survival. In this donor-host combination, 100% of the C3H/Hej corneal allografts are routinely rejected by immunologically naive CB6F1 hosts. During the past 3 years we have orthotopically grafted 33 untreated CB6F1 mice with C3H/Hej corneal grafts.
FIGURE 2. Longevity of oral tolerance. CB6F1 mice were given 1 dose per day for 10 days of cholera toxin B (CTB)-conjugated or nonconjugated C3H/Hej corneal epithelial cells (2 × 10^6 cells/dose). Orthotopic C3H/Hej corneal allografts were applied 30 or 60 days after the 10th oral inoculum. There were 10 to 11 mice per group. The difference between the number of cells with CTB on day 30 and cells with CTB on day 60 was not significant (P > 0.05).

All 33 grafts were rejected with a mean survival time of 21 ± 7 days. We used these historic controls as a reference point for gauging the efficacy of the various oral immunization experiments. Thus, 100% of the C3H/Hej corneal grafts are routinely rejected by untreated CB6F1 hosts; however, only 10% of the corneal grafts were rejected by mice immunized orally with 10 daily doses of 2 × 10^6 corneal cells (Fig. 1). However, reducing the oral inoculum by 50% resulted in a modest decrease in corneal allograft survival; 70% of the allografts remained clear compared with 0% in untreated control mice and in hosts orally immunized with 1 × 10^6 corneal cells (Fig. 1). The results suggest that increasing the cell inoculum used for oral immunization results in a proportionate increase in corneal allograft survival.

Longevity of Corneal Graft Enhancement

Determining the longevity of orally induced corneal graft enhancement is a crucial question that bears on the clinical feasibility of oral immunization. The present experiments were designed to determine whether a single 10-day course of oral immunization would induce long-lived, downregulation of host immune responses to subsequent challenge with donor-specific corneal allografts. The effect of CTB in promoting long-term enhancement of corneal survival was also evaluated. Groups of CB6F1 mice were immunized with 10 daily oral inocula containing CTB-conjugated C3H/Hej corneal cells (2 × 10^6 cells/dose) or nonconjugated C3H/Hej corneal cells (2 × 10^6 cells/dose). Orthotopic corneal allografts were applied 30 or 60 days after the 10th oral inoculum. The results indicated that orally induced allograft tolerance decayed over time. In previous experiments, 10 daily doses of 2 × 10^6 CTB-conjugated donor cells resulted in approximately 90% graft survival if the corneal grafts were applied 1 day after the 10th oral inoculum. However, delaying corneal transplantation 60 days resulted in a steep reduction in graft survival (Fig. 2). Instead of 90% graft survival, only 36% of the grafts remained clear. The presence of CTB did not prevent the decay of oral tolerance. Animals treated in a similar manner with corneal cells not
conjugated with CTB had a graft rejection rate that was insignificantly different from that of the group treated with CTB-conjugated corneal cells ($P > 0.05$). Although oral tolerance diminished with time, it is noteworthy that graft survival in all the groups was markedly better than in untreated control mice, which typically experience a 100% rejection rate.

**Efficacy of Fixed Donor Cells in Corneal Graft Enhancement**

The gradual decay of orally induced tolerance is similar to other categories of immune responses that diminish over time. Periodic "booster" immunizations are often needed to sustain long-term memory and immunity. Thus, it would be desirable to have a stable source of donor cells for periodic oral immunization. This could be satisfied with long-term cell cultures, but the risk of inadvertent laboratory contamination of cell cultures increases in long-term culture settings. However, preserving donor cells in mild fixatives could be a desirable solution that circumvents the risk of contamination during tissue culture and is significantly less expensive and laborious. Moreover, fixatives such as paraformaldehyde are excellent disinfectants that purge the oral inocula of potential pathogens. This proposition was tested by fixing CTB-conjugated corneal cells with 1% paraformaldehyde for 10 minutes at room temperature. After extensive washing in HBSS, the fixed and non-fixed CTB-conjugated cells were administered orally for 10 days as in the previous experiments. The results show that fixation did not adversely affect orally induced graft enhancement. Only 14% of the hosts given paraformaldehyde-fixed donor cells went on to reject their allografts (Fig. 3). This was not significantly different from hosts treated with nonfixed corneal cells ($P > 0.05$).
FREE CTB

CONJUGATED CTB

0 10 20 30 40 50 60 70
Figure 4. Effect of free cholera toxin B (CTB) on the induction of oral tolerance. CB6F1 mice were immunized orally with 1 dose per day for 10 days of CTB-conjugated C3H/Hej corneal cells. Another group of mice was immunized with C3H/Hej corneal cells and 20 μg free CTB. All mice received C3H/Hej orthotopic corneal grafts 1 day after the final oral inoculum. There were 11 animals in each group. The difference between the two groups was significant (P = 0.0002).

Effect of Free CTB on Orally Induced Tolerance

The results reported in the present study and in a previous study have shown the importance of CTB in enhancing oral tolerance.11 In an effort to simplify the oral immunization protocol, we wondered whether it would be possible to eliminate the need for the in vitro conjugation procedure and simply coadminister CTB with the donor cells at the time of oral immunization. Accordingly, CB6F1 mice were immunized orally with 10 daily doses of $2 \times 10^6$ CTB-conjugated C3H/Hej corneal cells or $2 \times 10^6$ nonconjugated C3H/Hej corneal cells coadministered with 20 μg free CTB. A dose of 20 μg CTB was selected, because it represents the amount of CTB that is used to conjugate $2 \times 10^6$ corneal cells (i.e., 100 μg CTB per $1 \times 10^7$ corneal cells). Mice were challenged with orthotopic C3H/Hej corneal grafts 1 day after the 10th oral inoculum. The results indicate that conjugation was necessary for CTB to augment oral immunization (Fig. 4). Coadministration of free CTB with donor cells resulted in a 45% survival rate of corneal allografts, which is the same degree of graft enhancement achieved by oral immunization with nonconjugated donor cells.10,11

Effect of Oral Tolerance on Corneal Allografts Secured with Running Sutures

We and others have used rodent models of keratoplasty in which interrupted sutures are removed 7 to 14 days after transplantation. Because keratoplasty in humans often involves the use of running sutures that are left in place for prolonged periods, we sought to determine whether the presence of a running suture would diminish the efficacy of orally induced tolerance. Untreated mice and mice given 10 daily doses of CTB-conjugated C3H/Hej corneal cells received orthotopic
Efficacy of oral tolerance in preventing the rejection of orthotopic corneal grafts sewn with running sutures. CB6F1 mice were immunized orally with 1 dose per day for 10 days of cholera toxin B-conjugated C3H/Hej corneal cells. One day after the 10th oral inocula, C3H/Hej corneal grafts were transplanted orthotopically, using a single running suture. Controls consisted of untreated CB6F1 mice that received C3H/Hej orthotopic corneal grafts sewn into place with running sutures. There were 21 mice in the control group and 14 in the orally immunized group. The difference between the two groups was significant ($P = 0.0004$).

Under normal circumstances topical corticosteroids are the only immunosuppressive agents needed to achieve long-term acceptance of corneal allografts. However, certain risk factors jeopardize corneal graft survival, even in the face of topical corticosteroids. In humans and experimental animals, the risk of rejection soars in recipients who have rejected previous corneal grafts or who have vascularized graft beds. In rodent models of keratoplasty, the presence of donor-derived LCs in the corneal allograft greatly increases the risk for immunologic rejection. The efficacy of oral immunization in preventing corneal graft rejection in high-risk hosts was evaluated. Three categories of high-risk hosts were tested: hosts that had previously rejected a corneal allograft, hosts with prevascularized graft beds, and hosts that received corneal allografts containing donor-derived LCs.

To evaluate the efficacy of oral immunization in hosts that had rejected previous corneal allografts, CB6F1 mice first received heterotopic C3H/Hej corneal allografts placed onto sub
PREIMMUNIZED HOSTS

CONTROL

ORAL CELLS

DAYS POST TRANSPLANTATION

% GRAFT SURVIVAL

0 10 20 30 40 50 60 70

0 20 40 60 80 100

Figure 6. Oral tolerance prolonged corneal graft survival in preimmunized hosts. CB6F1 mice were immunized with two heterotopic C3H/Hej corneal grafts. Fourteen days later, one group of mice was immunized orally with 1 dose per day for 10 days of cholera toxin B-conjugated C3H/Hej corneal cells. All animals received orthotopic C3H/Hej corneal grafts 25 days after the application of the initial heterotopic corneal allografts. There were 10 animals in the control group and 11 in the experimental group. The difference between the experimental and control groups was significant ($P = 0.00009$).

dermal graft beds on the lateral thorax. We elected to use heterotopic corneal allografts, rather than orthotopic corneal allografts for these experiments for two reasons. Heterotopic corneal grafts are significantly easier to apply than orthotopic corneal grafts. More important, corneal grafts are more immunogenic when placed heterotopically onto subdermal graft beds than when placed orthotopically. Therefore, sensitizing hosts with heterotopic, rather than orthotopic corneal allografts, is a more rigorous test for evaluating the efficacy of oral immunization. Each CB6F1 mouse received two heterotopic C3H/Hej corneal allografts on day 0. Two weeks later, one group was given 10 daily doses of C3H/Hej cornea cells. Orthotopic C3H/Hej corneal allografts were transplanted in both groups of mice, and graft survival was assessed. As expected, all the corneal grafts underwent swift rejection in the preimmune mice that were not subjected to oral antigen treatment. By contrast, oral immunization resulted in the long-term survival of 82% of the corneal grafts (Fig. 6).

The capacity of oral immunization to promote the survival of corneal allografts placed into prevascularized graft beds was tested next. Three interrupted sutures (11-0 nylon) were placed in the central corneas of CB6F1 hosts. Oral immunization with C3H/Hej corneal cells was initiated on the same day that the sutures were placed in the cornea and was continued for a total of 10 daily doses. Intense corneal neovascularization was apparent 7 to 10 days later. On day 10, orthotopic C3H/Hej corneal allografts were transplanted to the vascularized corneal graft beds of the CB6F1 hosts. The results (Fig. 7) show that all the grafts placed in vascularized graft beds underwent rejection. Although the tempo of corneal graft rejection was not accelerated in hosts with prevascularized graft beds, the intensity of graft edema was approximately 50% more than that
PREVASCULARIZED GRAFT BEDS

Figure 7. Effect of oral tolerance on grafts implanted in prevascularized graft beds. Corneal neovascularization was induced in CB6F1 mice by placing three interrupted 11-0 nylon sutures in the central corneas. One group of mice was given 1 dose per day for 10 days of cholera toxin B-conjugated C3H/Hej corneal cells. Eleven days later both groups of mice received orthotopic C3H/Hej corneal allografts. There were 8 animals in the control group and 11 in the experimental group. The difference between control and experimental groups was significant ($P = 0.0006$).

We have shown that the presence of donor-derived LCs renders orthotopic and heterotopic corneal allografts highly immunogenic and dramatically increases the incidence of immunologic rejection. Therefore, we tested the effect of oral immunization in promoting the survival of LC-containing grafts. The central corneas of C3H/Hej mice were treated with sterile latex beads as a means of inducing the centripetal migration of peripheral LC into the central corneal epithelium. Seven to 10 days later, the avascular latex bead–treated corneas were removed and transplanted to normal CB6F1 mice or CB6F1 mice that had been treated with 10 daily doses of C3H/Hej corneal cells conjugated with CTB. As in the previous experiments, all the corneal grafts underwent rejection in the untreated hosts. By contrast, 80% of the corneal grafts remained clear in hosts that were subjected to oral immunization with donor corneal cells (Fig. 8). Thus, oral immunization with donor corneal cells prevents rejection and promotes long-term corneal graft survival in all three categories of high-risk hosts. The present results along with previous findings provide insights into the conditions for enhancing corneal allograft survival by oral immunization (Table 1).

DISCUSSION

Previous studies have shown the efficacy of oral immunization in preventing corneal graft rejection in mouse and rat models of penetrating keratoplasty. The antigen specificity of oral tolerance combined with its apparent lack of toxicity makes it an attractive candidate for use in human patients undergoing...
keratoplasty. However, before contemplating clinical studies, it is crucial to determine optimal conditions for enhancing corneal graft survival by oral immunization. The experiments described here were the first step in this direction. The results reiterate the potent effects of CTB in augmenting oral tolerance and preventing corneal graft rejection. The beneficial effects of oral immunization incure closely with the number of cells in the oral inoculum. Graft survival climbs to 90% when $2 \times 10^6$ donor cells are administered in each oral inoculum. It is important to evaluate the effect of using greater numbers of cells in the daily inocula. However, the prospect of increasing the oral inoculum 1 log higher is not feasible at the present time.

With the present culture system, we were able to obtain approximately $1 \times 10^7$ corneal cells per 75-cm$^2$ culture flask. Increasing the oral inoculum to $2 \times 10^7$ cells/mouse would require two flasks daily for each mouse. Experiments typically require 10 mice per group. Thus, a typical 10-day experiment would require 200 flasks of corneal cells per experimental group! It is also possible that 10 daily oral immunizations is not the optimal number of doses and that maintaining the standard dose of cells per inoculum while increasing the number of daily immunizations will augment graft survival even more.

The longevity of graft enhancement is a crucial factor in considering the clinical feasibility of oral immunization. The present results and results reported elsewhere indicate that a single 10-day treatment regimen initiated before corneal transplantation permits the long-term survival of 80% to 90% of the corneal allografts, even in high-risk settings. However, the experiments summarized in Figure 2 reveal that the tolerizing effect decayed over time and that a significant number of grafts underwent rejection when they were transplanted 30 to 60 days after administration of the 10th oral inoculum. On the surface, these appear to be contradictory findings. However, we suspect that once a corneal graft has healed and resides on
an avascular bed, it is sequestered from the afferent arm of the immune apparatus and does not provoke an immune response. Although the graft is antigenic, it is not immunogenic. This hypothesis is supported by studies in experimental animal models of keratoplasty in which long-term corneal grafts remain clear until the host is immunized systemically with donor alloantigens.30,31 After systemic immunization, the previously healthy corneal allografts underwent immunologic rejection. Thus, the corneal allografts did not provoke an immune response; nonetheless, they were vulnerable to the host’s immunologic effector elements once the immune system was aroused.

Perhaps the most surprising and promising finding of the present study is the capacity of paraformaldehyde-fixed corneal cells to induce oral tolerance and prevent corneal allograft rejection as effectively as viable cells. This observation raises the possibility of culturing large numbers of donor cells and preserving them for future use in booster immunizations or during crisis episodes. Although it is not feasible to culture donor corneal cells for use in clinical studies, it is possible to obtain and culture significant quantities of skin from the same donor who provides the corneal button. We have recently cultured human skin and found that human keratinocytes grow exceedingly well in vitro and are amenable to conjugation with CTB. Using fluoresceinated CTB, we have found that CTB remains conjugated with paraformaldehyde-fixed keratinocytes for at least 120 days (unpublished data). Moreover, keratinocytes are as effective as corneal cells in inducing oral tolerance and enhancing corneal graft survival in mice.10 The efficacy of paraformaldehyde-fixed cells in promoting corneal graft survival after prolonged storage is of paramount importance and is under investigation. The prospect of eliminating the in vitro conjugation step in the preparation of the oral inocula was ruled out in the present study. Administering free CTB with nonconjugated donor cells did not produce the same effect as using cells that were conjugated with CTB in vitro before oral immunization.

Sutures induce corneal neovascularization, which increases the risk of rejection.72,25 However, oral immunization was highly effective in preventing the rejection of corneal allografts held in place with running sutures that were not removed.

Oral immunization had a remarkably beneficial effect in all three categories of high-risk hosts; in each case, 80% of the grafts survived compared with 0% graft survival in the respective untreated control mice. We have previously shown that oral immunization with nonconjugated C3H/Hej corneal cells does not prevent the rejection of C3H/Hej corneal grafts in CB6F1 mice that have been previously immunized with C3H/Hej heterotopic corneal grafts.10 However, the inability of oral immunization with nonconjugated cells to affect graft survival in preimmune hosts could be overcome with CTB conjugation. These findings also emphasize that oral immunization with alloantigens reprograms the immune system and desensitizes the previously immunized host.

Numerous investigators have examined the efficacy of oral tolerance in mitigating a variety of autoimmune diseases in experimental animals and patients.8,9 Although many of the animal studies report impressive amelioration of experimental autoimmune disease, the results of clinical studies on humans have been less impressive.9,32 There are many reasons that account for this disparity between experimental models and the human counterpart. However, one compelling explanation that comes to mind is the likelihood that multiple autoantigens incite human autoimmune diseases and may be different from the antigenic preparation administered in the therapeutic oral inoculum. Moreover, oral immunization with autoantigens is initiated after the disease process has begun. By contrast, in the case of corneal allografts, oral immunization can be initiated before the unwanted immune response has been elicited. The oral inoculum contains all the antigenic components that provoke the rejection process. These attributes along with the nontoxic nature of oral immunization make it an attractive candidate for clinical evaluation in high-risk patients undergoing keratoplasty.

### References


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**Table 1. Optimal Conditions for Enhancing Corneal Graft Survival by Oral Tolerance**

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<tr>
<th>Parameter</th>
<th>Optimal Effect</th>
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<td>Cell type</td>
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CTB, Cholera toxin B; MHC, major histocompatibility complex; LC, Langerhans’ cells.

*Paraformaldehyde-fixed corneal cells are preferred over viable cells because the fixative permits storage of donor cells and acts as a disinfectant for purging potential pathogens.


