Immunologic Phenotype of Hosts Orally Immunized with Corneal Alloantigens

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PURPOSE. To evaluate the immunologic phenotype of hosts tolerized by oral administration of corneal alloantigens.

METHODS. CB6F1 mice were tolerized by oral administration of allogeneic C3H/Hej corneal epithelial and endothelial cells before receiving heterotopic C3H/Hej corneal allografts. C3H-specific cytotoxic T-lymphocyte (CTL), delayed-type hypersensitivity (DTH), and mixed-lymphocyte responses were evaluated in orally tolerized and control mice. Cytokine profiles of Peyer’s patch cells from orally tolerized mice were determined by enzyme-linked immunosorbent assay and mink lung cell culture bioassay.

RESULTS. Oral administration of corneal cells produced a profound inhibition of allospecific CTL, DTH, and mixed-lymphocyte responses. Conjugation with the B subunit of cholera toxin markedly increased the tolerizing activity of corneal endothelial cells, so that a single dose of cholera toxin-conjugated corneal cells inhibited alloimmune responses to the same degree as 10 doses of corneal cells unconjugated with cholera toxin. Peyer’s patch cells from orally tolerized mice produced reduced quantities of interferon-γ and interleukin-2 but produced increased amounts of transforming growth factor-β and interleukin-10 compared with concentrations in normal control animals.

CONCLUSIONS. Oral administration of cholera toxin-conjugated corneal cells produces a dose-dependent inhibition of allospecific CTL, DTH, and mixed-lymphocyte responses. Orally induced inhibition of cell-mediated immune responses to corneal alloantigens is correlated with a sharp increase in the secretion of transforming growth factor-β and interleukin-10 and a concomitant suppression of interleukin-2 and interferon-γ. The well-recognized immunosuppressive characteristics of transforming growth factor-β and interleukin-10 are suggestive that orally induced tolerance to corneal alloantigens is mediated by these cytokines. (Invest Ophthalmol Vis Sci. 1998;39:744–753)
tering antigens stimulates cytotoxic T-lymphocyte (CTL) responses, whereas others have reported inhibition of CTLs.\textsuperscript{22,23} Thus, the cytokine profile and effector cell phenotype of orally tolerized hosts can vary dramatically, depending on the type of antigen used and the manner in which it is delivered.

In the present study, we sought determine which cell-mediated immune effector elements were inhibited by oral administration of alloantigenic corneal cells. Investigations were performed to ascertain the T-cell cytokine profile of orally tolerized hosts and thereby to provide insights into the mechanisms for improvement in corneal graft survival time.

**METHODS**

**Mice**

Female C3H/Hej (H-2,\textsuperscript{b}), NZB (H-2,\textsuperscript{d}), FVB/n (H-2,\textsuperscript{a}), and C66F1 (H-2,\textsuperscript{bo}) 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.

**Corneal Cell Cultures**

Tissue-Cultured NZB and 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{24,25} and were propagated in minimum essential medium (MEM) supplemented with 10% fetal calf serum. After the primary cultures were established, these cells were immortalized with human papilloma virus genes E6 and E7 using the disabled recombinant retroviral vector pLXSN16E6/ E7.\textsuperscript{26} These cells proliferate indefinitely while maintaining their original morphologic characteristics. Furthermore, the cells express the same histocompatibility antigens as do their nontransformed counterparts.\textsuperscript{11}

**Oral Tolerance Induction**

Cultured murine corneal cells and spleen cells were used for inducing oral tolerance. In some experiments, the nontoxic B subunit of cholera toxin was conjugated to C3H/Hej and NZB corneal epithelial and endothelial cells before oral administration. In other experiments, the B subunit of cholera toxin was also conjugated to C3H/Hej, NZB, and FVB/n spleen cells. CTB subunit (Sigma Chemical, St. Louis, MO) was conjugated to cells by incubating 100 \( \mu \)g of CTB with a cell suspension containing \( 1 \times 10^7 \) spleen cells or 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. The cell suspensions were incubated for 2 hours at 37°C with frequent shaking and were washed three times in Hanks' balanced salt solution. The efficiency of the CTB conjugation procedure was confirmed by incubating corneal cell suspensions with 100 \( \mu \)g of fluorescein isothiocyanate CTB (Sigma) using the same protocol and viewing the conjugated cells by fluorescent microscope. For each oral immunization, \( 2 \times 10^5 \) spleen cells or a mixture of \( 1 \times 10^6 \) epithelial cells plus \( 1 \times 10^6 \) endothelial cells was administered directly into the stomach through a gavage tube.

**Preparation of a Culture Supernatant**

Peyer's patch cells (\( 5 \times 10^9/\text{ml} \)) from orally immunized mice or untreated control mice were cultured for 24 or 48 hours in the presence of x-irradiated (3000 cGy) C3H/Hej spleen cells (\( 2 \times 10^6/\text{ml} \)) and syngeneic antigen-presenting cells (\( 2 \times 10^5/\text{ml} \)) in complete Rose Park Memorial Institute 1640 medium (RPMI) containing 10% fetal calf serum (Hyclone Labs, Logan, UT), 2 mm l-glutamine (JRH Biosciences,Lenexa, KS), 1 mM sodium pyruvate solution (JRH Biosciences), 10 mM Hepes buffer solution (JRH Biosciences), 1% penicillin-streptomycin-fungizone solution (Bio Whittaker, Walkersville, MD), 1% non-essential amino acids solution (Bio Whittaker), and 5 \( \times 10^{-5} \) M 2-mercaptoethanol (Sigma). After incubation, the culture fluid was harvested and centrifuged at 1000 rpm for 5 minutes and kept at \(-20^\circ \text{C} \) until used.

**Assessment of Cytokine Activity**

The levels of IL-2, IL-4, IL-10, and IFN-\( \gamma \) in culture supernatants were measured by double monoclonal antibody (mAb) sandwich enzyme-linked immunosorbent assays (ELISA).\textsuperscript{27} Primary and secondary mAbs for each cytokine were: IL-2, JeS6.1A12 (rat IgG\(_{2a}\)) and JE56-5H4 (rat IgG\(_{2a}\)); IL-10, JE55.2A511 (rat IgG\(_{1}\)) and SXC1 (rat IgM); IFN-\( \gamma \), R46A2 (rat IgG\(_{1}\)) and XMG1.2 (rat IgG\(_{2a}\)) and IL-4, 11B11 (rat IgG\(_{1}\)) and BVG6.24G2 (rat IgG\(_{2a}\)). Activity in culture supernatants was compared with that in cytokine standards: rIL-2, rIL-10, rIL-4, and rIFN-\( \gamma \). Hybridomas producing anti-IL-2 (JE61A12), anti-IL-10 mAb (JE55.2A511), and anti-IFN-\( \gamma \) mAbs (XMG 1.2) were kindly provided by John Abrams (DNAX, Palo Alto, CA). Recombinant IL-10 and IL-4 were also provided by John Abrams. Hybridomas producing anti-IFN-\( \gamma \) mAbs (R46A2) and anti-IL-4 mAbs (11B11 and BVG6.24G2) were purchased from American Type Culture Collection (ATCC, Rockville, MD). Biotinylated anti-IL-2, anti-IL-10, recombinant IL-2, and recombinant IFN-\( \gamma \) were purchased from PharMingen (San Diego, CA). Enhanced protein-binding ELISA microplates (Baxter Diagnostics, McGraw Park, IL) were coated with 50 \( \mu \)g of each primary mouse mAb (10 \( \mu \)g/ml). The plates were covered and incubated at 4°C overnight. Each well was blocked with 200 \( \mu \)l phosphate-buffered saline (PBS) containing 20% FBS. Plates were incubated at room temperature for 2 hours. Standards and samples were added at 25 \( \mu \)l per well and were incubated at 37°C for 1 hour. Biotinylated rat anti-mouse secondary mAb (1.0 \( \mu \)g/ml) was added at 50 \( \mu \)l well and was incubated at 37°C for 30 minutes. Seventy-five microliters of peroxidase-conjugated streptavidin (1:4000) was added to each well and was incubated at 37°C for 25 minutes. Plates were washed with 0.05% Tween 20 in PBS (Sigma) between each step. Finally, 1 mg/ml 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS; Sigma) substrate with 0.03% \( \text{H}_2\text{O}_2 \) was added at 100 \( \mu \)l to each well and allowed to develop at room temperature for 5 to 20 minutes. Plates were read with a microplate reader (Molecular Devices; ThermoMax, Menlo Park, CA) at an optical density of 405 to 490 nm.

**Transforming Growth Factor-\( \beta \) Bioassay**

The level of TGF-\( \beta \) in the culture supernatants was evaluated by bioassay, according to a method described elsewhere,\textsuperscript{28} with minor modifications. Mink lung epithelial cells (ATCC) were plated in 96-well microtiter plates (no. 3596; Costar Corporation, Cambridge, MA) at \( 1 \times 10^4 \) cells/well and allowed to adhere for 30 minutes. Recombinant human TGF-\( \beta \) (Genzyme, Cambridge, MA) was diluted in culture medium to concentrations ranging from 0.002 ng/ml to 10 ng/ml. Aliquots (50 \( \mu \)l) of Peyer’s patch cell culture supernatants and TGF-\( \beta \) standards were transferred in triplicate into separate wells with
The protein concentration was determined by the Bradford enzyme-linked immunosorbent assay. Heterotopic corneal transplantation of such Langerhans cell-bearing corneal allografts is an effective method for inducing CTL and delayed-type hypersensitivity (DTH) alloimmune responses in mice.

**Generation of Hyperimmune Alloantiserum**

Hyperimmune alloantiserum directed against C3H allografts was generated by immunizing 20 CB6F1 (H-2k) mice with three subcutaneous injections of 1 X 10^7 C3H spleen cells administered at weekly intervals. The initial subcutaneous cell inoculum was emulsified in an equal volume of complete Freund's adjuvant (CFA). Subsequent subcutaneous cell injections did not use complete Freund's adjuvant. Mice were exsanguinated 1 week after the third subcutaneous cell injection.

**Enzyme-Linked Immunosorbent Assay**

A conventional ELISA was used to detect alloantibodies directed at major and minor histocompatibility antigens. C3H corneal cells (5 X 10^6 cells/ml) were frozen at -70°C, rapidly thawed three times, and centrifuged 20 minutes at 3000 rpm. The protein concentration was determined by the Bradford assay. The aqueous soluble antigen was diluted with ELISA buffer (0.1 M Na_2CO_3 and 0.1 M NaHCO_3; pH 9.6) to provide 100 μg protein/ml. The antigen was coated onto ELISA plates (10 μg protein/well) overnight at 4°C. Plates were washed three times with PBS containing 0.5% Tween 20. Appropriate test sera were diluted in PBS containing 1% bovine serum albumin and 0.5% Tween 20, and by incubating the plates for 20 minutes at 37°C. Wells were washed three times with PBS, and 50 μl 1:1000 dilution of each serum sample was added to individual wells. Each serum sample was tested in triplicate. Plates were incubated for 2 hours at 37°C. Plates were washed three times with PBS and 50 μl horseradish peroxidase-conjugated goat antibody directed against mouse IgG (Accurate Chemical, Westbury, NY) was added to individual wells and was incubated 2 hours at 37°C. Plates were washed three times with Tween 20-PBS solution and 100 μl 0.1 M ABTS and 0.1 M citric acid monohydrate (pH 4.2), containing 10 μl 30% H_2O_2/10 ml ABTS was added to each well. Plates were incubated in the dark for 30 minutes at room temperature, and the reaction was arrested by the addition of 100 μl stop buffer (5.0% sodium dodecyl sulfate). Substrate degradation was evaluated colorimetrically by a spectrophotometer (MR700; Dynatech, Baton Rouge, LA) with an absorbance band of 405 nm. Negative controls consisted of normal mouse sera. Sera were tested in wells containing irrelevant histocompatibility antigens (i.e., BALB/c; H-2.4 corneal cell extract).

**Cell-Mediated Cytotoxicity Assay**

CB6F1 mice were killed 7, 14, 21, and 28 days after receiving heterotopic corneal allografts. Single-cell suspensions were prepared from spleens removed from individual animals and were used as effector cells in conventional in vitro CTL assays, according to a previously described method. Effector lymphocytes were boosted in vitro for 48 hours at 37°C with γ-irradiated (3000 cGy) C3H/Hej stimulator spleen cells. After in vitro boosting, the effector cells were washed and resuspended in RPMI medium, and 100 μl various concentrations of the effector cell suspensions was added to round-bottomed, 96-well microtiter plates. Chromium-labeled C3H/Hej concanavalin A blasts (1 X 10^4 cells) were added to the various wells to produce effector-to-target ratios ranging from 100:1 to 12.5:1. Plates were centrifuged 2 hours at 100g and were incubated at 37°C for 4 hours in a humidified 5% CO_2 atmosphere. Plates were centrifuged, 100 μl of each supernatant was collected, and the counts per minute were determined by a gamma counter (Tracor Analytical, Atlanta, GA). Cytotoxicity was calculated according to the formula:

\[
\% \text{ Specific cytotoxicity} = \frac{\text{Exp. cpm} - \text{Spontaneous release cpm}}{\text{Maximum release cpm} - \text{Spontaneous release cpm}} \times 100
\]

**Delayed-Type Hypersensitivity Assay**

CB6F1 mice were orally tolerized with 1, 5, or 10 doses of CTB-conjugated C3H/Hej, nonconjugated C3H/Hej, or NZB cornal cells. Mice were immunized 1 day after administration of the final oral inoculum by applying two heterotopic C3H/Hej allografts to the lateral thorax of each mouse, as described earlier. Delayed-type hypersensitivity responses to C3H/Hej alloantigens were measured by a conventional footpad swelling assay 7, 14, 21, and 28 days later. The positive control group consisted of normal CB6F1 mice that were grafted heterotopically but were not administered alloantigens. Normal age-matched CB6F1 mice served as negative control subjects. Both hind footpads of each mouse were measured with an engineer's micrometer (Mitutoyo, Tokyo, Japan) immediately before footpad challenge. An eliciting dose of 1 X 10^7 γ-irradiated (3000 cGy) C3H/Hej splenocytes suspended in 25 μl Hanks' balanced salt solution was injected into the subcutaneous tissue of the right hind footpad. The left hind footpad served as a background control and received 25 μl Hank's balanced salt solution without splenocytes. Both footpads were measured 24 hours later, and the difference in footpad swelling size was used as a measure of DTH. Results were expressed as specific footpad swelling, which equals: ([24 hours right hind foot measurement - 0 hours right hind foot measurement] - [24 hours left hind foot measurement - 0 hours left hind foot measurement]) × 10^{-4} ± SD in inches. Student's t-test was used to evaluate the statistical significance of the results.

**Lymphocyte Proliferation Responses**

CB6F1 mice were killed 14 days after receiving heterotopic corneal allografts, and single-cell suspensions were prepared from the pooled spleens. Mononuclear cells were cultured in triplicate for 72 hours in flat-bottomed microtiter plates at a
RESULTS

Prevention of the Induction of Cell-Mediated Immune Responses

We have previously shown that oral administration of corneal cells dramatically reduces the incidence of orthotopic corneal allograft rejection in mice.11,13 The desensitizing properties of orally administered corneal cells can be markedly improved by conjugating the cells with CTB.19,22 Therefore, it was important to determine which cell-mediated immune responses were affected by oral antigen administration and whether there were quantitative and qualitative differences in the cell-mediated immune responses in hosts administered CTB-conjugated cells versus those administered nonconjugated cells.

CB6F1 mice were administered either 1, 5, or 10 doses of CTB-conjugated or nonconjugated corneal cells (2 × 10^6 cells/dose) 1, 5, or 10 days before receiving heterotopic C3H/Hej corneal grafts. Delayed-type hypersensitivity, mixed-lymphocyte, and CTL responses were assessed 14 days after heterotopic corneal transplantation. In the results, a dose-dependent effect was shown in inhibiting DTH responses (Fig. 1). As few as five doses of nonconjugated corneal cells inhibited the induction of DTH response in heterotopically grafted mice. Conjugation with CTB significantly improved the efficacy of oral tolerance, compared with tolerance induced by oral administration of nonconjugated cells. A single dose of CTB-conjugated corneal cells was as effective as 10 doses of nonconjugated cells in downregulating the DTH responses in heterotopically grafted mice. Similar results were found in CTL assays. Maximum inhibition of anti-C3H CTL responses was recorded in hosts administered 10 doses of CTB-conjugated corneal cells (Fig. 2). Oral antigen administration also prevented alloantigen stimulation of lymphocyte proliferation. A single dose of CTB-conjugated cells resulted in almost complete inhibition of anti-C3H mixed-lymphocyte response (Fig. 3). The inhibition of the anti-C3H lymphocyte proliferation responses was antigen specific, in that CB6F1 mice administered CTB-conjugated or nonconjugated NZB corneal cells had normal lymphocyte proliferation responses to C3H/Hej alloantigens. These results indicate that the downregulation of the lymphocyte proliferation responses was not a consequence of nonspecific effects of CTB.

We have shown that orthotopic C3H corneal allografts undergo rejection in CB6F1 mice within 18 to 20 days of transplantation.11,13 It was important to determine the cell-mediated and humoral immune responses to the donor's alloantigens in control and orally immunized mice during this period. Accordingly, mice received five daily oral immunizations with CTB-conjugated C3H corneal cells. One day later, mice were immunized with two heterotopic C3H corneal allografts. Cytotoxic T-lymphocyte,
Administration of Antigen

DTH, mixed-lymphocyte and serum antibody responses to C3H alloantigens were determined 7, 14, 21, and 28 days later. The results to this point indicated that oral administration of all three forms of cell-mediated immune responses were significantly suppressed at all time points (Figs. 4, 5, 6). By contrast, oral immunization had no effect on alloantibody responses (Fig. 7).

Desensitizing Preimmune Hosts with Oral Administration of Antigen

The results to this point indicated that oral administration of alloantigen was highly effective in preventing the induction of alloimmune responses. However, it was important to determine whether antigen administration would also suppress ongoing immune responses. Accordingly, groups of C6F1 mice were immunized with heterotopic C3H/Hej corneal allografts. Groups of mice were injected with a single oral inoculum consisting of 2 x 10^6 NZB or C3H/Hej corneal cells that were conjugated with CTB. On the same day as oral immunization, mice were immunized with two heterotopic C3H/Hej corneal allografts. Mixed-lymphocyte responses to C3H/Hej alloantigens were assessed 14 days later. Results are expressed as mean ± SEM.

Effect of Oral Antigen Administration on Cytokine Secretion in T Helper Cells Types 1 and 2

Previous studies have suggested that corneal allograft rejection is a Th (T helper cell) 1-mediated process. Moreover, strategies that preferentially activate Th2 responses and downregulate donospecific Th1 alloimmune responses promote increased corneal allograft survival time.33-35 In some antigen systems, oral tolerance is correlated with the production of IL-4 or IL-10,34-36 whereas in others, antigen administration elicits an upregulation of IFN-γ production by Peyers's patch cells.19 Thus, the role of specific Th cytokines in oral tolerance varies, depending on the antigen system and the model used in the investigation. Accordingly, the cytokine production by Peyers's patch cells was determined in orally tolerized and normal mice. C6F1 mice received 10 daily doses of CTB-conjugated C3H/Hej cells. Mice were killed 1 day after the 10th oral inoculum. The Peyer's patch cells were removed, and single cell suspensions were prepared. Peyers's patch cell cultures from orally tolerized and normal mice were stimulated with γ-irradiated C3H/Hej spleen cells for 24 or 48 hours. Supernatants were collected and assessed for the presence of IL-2, IL-4, IL-10, and IFN-γ by a conventional ELISA. Production of TGF-β was determined by bioassay. The results from a typical assay are shown in Figure 10 and indicate that oral administration of C3H/Hej alloantigens had little effect on IL-4 secretion. By

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of antigen downregulates CTL response, whereas in other reports, findings show significant increases in CTL response. Production of IFN-γ is elevated in mice administered myelin-basic protein, but it is profoundly suppressed in animals orally tolerized with ovalbumin. The results of the present study indicate that oral administration of corneal alloantigens induces a remarkable inhibition of three categories of cell-mediated alloimmune responses. The mucosal adjuvant CTB significantly improved the efficacy of orally induced tolerance to the extent that a single of dose of CTB-conjugated corneal cells suppressed CTL, DTH, and mixed lymphocyte proliferation responses as effectively as did 10 doses of non-conjugated corneal cells. The orally induced downregulation of CTL, DTH, and mixed lymphocytes persisted for at least 28 days. Antigen administration also inhibited ongoing alloimmune responses. Mice that were administered corneal cells 7 days after immunization with heterotopic corneal allografts displayed dramatically reduced CTL and DTH responses to donor alloantigens.

The results of the present study and of previous investigations involving this model indicate that orally induced tolerance is alloantigen specific and can be induced with keratinocytes, lymphoid cells, or transformed corneal cells. That transformation with human papilloma E6 and E7 oncogene alters corneal cells in a manner that renders them capable of inducing nonspecific suppression of alloimmune responses is unlikely, because corneal cells from unrelated mouse strains (FVB/n and NZB), transformed with the E6-E7 oncogene in the same manner as C3H/Hej corneal cells, did not induce down-

**DISCUSSION**

Although cyclosporin A and other recently developed immunosuppressive drugs have revolutionized transplantation, they can produce serious side effects and produce antigen-nonspecific immune suppression, thereby rendering the host susceptible to opportunistic infections. By contrast, orally induced tolerance produces profound suppression of alloimmunity that is antigen specific and thus does not disarm the host’s capacity to resist infectious agents. Moreover, the risk for provoking autoimmunity by antigen administration is minimal.

Conjugation of CTB was used in the present study because of CTB’s potency as a mucosal adjuvant and its profound capacity to improve oral tolerance. For example, conjugating CTB to myelin-basic protein reduces the amount of antigen needed to mitigate experimental autoimmune encephalitis several hundred times.

The underlying mechanisms of orally induced tolerance remain poorly understood and vary depending on the antigens used. In some studies, findings show that oral administration
In the present study, the elevated IL-10 levels in mice administered corneal alloantigens is consistent with results of studies employing oral tolerance for mitigating autoimmune diseases in rodents. The increased IL-10 levels are known to cross-regulate Th1 responses. However, it remains to be confirmed whether the increased regulation of alloimmune responses to C3H/Hej alloantigens after oral administration to CB6F1 hosts (Figs. 3, 8).

The apparent inability of oral immunization to affect alloantibody responses is consistent with results in previous studies, which have demonstrated that it is much more difficult to suppress humoral immune responses by oral immunization than to suppress cell-mediated immune responses. That is, 100 times more antigen is needed for oral immunization to suppress antibody responses than is needed to suppress T-cell responses.

There is considerable variation in the literature regarding the cytokine profile in orally tolerized animals. In some reports, a role for IL-4 is suggested, yet findings in other studies demonstrate the successful induction and expression of oral tolerance in IL-4 knockout mice. Moreover, the role of IL-4 varies, even when the same antigen is used with only minor modifications. In the case of experimental autoimmune uveoretinitis, mice orally tolerized with five doses of interphotoreceptor retinoid-binding protein (IRBP) do not produce increased amounts of IL-4, even though the autoimmune manifestations of experimental autoimmune uveoretinitis are significantly blunted. However, if exogenous IL-2 is administered during the course of IRBP administration, IL-4 secretion by Peyer’s patch cells is increased approximately 20-fold compared with IL-4 secretion in control subjects. The role of IL-10 in oral tolerance is similarly confused. Although investigators in several studies have suggested that the production of IL-10 is increased during oral tolerance, others have reported suppression of this cytokine after antigen administration. Moreover, oral tolerance can be induced in mice depleted of IL-10.

**Figure 7.** Effect of oral immunization on anti-C3H/Hej antibody responses. CB6F1 mice (n = 5) received 10 daily oral inocula of CTB-conjugated C3H/Hej corneal cells. Mice received two heterotopic C3H/Hej corneal allografts 1 day after the 10th oral immunization. Anti-C3H/Hej IgG antibody responses were assessed by enzyme-linked immunosorbent assay 7, 14, and 28 days later. Results are expressed as mean ± SEM.

**Figure 8.** Effect of oral immunization with corneal alloantigens on delayed-type hypersensitivity responses in previously immunized mice. CB6F1 mice (three groups of five mice each) received two heterotopic C3H/Hej corneal allografts on day 0. A single oral inoculum of CTB-conjugated C3H/Hej corneal cells was administered on either day -7, day 0, or day +7. Delayed-type hypersensitivity responses to C3H/Hej alloantigens were assessed 14 days after heterotopic corneal transplantation. (A) Results from experiments involving oral immunization with corneal cells on the days indicated. (B) Anti-C3H/Hej delayed-type hypersensitivity responses in CB6F1 mice immunized orally with CTB-conjugated spleen cells from NZB or FVB/n donors administered on day -7. Results are expressed as mean ± SEM.
Immune Phenotype in Oral Tolerance

FIGURE 9. Effect of oral immunization with corneal alloantigens on cytotoxic T-lymphocyte responses in previously immunized mice. CB6F1 mice (three groups of five each) received two heterotopic C3H/Hej corneal allografts on day 0. A single oral inoculum of CTB-conjugated C3H/Hej corneal cells was administered on day −7, day 0, or day +7. Cytotoxic T-lymphocyte responses to C3H/Hej alloantigens were assessed 14 days after heterotopic corneal transplantation. (A) Results from experiments involving oral immunization with corneal cells on the days indicated. Results are expressed as mean ± SEM.

FIGURE 10. Interleukin-4 production by Peyer’s patch cells from orally tolerized mice. CB6F1 mice (two groups of five each) received 10 daily doses of CTB-conjugated C3H/Hej corneal cells. Peyer’s patch cells were collected 1 day after the 10th oral inoculum of corneal cells and were stimulated in vitro with γ-irradiated C3H/Hej lymphoid cells, and culture supernatants were collected 24 and 48 hours later. The presence of interleukin-4 was determined by enzyme-linked immunosorbent assay.

FIGURE 11. Interleukin-10 production by Peyer’s patch cells from orally tolerized mice. CB6F1 mice received 10 daily doses of CTB-conjugated C3H/Hej corneal cells. Peyer’s patch cells were collected 1 day after the 10th oral inoculum of corneal cells. Peyer’s patch cells were stimulated in vitro with γ-irradiated C3H/Hej lymphoid cells, and culture supernatants were collected 24 and 48 hours later. The presence of interleukin-10 was determined by enzyme-linked immunosorbent assay.

production of IL-10 is adequate to account for the suppression of allospecific cell-mediated immunity and the prevention of corneal allograft rejection.

The marked reduction in IFN-γ and IL-2 and the concomitant increase in TGF-β synthesis are consistent with downregulation of Th1 immune responses and improvement of graft survival time. In numerous studies, investigators have demonstrated a close correlation between successful induction of oral tolerance and increased production of TGF-β. Moreover, it is
well known that corneal allograft rejection is a Th1-mediated immune process. We propose, therefore, that oral administration of CTB-conjugated corneal cells elicits the generation of a cytokine network in which TGF-β and IL-10 production is upregulated. The abundance of TGF-β and IL-10 prevents the activation and clonal expansion of Th1 effector cells. This in turn favors the survival of corneal allografts. Gaining a better understanding of the underlying mechanism of oral tolerance to corneal alloantigens may permit implementation of this novel strategy in high-risk patients.

References