Evidence that Retinal Pigment Epithelium Functions as an Immune-Privileged Tissue

Hartmut Wenkel and J. Wayne Streilein

PURPOSE. Tissues derived from immune-privileged sites sometimes possess special characteristics that promote their own survival when transplanted to a nonprivileged site. This study was undertaken to evaluate whether retinal pigment epithelium (RPE) behaves as an immune-privileged tissue when transplanted extraocularly.

METHODS. RPE grafts were prepared from eyes of neonatal C57BL/6 or C57BL/6 gld/gld (deficient in CD95 ligand expression) mice. These grafts (or conjunctival grafts as positive controls) were transplanted into the anterior chamber, the subretinal space, the subconjunctival space, and underneath the kidney capsule of histoincompatible BALB/c mice. Transplant survival was evaluated by histology at selected time points after engraftment. Recipients were tested for acquisition of C57BL/6-specific delayed-type hypersensitivity (DH) and for the ability to suppress DH.

RESULTS. Allogeneic neonatal RPE grafts from normal donors showed significantly enhanced survival at all graft sites compared with conjunctival grafts. However, allogeneic RPE cell grafts from gld/gld mice were rapidly rejected after transplantation beneath the kidney capsule. Allogeneic RPE grafts placed in extraocular sites induced systemic DH directed at donor alloantigens, whereas RPE allografts placed intraocularly induced suppression of systemic DH.

CONCLUSIONS. Allogeneic neonatal RPE grafts, through constitutive expression of CD95 ligand, promote their own survival at heterotopic sites. Paradoxically, these grafts also display immunogenicity. Thus, neonatal RPE tissue owes its immune privilege to the capacity to prevent immune rejection rather than to inhibit sensitization. (Invest Ophthalmol Vis Sci. 2000;41:3467–3473)

Our laboratory has been studying the immunobiology of retinal tissues as transplants. One aspect of these studies relates to the immune status of the subretinal space as the orthotopic site for transplants of both neuronal retina and retinal pigment epithelium (RPE). Recently, we were able to show that the subretinal space is an immune-privileged site that allows prolonged survival of allogeneic tumor cells compared with the survival of the same tumor cells at a conventional site.1,2 Another aspect of our studies relates to the immune status of retinal tissue itself—that is, whether it is immune privileged. Particularly, we are interested in knowing whether retinal pigment epithelium acts as an immune-privileged tissue. To date, evidence bearing on this issue has been complicated by the fact that grafts of RPE have usually been placed within the eye, a site that displays its own immune privilege.3–14 Tissues with no inherent immune privilege are often able to survive within the eye,15–16 and therefore examining the fate of RPE grafts in the eye cannot separate graft-derived factors from site-derived factors.

Testis grafts containing Sertoli cells and epithelium-deprived corneal grafts have recently been shown to possess inherent and unique properties usually associated with immune-privileged tissues.17–19 Allogeneic testis grafts placed beneath the kidney capsule are not rejected, whereas allogeneic grafts of skin and islets of Langerhans are promptly destroyed at this site.17,18 Similarly, epithelium-deprived corneal allo-grafts survive indefinitely when placed beneath the kidney capsule.19 Sertoli cells within testis and endothelium of the cornea constitutively express CD95L, and in both cases, expression of CD95L has been shown to protect grafts placed beneath the kidney capsule from immune rejection.17,19 Many cells within the eye other than those in corneal endothelium express CD95L, including cells in the RPE.20–22 This raises the possibility that RPE cells, as a tissue, may possess their own inherent immune privilege.

We report the results of a series of studies that examine the fate of allogeneic neonatal RPE transplants placed at heterotopic (extraocular) sites and evaluate the ability of RPE grafts placed heterotopically as well as intraocularly for their capacity to sensitize recipients to donor alloantigens. The results reveal neonatal RPE cells to be immune-privileged tissues, even though they display the capacity to induce allosensitization when grafted extraocularly.

METHODS

Animals

Adult female BALB/c, C57BL/6, and C57BL/6 gld/gld (B6.gld) mice, aged 6 to 8 weeks, were obtained from the animal facilities at the Schepens Eye Research Institute or from Jack-
son Laboratories (Bar Harbor, ME). Mice were maintained in a common room of a vivarium. Inoculations, injections, clinical examinations, and enucleations were conducted under anesthesia induced by intraperitoneal injection of ketamine (Ketalar; Parke Davis, Paramus, Nj) at 0.075 mg/g body weight, and xylazine (Rompun; Phoenix Pharmaceutical, St. Joseph, MO) at 0.006 mg/g body weight. All experimental procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Five mice were used for each experimental group, and experiments were repeated at least twice with similar results.

Preparation of Neonatal RPE Cells

For isolation of RPE cells as an intact monolayer a modification of the technique of Chang et al. was used.3-23 Within 24 hours after birth, neonatal C57BL/6 mice were decapitated, and the eyes were enucleated. For this purpose the still-closed eyelids were opened with scissors, and the eyes were separated from the optic nerve using forceps. After incubation for 20 minutes at 37°C in Hanks’ balanced salt solution (HBBS) with 2% dispase (Grad II; Boehringer–Mannheim, Indianapolis, IN), the eyes were intensely rinsed with Dulbecco’s modified Eagle’s medium (DMEM). Under a dissection microscope, the eyes were opened along the limbal region, and the anterior segment was discarded. Sclera and choroid were carefully separated from the RPE cells, and the lens was removed. After incubation in HBBS for 15 minutes, the RPE cell layer could be removed easily from the neural retina and immediately used for transplantation.

Preparation of Neonatal Conjunctival Grafts

Neonatal conjunctiva was excised from freshly enucleated eyes of neonatal C57BL/6 mice within 24 hours after birth. The conjunctiva was separated from the globe with surgical scissors and was intensely rinsed in HBSS. Small pieces of excised conjunctiva (approximately 5 × 5 mm) were immediately used for transplantation.

Intraocular Injections

For anterior chamber (AC) and subconjunctival injections, a 0.3-mm penetrating wound was made in the peripheral portion of the cornea, 1 mm posterior to the limbus, or in the fornix of the conjunctiva, respectively, using a 30-gauge needle. For injections in the subretinal space, the temporal conjunctiva was opened parallel to the limbus, and the eye was rotated to expose the posterior part of the sclera. A 0.3-mm tangential sclerotomy was made with a 30-gauge needle, a retinal bleb was created with 0.5 μl HBSS, and then the intact sheets of RPE cells were injected into the wound.1,2 Careful evaluation of the transplantation method described by Jiang et al.3 and Jiang and Streilein4 indicates that RPE sheets injected with great pressure through glass needles are disrupted, and many cells in the injected tissue die. Therefore, we modified the method to obtain an intact sheet of RPE as a graft. For subconjunctival transplantation, RPE sheets were carefully placed underneath the conjunctiva through the penetrating wound to ensure grafting of an intact sheet of RPE.

Transplantation under the Kidney Capsule

After anesthesia, the back of the animals was opened along a line parallel to the spinal cord. Under visualization with a dissecting microscope, the peritoneum was opened, and the kidney was identified and moved outside the peritoneum. A small incision was made into the kidney capsule using a 30-gauge needle, and intact RPE cell sheets were placed underneath the kidney capsule. The kidney was returned to its physiological position, and the wound was closed using a surgical stapler.

Assay for DH

To assess delayed-type hypersensitivity (DH), an ear-swelling assay was used as previously described.1,3 Briefly, 5 × 10^5 C57BL/6 spleen cells (irradiated with 2000 R) were injected into the left ear pinna of the mice. The right ear served as an untreated control. Both ear pinnae were measured immediately before injection and 24 hours later with an engineer’s micrometer (Mitutoyo, Tokyo, Japan). The measurements were performed as triplicates. Results were expressed as specific ear swelling = (24-hour measurement − 0-hour measurement) experimental ear − (24 hour measurement − 0-hour measurement) negative control ear × 10^-3 mm. A two-tailed Student’s t-test was used and significance assumed if P < 0.05. Mice subcutaneously immunized 1 week previously with 2 × 10^5 C57BL/6 spleen cells served as positive controls.

Histology

Tissue for histologic evaluation was immediately fixed in 10% buffered formalin and embedded in paraffin. Five micron sections were cut, and tissue was stained with hematoxylin-eosin and periodic acid–Schiff. Grafts were then evaluated by light microscopy. Microscopic examination of sections prepared from all five samples of each panel tested revealed similar histologic patterns. Figures 1 through 5 are representative photomicrographs.

RESULTS

Fate of Neonatal Retinal Pigment Epithelial Cell Grafts at Heterotopic Tissue Sites

We first investigated the vulnerability to rejection of allogeneic neonatal RPE cell grafts implanted as intact sheets at heterotopic sites (subconjunctival space and beneath the kidney capsule) that are known not to be immune privileged. RPE tissue grafts were prepared from eyes of C57BL/6 mice within the first 24 hours after birth. These grafts were then placed in the subconjunctival space or beneath the kidney capsule of C57BL/6 (syngeneic) and BALB/c (allogeneic) mice. For comparison, allogeneic conjunctival grafts were obtained from neonatal C57BL/6 donor eyes, then similarly implanted at these heterotopic sites. Panels of recipients (five each at each time point) were killed at 1, 2, 4, 8, and 12 weeks after grafting, and the graft sites were prepared for histologic analysis. Syngeneic neonatal RPE cells implanted beneath the kidney capsule and into the subconjunctival space survived throughout the observation interval in all examined animals (Fig. 1).

In similar fashion, allogeneic RPE grafts were accepted in the subconjunctival space and beneath the kidney capsule of all BALB/c mice (Figs. 2, 3A, 3B). Apart from occasional lymphocytes in the stroma surrounding these grafts (2/10 grafts after 12 weeks), no inflammatory infiltrate was observed. The grafted RPE cells retained their pigment and formed typical monolayers (Figs. 2, 3A). In some instances (6/50 grafts), the
RPE sheets were folded during the grafting process, and this produced pseudocysts that were also devoid of inflammatory cell infiltrates (Fig. 3B). By contrast, all allogeneic conjunctival grafts underwent rejection at both heterotopic sites. Massive inflammatory cell infiltrates were observed at 1 week after transplantation, and by 2 weeks, virtually all recognizable conjunctival tissue had been destroyed (Figs. 4A, 4B). These results indicate that allogeneic neonatal RPE displays properties usually ascribed to immune-privileged tissues, in that they resist rejection when placed at sites in the body where conventional tissue grafts are promptly rejected.

**Fate of Neonatal RPE Cell Grafts from B6.gld Mice at Heterotopic Tissue Sites**

It has recently been reported that Sertoli cells within the normal testis constitutively express CD95 ligand (CD95L), and that expression of this molecule protects allogeneic testis grafts from rejection beneath the kidney capsule.\(^\text{17}\) CD95L is also constitutively expressed on many ocular tissues, including RPE.\(^\text{20–22}\) We next tested whether expression of CD95L on neonatal RPE tissue is important in the ability of RPE grafts to survive at this heterotopic site. RPE tissue was removed from eyes of neonatal C57BL/6 gld/gld mice (B6.gld) and implanted beneath the kidney capsule of BALB/c mice. When these graft sites were examined histologically at 1 week, massive leukocytic infiltrates were observed in all grafts. At 2 weeks after grafting, all the RPE grafts were completely destroyed (Figs. 5A, 5B). These results indicate that constitutive expression of CD95L on allogeneic neonatal RPE cells protected the grafts from rejection at a heterotopic site, implying that CD95L makes a major contribution to the immune-privileged status of neonatal RPE.

**Immunogenicity of Allogeneic Neonatal RPE Grafts Placed at Various Tissue Sites**

Fetal RPE cells express readily detectable levels of class I alloantigens on their surfaces.\(^\text{24–25}\) and they probably also express minor histocompatibility antigens. Despite their remarkable ability as grafts to avoid rejection at heterotopic sites, we were interested in knowing whether allogeneic neonatal
RPE allografts were capable of sensitizing recipient mice when implanted at heterotopic and orthotopic sites. To examine this issue, RPE tissue grafts were prepared from eyes of neonatal C57BL/6 donors, and these grafts were implanted at heterotopic sites (beneath the kidney capsule, subconjunctivally, into the anterior chamber) and an orthotopic site (subretinal space). BALB/c mice bearing allogeneic neonatal RPE grafts at these sites for 4 weeks were tested for acquisition of delayed hypersensitivity directed at C57BL/6 alloantigens. Irradiated C57BL/6 spleen cells ($5 \times 10^5$) were injected into the ear pinnae of graft recipients and into the ear pinnae of positive control BALB/c mice that were immunized subcutaneously 1 week previously with C57BL/6 spleen cells. Ear-swelling responses measured 24 hours later in a representative experiment (of three) are presented in Figure 6.

After implantation of neonatal allogeneic RPE cells into the subconjunctival space and beneath the kidney capsule, significantly increased ear-swelling responses compared with negative control were observed for recipient mice. By contrast, mice bearing allogeneic neonatal RPE grafts in the anterior chamber or in the subretinal space displayed ear-swelling responses no different from those in negative controls. These findings indicate that allogeneic neonatal RPE cells are able to sensitize recipient mice if the grafts are placed at non-immune-privileged sites, but not if the grafts are placed at sites known to be immune privileged.

**Capacity of Allogeneic Neonatal RPE Grafts to Suppress DH**

Transplantation of histoincompatible cells into the immune-privileged compartments of the eye usually leads to the induction of a deviant immune response to antigens expressed on the donor tissue. Suppressed donor-specific DH is one manifestation of this deviant response.1,26 We next examined whether immune-privileged tissues such as neonatal RPE grafts were capable of promoting suppression of DH when grafted intraocularly and when placed at extraocular sites. We transplanted neonatal C57BL/6 RPE cells into BALB/c mice at various sites: in the subconjunctival space, beneath the kidney capsule, in the anterior chamber, and in the subretinal space. Four weeks after transplantation the recipients were immunized subcutaneously with C57BL/6 spleen cells and assayed for donor-specific DH 1 week later. As revealed by the results of a representative experiment displayed in Figure 7, DH was significantly impaired in mice bearing intraocular RPE grafts, compared with positive control animals. By contrast, mice bearing allogeneic RPE transplants within the conjunctiva or under the kidney capsule showed vigorous DH responses, comparable with those of positive controls. These results indi-

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**FIGURE 4.** Histopathologic appearance of C57BL/6 conjunctival grafts 1 (A) and 2 (B) weeks after transplantation underneath the kidney capsule of allogeneic BALB/c mice. Massive cellular infiltration with destruction of the conjunctival grafts. Hematoxylin-eosin; original magnification, $\times 240$.

**FIGURE 5.** Histopathologic appearance of C57BL/6 gld/gld RPE cell grafts 1 (A) and 2 (B) weeks after transplantation underneath the kidney capsule of allogeneic BALB/c mice. Massive cellular infiltration of the RPE cells grafts with almost complete destruction. Periodic acid–Schiff stain; original magnification, $\times 480$.
cate that implantation of neonatal allogeneic RPE cells into ocular immune-privileged sites (anterior chamber, subretinal space) suppressed DH in recipients, whereas similar grafts implanted at non-immune-privileged sites (subconjunctival space and beneath the kidney capsule) did not. Thus, in these experiments the capacity to suppress DH rests with the immune-privileged status of the graft site, rather than with the tissue as a graft.

DISCUSSION

The technical feasibility of transplanting suspensions and sheets of RPE into the subretinal space has been adequately documented. There is evidence in both experimental animals and in humans that such transplants can survive and even retard or reverse retinal degeneration. In most published reports, the transplanted RPE are histoincompatible with the recipient and have the potential of inducing immunity and of undergoing immune rejection. Therefore, it is important to define the immune properties of RPE and to understand the vulnerability of this highly specialized tissue to immune rejection. To that end, the results of our experiments indicate that neonatal RPE tissue displays inherent immune privilege. When placed beneath the kidney capsule, allogeneic neonatal RPE grafts survived for longer than 12 weeks, retained pigment intracellularly, and were devoid of evidence of inflammation. Moreover, in a fashion similar to two other immune-privileged tissues—testis and cornea—neonatal RPE allografts survived at this heterotopic site because they expressed CD95L. These results indicate the important role that CD95L plays in creating and maintaining immune-privileged tissues.

Although our results are similar to those reported in certain previous studies, they are not similar to those in all studies. For example, our allogeneic neonatal RPE sheets placed underneath the kidney capsule and subconjunctivally survived indefinitely, whereas allogeneic and even syngeneic RPE grafts have been reported to be rapidly destroyed in the subconjunctival space. The method of graft placement...
seems to play a key role in the graft’s success. For example, Grisanti et al.\(^3\) used fetal cultured RPE cells that were destroyed in the subconjunctival space of syngeneic mice. These cells were not only cultured and labeled with bromodeoxyuridine, but were injected as a single-cell suspension. Our results showing that intact sheets of fetal RPE survived as allografts indefinitely beneath the kidney capsule and subconjunctively emphasize the crucial role that integrity of the RPE layer plays in ensuring graft survival. We suspect that the reason Jiang et al.\(^3\) and Jiang and Streilein\(^4\) found that allogeneic RPE grafts were destroyed in the subconjunctival space reflects the non-specific disruption of the RPE monolayer and death of some of the cells that occurred during injection, rather than the ability of the RPE to induce an immune response that destroys it. Experiments to answer this interesting and important aspect are under way.

However, our results clearly indicate that the neonatal RPE grafts possessed immunogenic potential. On the one hand, allogeneic neonatal RPE grafts placed in the anterior chamber or in the subretinal space induced suppression of systemic DH, one characteristic of immune deviation. Because this phenomenon is an antigen-specific modification of the systemic immune response, we can conclude that the neonatal RPE grafts used in our experiments expressed relevant alloantigens. On the other hand, allogeneic neonatal RPE grafts placed beneath the kidney capsule or subconjunctively induced DH that was elicited by donor cells. Such an outcome could only occur if the graft expressed donor alloantigens in a form capable of inducing sensitization. We conclude that allogeneic neonatal RPE tissue is immunogenic, that this property places the tissue as a graft at risk of immune rejection, and that the constitutive expression of CD95L protects the graft from that destructive outcome.

We were somewhat surprised to find that allogeneic neonatal RPE grafts placed beneath the kidney capsule were accepted, yet induced systemic donor-specific immunity. In this regard, they differ from allogeneic, epithelium-deprived cornea grafts placed beneath the kidney capsule. Not only are these cornea grafts accepted indefinitely, but they fail to induce immunity against donor major histocompatibility complex (MHC) antigens.\(^9\) As is the case with RPE grafts, CD95L-deficient cornea grafts of this type are promptly rejected beneath the kidney capsule, but only recipients of CD95L-deficient corneas acquire immunity directed at donor MHC (but not minor histocompatibility) antigens. We are at a loss to explain this difference in immunogenicity between heterotopic neonatal RPE grafts and cornea grafts, but we suspect that it arises from differences in physiologic properties of RPE cells and corneal endothelial cells.

It is of interest that allogeneic neonatal RPE grafts placed heterotopically did not promote suppression of DH to donor alloantigens. Only intraocular RPE transplants induced suppression of DH, a result that has been described.\(^3,4\) We are forced to conclude that the capacity to facilitate suppression of DH is a property of an immune-privileged site, but not of an immune-privileged tissue. Why should that be the case? Although CD95L expression is clearly important in protecting heterotopic RPE grafts from immune rejection, RPE cells possess other features that might also contribute to the immune-privileged phenotype. As a tissue the RPE is devoid of bone marrow-derived cells that could function as passenger leukocytes (and thereby promote alloimmunity).\(^32\) As a cell layer, RPE cells secrete immunomodulatory factors (such as transforming growth factor-β) that have the potential to create a local immunosuppressive microenvironment.\(^32-36\) Moreover, RPE cells have recently been shown capable of inducing apoptosis among activated T cells.\(^22\) Perhaps these features have no relevance when RPE grafts are placed at heterotopic sites (such as the kidney capsule), but they may be important when RPE grafts are placed in the subretinal space. This may be especially true if the subretinal space has been ravaged by degenerative and/or inflammatory diseases. Thus, in the long run, the immune-privileged status of RPE may promote the tissue’s survival as a graft for reasons beyond mere expression of CD95L.

**Acknowledgments**

The authors thank Jacqueline Doherty for expert managerial assistance and Marie Ortega for care of the animals.

**References**


