Retinal Pigment Epithelial Transplants and Retinal Function in RCS Rats

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Purpose. To determine if retinal pigment epithelium (RPE) transplantation maintains visual function in Royal College of Surgeons (RCS) strain of rats.

Methods. Twelve RCS rats received RPE transplants at 16 to 20 days after birth. The retinas were studied electrophysiologically and histologically from 3 to 10 months after transplantation and compared with 11 RCS controls and 11 normal rats of comparable ages. A microelectrode was guided to the transplant site visible by its pigmentation in the albinotic RCS retina to detect responses.

Results. Spontaneous ganglion cell activity was present in all retinas. Ganglion cell responses to light were detected in 9 of the 12 transplant eyes but not in any of the 11 controls. 96, 44, 140 units were encountered and 30%, 0%, 97% were driven by light respectively in transplant, control, and normal retinas. In transplants 36%, 29%, and 28% were driven at 3 to 4, 6 to 7, and 10 months after transplantation, respectively. Intraretinal ERGs with both a- and b-waves were recorded in 5 of the 8 transplants studied. None of the RCS controls studied had an IERG. The average IERG was 2.5 μV (SD = 1.9) in transplants and 59 μV (SD = 19) in normal retinas. The electrode track was traced to the transplant site in six of the seven retinas that were responsive to light and examined histologically.

Conclusion. RPE transplants to RCS rats maintain retinal function in the transplant site for long periods of time. Invest Ophthalmol Vis Sci. 1993;34:3068-3075.

Transplantation of normal retinal pigment epithelium (RPE) can prevent photoreceptors from degenerating in the Royal College of Surgeons (RCS) strain of rats.1-2 Because transplanted RPE was known to resume phagocytosis of host outer segments3 and because the inability of the RCS rat's RPE to phagocytize outer segments was responsible for the retinal degeneration,4 it was reasonable to expect that RPE transplants might ameliorate this disease.5 This hypothesis may be too simple, however, because photoreceptors are saved at some distance from the transplanted RPE7,8 and the local administration of a trophic factor, basic fibroblastic growth factor,9 to some degree sham surgery10 can also save these photoreceptors from degenerating.

The surviving photoreceptors have been shown to retain several anatomic characteristics necessary for their normal function. Their outer segments grow at a normal rate11 and are being phagocytized by the transplanted RPE7,8 although their length appears to be shorter than normal.11 These photoreceptors have outer segments, which are surrounded by an extracellular matrix11 and contain visual pigment protein, opsin,12 and have inner segments with membrane Na+/K+ ATPase.12

Until now, however, there has been no evidence that the receptors respond to light and transmit their signals to other retinal neurons, which is essential for vision. In earlier experiments, we examined this question using the corneal electroretinogram (ERG) but we were unable to demonstrate any response despite the presence of surviving photoreceptors.8 We interpreted this to indicate that the number, the status, or both, of the photoreceptors saved was inadequate to generate a detectable corneal ERG. In this paper, we
examine this hypothesis by introducing a microelectrode directly into the transplant site to record local ganglion cell activity and the intraretinal ERG (IERG). A brief abstract describing our results has been published.13

MATERIALS AND METHODS

Electrophysiologic studies were performed on three groups of congenic rats. Twelve were tan-hooded, pink-eyed (p/p) RCS mutants that received RPE transplants in one eye and were studied physiologically and then sacrificed—4 at 3 to 4 months, 4 at 6 to 7 months, and 4 at 10 months after transplantation. Eleven were pink-eyed RCS mutants that received no transplants, and eleven were congenic normal pigmented (p/+) rats, both of which were studied at comparable ages to those receiving transplants.

Controls in which only sham surgery alone was performed were not done because the sham effect is not as effective in saving photoreceptors as RPE transplants,14 a phenomenon we have also noticed (unpublished observations, 1992). The RPE transplantation was performed at 16 to 20 days after birth, when photoreceptor degeneration is just beginning. Normal donor rats (2 to 3 weeks old) congenic to the host RCS rats were killed with nembutal, and their eyes were removed and bathed in Hank’s solution. The anterior segment, including the lens and vitreous, was removed, and the neural retina dissected away with the aid of a surgical microscope. The eye cup was transferred to a Hank’s solution without Ca++ and Mg++ and containing 0.25% trypsin and kept at 37°C for 40 minutes to dissociate the RPE layer. The dissociation was stopped by washing the eye cup with minimum essential medium (MEM) solution containing 20% fetal bovine serum. RPE cells were then dislodged from Bruch’s membrane using gentle trituration and pipetted into a test tube. A small aliquot of this solution was examined in a hemacytometer for purity and the number of RPE cells/ml measured. The cell suspension was centrifuged at 1000 RPM for 5 minutes. The supernatant was discarded and the concentrated RPE cell pellet resuspended in 2 to 3 μl MEM solution and pipetted into a Petri plate. A glass pipette with a tip diameter of 120 to 130 μm containing a balanced salt solution was used to suck up this solution after first drawing up an air bubble to block back diffusion.

The RPE cell solution was injected into the subretinal space transchoroidally through a small scleral incision along the equator of the eye. After injection, the retina was examined by rotating the rat and observing the fundus through the dilated pupil while compensating for corneal curvature with a hand-held contact lens. Optionally, a small retinal detachment containing the bolus of pigmented cells was seen. The rats were anesthetized with ketamine (80 mg/kg, intraperitoneally) and xylazine (10 mg/kg, intraperitoneally). Only one eye received the transplants; the other eye was untouched. One transchoroidal transplantation usually produces several distinct patches of pigmentation in the retina of the host because of the unpredictable diffusion of the transplant cells in the subretinal space.

The electrophysiologic experiments were performed at least 3 months after transplantation using the same method of anesthesia. An insulated tungsten microelectrode was introduced into the eye through a scleral incision at a point opposite the transplant site, which could be easily observed in the fundus through the dilated pupil as subretinal pigmented areas beneath the retinal vessels (Fig. 1). The trajectory of the microelectrode was observed in the vitreous and corrected around its entrance point into the eye to guide it to the transplant site. Because its pivotal point was preset to be at the electrode’s entrance into the eye, no mechanical movement of the eye resulted from a change in its trajectory (Fig. 2). About 10 or more penetrations were made in or near the transplant site in each eye or in comparable areas selected in the control eyes. Impulses of retinal ganglion cells at or just below the surface of the retina were examined and monitored on an oscilloscope and with a loudspeaker. We adjusted the microelectrode to isolate the responses of only one, or occasionally two, distinguishable units to full-field light stimuli. Photographs of selected responses were obtained using a Polaroid (Cambridge, MA) camera and a storage oscilloscope. Light stimuli were obtained from a Leitz (Wetzlar, Germany) projector with an electronically controlled shutter. The duration of the pulse was usually 100 ms in duration, but this was often changed to determine if a unit was responding to the on- or the off-phase of the light pulse. The light was led to the pupil of the rat by a fiber-optic bundle, 1 cm in diameter, which completely filled the pupil plane with light. The energy and the wavelength of the light was changed with calibrated neutral density and waveband selective absorption filters (Wratten, Eastman Kodak, Rochester, NY). Four different wave bands were used with their nominal peak transmissivity at, 450 nm (blue), 534 nm (green), 593 nm (yellow), and 633 nm (red). These are Wratten filters 97, 61, 21, and 29, respectively. The energy transmitted by each spectral filter was measured at the output of the fiber-optic bundle using a Tetronix (Beaverton, OR) JE512 irradiance probe. By this means, the spectral sensitivity of units as well as the electroretinogram (ERG) could be determined based on a constant threshold response criterion.

Vitreal and intraretinal ERGs were also recorded from the same microelectrode facilitated by a Nicolet (Madison, WI) CA-1000 averaging computer. Gan-
glion cell activity, either light driven or spontaneous, was our guide to putting our microelectrode in the optimum position to detect the intraretinal ERG (IERG). The IERG becomes maximal just after the impulses of ganglion cells can no longer be detected. Without this clue, it is difficult to sense one's position in an otherwise unresponsive retina. About 10 to 100 responses to the same stimulus were usually averaged, depending on the amplitude of the signal, to improve the signal-to-noise ratio of each response and recorded by an X-Y plotter. A response of 0.1 \( \mu \)V was easily detectable. Each rat retina was partially light adapted during the recording session although in some cases we examined the same unit or the same ERG for about 1 hour, during which time the retina became more dark adapted. Because each rat was examined in the same way, the comparison of responses in different rats involved quasi-identical states of retinal adaptation.

After every physiological experiment, the rat was sacrificed by an overdose of anesthesia. The eyes were removed and placed in a buffered solution of 3% glutaraldehyde and kept at 4°C for at least 24 hours. Then the eyes were washed, and the section of the retina containing the transplant site was cut out with the aid of a surgical microscope and processed for light and, in selected samples, for electron microscopy. Semi-serial sections were made through the transplant site to identify the microelectrode track and determine the effects of the transplants on photoreceptor survival. We were unable to examine 2 of the 9 light responsive transplant retinas because of an error in the embedding procedure. One unresponsive retina that had received a transplant was also examined.
RESULTS

Ganglion Cell Responses

The impulses of ganglion cells were detected in most retinal regions and stable recordings could usually be maintained for as long as 30 to 60 minutes, implying that there was very little eye movement in these anesthetized rats. Spontaneous activity was present in both normal, transplant, and control eyes. In the normal retina, almost every ganglion cell encountered by its spontaneous activity could also be driven by full-field light flashes.

Figure 3 shows ganglion cell responses recorded from the normal retina. The cell was inhibited by the light stimulus and excited when the stimulus went off. This response could be recorded over at least three logarithmic units of light intensity; as light intensity decreased, the duration of the inhibition decreased. We determined the action spectra of such a ganglion cell based on a constant near-threshold response criterion to four different spectral stimuli (see Fig. 4). The response appears to be generated by a receptor system most sensitive to greenish-blue light, with a steep fall-off in sensitivity at long wavelengths. The responses to both spectral extremes (450 and 633 nm in Fig. 3) are similar, implying a similar receptor system is mediating these responses. We also encountered units responding to the on-phase of the light; such a unit is visible to the strongest flash at 593 nm in Figure 3 and also in Figures 5 and 6. These on-units were the most frequently encountered. They tended to have tonic responses but were less sensitive than the off-units.

In 76 regions into the retinas of 11 normal rats, we encountered 140 units (1.84 units per penetration examined), of which 133 were responsive to light. In 94 regions into the retinas of 11 control RCS rats, we encountered 44 units (0.47 units per penetration examined), of which none were responsive to light. We detected ganglion cells responsive to light in 9 of the 12 transplant eyes studied. In 156 regions into the transplant sites of these eyes, we encountered 96 units...
TRANSPLANT (11 mos)

CONTROL (6 mos)

FIGURE 5. Ganglion cell responses from RCS rats. The upper trace shows responses to the light stimulus from two cells in a transplant area of an RCS rat 11 months of age. The arrow points to impulses of smaller amplitude from an on-cell; the large impulses (asterisk) are from an off-cell, inhibited by the light pulse, and excited by the termination of the light. Differences in both amplitude and waveform can sometimes allow us to distinguish two or more different cells in the same recording. The lower trace shows the spontaneous activity of a ganglion cell in a control RCS rat, 6 months of age; this cell did not respond to the light stimulus. The light stimulus (100 ms in duration) is shown by the horizontal line (below).

(0.62 units per penetration examined), of which 29 were responsive to light. The responses detected at relatively long times were similar to those at earlier times after transplantation, implying that function remained stable for a long time. Figure 5 illustrates two light-responsive units in the transplant site compared to a spontaneously active but unresponsive unit in the control retina. One of the units (arrow) is excited by the on-phase of the stimulus, whereas the other (asterisk) is excited by the off-phase of the stimulus. Figure 6 illustrates such light-responsive units detected in transplants at different times after transplantation surgery.

All the units responsive to light within the transplant site were about a thousand-fold less sensitive than those in the normal retina, which made them difficult to examine with spectrally narrow stimuli. Figure 4 shows the spectral sensitivity of one unit detected in a transplant site 4 months after surgery, in which responses to green and yellow stimuli could be obtained. The sensitivity was greater to green than to yellow with the difference resembling that found for units in the normal retina.

Electroretinograms

We attempted to record ERGs from the vitreous of every rat but were only successful in detecting such responses in the normal eye. Figure 7 (left, above) illustrates a vitreal ERG from a normal rat. There is a large, positive b-wave followed by a larger late negativity; an initial negative a-wave is only seen with very strong stimuli. After penetrating the retina to a depth that is just beyond the level where ganglion cell responses are detectable, the IERG reverses in polarity and increases in amplitude. The strongest stimulus (593 nm) produces a small initial (positive) a-wave and a more oscillatory (negative) b-wave (Fig. 7, left, below). The action spectrum of the IERG resembles that of the ganglion cell responses both in sensitivity and shape (Fig. 4).

We detected an IERG response in all eight normal rats studied at positions similar to where the transplants were located. The average amplitude of the intraretinal b-wave was 59 μV (SD = 19) to a maximum yellow stimulus. We attempted to record the IERG in eight transplant eyes and were successful in five of

FIGURE 6. Ganglion cells responsive to light in the transplant area of different RCS rats at age 5, 8, and 11 months. The lowest trace is from the same rat whose responses are shown in Fig. 4; there are two different cells responding to the same stimulus here. The light stimulus (100 ms) is shown by the horizontal line (below).

FIGURE 7. Electroretinograms recorded from a normal rat (left) and from the transplant area of an RCS rat (right) at 11 months. The vitreal ERG is shown on the left (above) from the normal rat, the intraretinal ERG to blue (450 nm), yellow (593 nm), and red (633 nm) stimuli are shown on the left (below). There is no detectable vitreal ERG from the RCS rat but an IERG is detectable to all spectral lights. The duration of light stimulation is identical to those of the previous figures. A positive response produces an upward deflection. In the IERGs, the conventional a- and b-waves of the responses are reversed in polarity. The light stimulus is 100 ms in duration and begins with the trace.
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these attempts. The response amplitudes were much lower than those of the normal retina; the average amplitude was 2.5 μV (SD = 1.9). The peak (implicit) time of the b-wave was also slightly slower than that of the normal retina (Fig. 7, right). The IERG had both a- and b-wave components, and these were similar at different wavelengths of stimulation. An action spectrum of an IERG obtained from a transplant site 11 months after surgery is shown in Figure 4. It is similar to that of the ganglion cell response, having its highest sensitivity in the blue-green part of the spectrum.

In 14 regions in the retinas of three control RCS rats, we were unable to detect any IERG even though our sensitivity was sufficient to detect a 0.1 μV signal.

Histology

Eight RCS rat retinas examined electrophysiologically after transplantation were also studied histologically. Seven of these retinas had light-induced ganglion cell responses and four had IERG responses. One retina without detectable light-induced responses was also examined. Photoreceptor saving was detectable in all eight retinas. The rescued photoreceptors appear as small regions above or near transplanted RPE cells located within the subretinal space, either on or close to Bruch’s membrane, on top of the host RPE, or among the outer segments. The pigment granules identify the transplanted RPE in this otherwise albinotic retina. Figure 8 shows light micrographs of photoreceptor saving at 3 (A), 4 (B), 7 (C), and 10 (D) months after transplantation. In all cases, the photoreceptors are found in close proximity to the transplanted RPE. In Figure 8B, the magnification is low enough to show two patches of photoreceptor saving adjacent to the pigmented transplants; areas on both sides that are more distant from the transplanted RPE have no photoreceptor saving. An electromicrograph (Fig. 9) shows the intimate relationship of the outer segments of rescued photoreceptors to the pigmented transplant cells. All the rescued photoreceptor outer segments appear to be those of rods. Evidence of the electrode’s track was found within the transplant area in seven of the eight specimens by a break either in the inner limiting membrane (Fig. 8B, arrow) or in Bruch’s membrane and usually by concomitant signs of a small fresh hemorrhage (Figs. 8A–C, arrows).

Table 1 shows the relationship between the amount of photoreceptor saving and the strength of the light-induced electrical activity. Three of the four retinas with the most saving showed detectable light-induced ganglion cell responses and had IERG responses, whereas four of the five retinas with less photoreceptor saving had only ganglion cell activity but no detectable IERGs.

DISCUSSION

The results demonstrate that photoreceptors destined to degenerate were not only anatomically rescued by RPE transplant from this degeneration but also remained able to respond to light and transmit signals to higher-order retinal neurons. There are three parameters of function that these electrophysiologic experiments reveal. The presence of an IERG a-wave indicates that the photoreceptors are transducing light into an electrical signal. The presence of an IERG b-wave indicates that these light-induced photoreceptor responses also generate activity in second-order retinal neurons. The existence of light-evoked ganglion cell activity indicates that these signals are reaching the output layer of the retina, presumably through the synaptic activity of bipolar cells. The pattern of ganglion cell responses resembles that found in the normal rat retina, implying that the functional circuitry of these cells has not been markedly altered by the degeneration.

The major differences between the responses detectable in the transplant site and those detectable in the normal retina is sensitivity. The reduced sensitivity of the transplant responses can be explained by the relatively small numbers of photoreceptors saved and perhaps by their shorter than normal outer segments. This factor must be responsible for making it difficult to record the ERG in the vitreous or at the cornea. This is unfortunate because it prevents an easy, noninvasive way to follow the effects of transplantation on receptor function.

The prime evidence that these light-induced responses are in fact due to the transplants is the absence of any responses in the untreated control retinas. Previous studies, however, have found light-responsive units in the optic nerve15 and optic tract16 of RCS rats that are at least 5 months of age. Behavioral studies17 have also revealed some visual function in RCS rats 2 years of age. We were unable to find any light-responsive ganglion cells in the control RCS retinas. We detected 44 spontaneously discharging ganglion cells in

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FIGURE 8. Light micrographs of transplant sites in which electrical responses were detected and the microelectrodes track was identified at 3 (A), 4 (B), 7 (C), and 10 (D) months after transplantation. Some sign of the microelectrode's track can be seen by the evidence of recent subretinal hemorrhage in A, B, and C (white outlined arrows). In B, the microelectrode's track can also be identified by the break in the inner limiting membrane (black arrow).

Previous investigators of the optic nerve \textsuperscript{15} and optic tract \textsuperscript{16} have not commented on what fraction of the visual units they encountered were unresponsive. Based on our own results, it would seem that this fraction is relatively large in the adult RCS rat. In addition, the optic tract neurons detectable in 5-month-old RCS rats were 4 to 5 logarithmic less sensitive than normal units in the dark-adapted rat retina. \textsuperscript{16} Our experiments were performed in partially light-adapted retina. This enabled us to see the pigmented transplant site to which we guided our electrode; we used a similar examining light in the control RCS rat.

In the previous studies of RCS rats at 5 months of age, the action spectra of detectable units in the optic tract had shifted from rods to cones. \textsuperscript{16} We attempted to determine if there were more cone than rod responses in the transplant site. This proved difficult because of the reduced sensitivity of these responses. The evidence we did obtain indicated that the spectral sensitivity was approximately parallel to what we found in the normal retina and, therefore, no more influenced by cones than the normal responses were under similar conditions of retinal adaptation.

We were surprised to record so many spontaneously discharging ganglion cells in the control RCS retina because earlier reports on cat retina indicated that spontaneous ganglion cell activity ceased after the photoreceptors degenerated. \textsuperscript{18} This is obviously not true in the RCS rat, even at a relatively advanced stage of the disease. This supports recent histologic results that reveal the presence of ganglion cells and optic nerve fibers in patients with advanced forms of human retinal degenerations.\textsuperscript{19}

The major finding of this paper, however, is that the photoreceptors that are obviously being saved from degeneration by retinal epithelial cell transplantation are also able to respond to light and to commu-
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FIGURE 9. Electronmicrograph of a transplant area 3 months after surgery, illustrating the presence of numerous rod outer segments oriented toward a pigmented transplanted epithelial cell, which is on Bruch's membrane. The horizontal bar signifies 2 μm; the magnification is ×2475.

nicate with the part of the brain that controls sight. It adds support to the idea that retinal cell transplantation may be able to influence visual function in other degenerative retinal diseases.

Key Words
RPE transplants, RCS rats, electroretinogram, ganglion cell function

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