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Photoreceptor function of retinal transplants implicated by light-dark shift of S-antigen and rod transducin

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Abstract

The aim was to demonstrate functional properties of transplanted histologically normal photoreceptors. Subretinal intact-sheet transplants of fetal E17–E20 rat retinas to light-damaged albino rat eyes were fixed in light or dark, 2 to 42 weeks after transplantation, and stained immunohistochemically for certain phototransduction proteins. In light adapted transplants, transducin was predominantly found in inner segments of parallel-organized photoreceptors. Transducin shifted to the outer segments with dark-adaptation. S-antigen distribution was opposite to transducin. Rhodopsin distribution did not change. The shift of signal transduction proteins correlated to the light conditions indicates that normal phototransduction processes were established in photoreceptors of transplanted retinal sheets.

Keywords: Retinal transplantation; Photoreceptor; Rat; Light damage; Immunohistochemistry; Transduction

1. Introduction

Many retinal diseases result in the deterioration of the outer retina while often leaving retina to brain connections intact (Eisenfeld, LaVail & LaVail, 1984; Voaden, 1990; Papermaster & Windle, 1995). In these cases, it may be possible to repair the retina by implanting retinal cells into the subretinal space. We have shown that fetal retinal transplants survive, form most retinal cell types, contain many neurotransmitters (Aramant, Seiler, Ehinger, Bergström, Adolph & Turner, 1990a) and components needed for phototransduction (Aramant, Seiler, Ehinger, Bergström, Gustavii, Brundin et al., 1990b; Aramant & Seiler, 1994; Seiler & Aramant, 1994), and make intrinsic synaptic contacts (Adolph, Zucker, Ehinger & Bergström, 1994; Zucker, Ehinger, Seiler, Aramant & Adolph, 1994) (see Aramant & Seiler, 1996 for review). Perhaps more importantly, in a previous study we have shown that transplant cells can send out neuronal processes and form synapses in the host retina (Aramant & Seiler, 1995a).

To achieve optimally performing transplants we have developed a protocol in which fresh fetal tissue is transplanted as intact sheets (Aramant & Seiler, 1995b, 1996; Seiler & Aramant, 1998). Early transplantation attempts in which tissue was transplanted as cell aggregates or as cell suspensions, resulted in the formation of rosettes (Aramant, Seiler & Turner, 1988; Aramant et al., 1990b; Aramant & Seiler, 1991; Del Cerro, Ison, Bowen, Lazar & Del Cerro, 1991; Juliusson, Bergström, van Veen & Ehinger, 1993; Seiler & Aramant, 1995). Silverman has reported the presence of outer segments after transplantation of intact sheets of postnatal day 8 retina or mature photoreceptors (Silverman & Hughes, 1989; Silverman, Hughes, Valentino & Liu, 1992). Transplantation of retinal microaggregates of postnatal mouse retina resulted in the formation of small patches of transplant photoreceptors with outer segments (Gouras, Du, Kjeldbye, Yamamoto & Zack, 1994). However, embryonic tissue, rather than postnatal or mature retina, may have a greater potential to become

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integrated with the host retina because embryonic tissue is composed of pluripotent cells and its immunity is less developed (Widner & Brundin, 1988; Dunnett, 1990; Kupsch, Oertel, Earl & Sautter, 1995).

Most studies report only anatomical descriptions of the retinal grafts as evidence of potential function while few studies have directly shown evidence of functional activity following transplantation. Adolph recorded transient spike potentials and local ERGs from the surface of a subretinal graft in vitro (Adolph et al., 1994). Unfortunately these transplants were composed of rosettes rather than layers parallel to the host RPE. Thus, although capable of responding to light, these transplants were suboptimal because of abnormal retinal organization. In order to achieve long term restoration of visual function the transplant should develop normal cell layers and become integrated with the remaining healthy portions of the eye including both the RPE and the inner retina of the host.

For the first time, we present evidence that photoreceptors of a transplant that histologically resembles a normal retina exhibit distributional changes in photoreceptor proteins involved in the phototransduction cascade, and thus, are responding to light. To assess the viability and functional capacity of photoreceptors in transplants, we have used immunohistochemistry to describe distributional changes in photoreceptor proteins dependent on lighting conditions.

Photoreceptors respond to light through the process of phototransduction, which leads to nerve stimulation and visual perception (review in Koutalos & Yau, 1993). Light triggers a biochemical cascade beginning with the activation of rhodopsin within the outer segments of rod and cone photoreceptors. The S-antigen (arrestin), a soluble protein of 48 kDa, stops the phototransduction cascade (Zuckerman & Cheasty, 1986) by binding to the phosphorylated rhodopsin (Kuhn, Hall & Wilden, 1984) and thus inhibiting cGMP phosphodiesterase activation.

The events of phototransduction can be followed histologically by tracking the distribution of specific proteins. Following the onset of light in dark adapted rats, proteins such as the S-antigen and α-transducin involved in visual transduction undergo changes in intracellular distribution (Whelan & McGinnis, 1988). α-transducin is primarily localized in the outer segments of dark-adapted retinas. After light exposure, it rapidly redistributes to the inner segments (Brann & Cohen, 1987; Philp, Chang & Long, 1987; Roof & Heth, 1988). The intracellular localization of S-antigen immunoreactivity is also dependent on the state of light or dark adaptation and is opposite to that of transducin (Broekhuyse, Tolhuizen, Janssen & Winkens, 1985; Philp et al., 1987; Craft, Whitmore & Donoso, 1990; Nir & Ransom, 1993; Gropp, Huang & Aguirre, 1997). The S-antigen shift has also been detected in humans (Loeffler & Mangini, 1995). The outer segments of dark-adapted animals do not display the S-antigen, but the inner segments, cell bodies, and synaptic terminals are strongly labeled. After light exposure, there is an inversion of this pattern as the S-antigen is mobilized to inactivate rhodopsin. Although opsin mRNA levels fluctuate with the light cycle (McGinnis, Whelan & Donoso, 1992), no distributional changes of opsin, and thus, rhodopsin, have been observed (Whelan & McGinnis, 1988).

In this study, we show that the light-dependent shifts of transducin and S-antigen clearly occur in organized transplants indicating that the photoreceptors of intact-sheet transplants are responding to light. The presence and redistribution of photoreceptor signal transduction proteins provide evidence that such transplant photoreceptors can function normally.

2. Material and methods

2.1. Experimental animals

Two normal (not light-damaged, not transplanted) female adult Sprague-Dawley rats were used as controls. A total of 19 female adult albino Sprague-Dawley rats (weight: 200–250 g) were used as transplant recipients. Before transplantation, the recipient rats were light-damaged (see below). We used this model in an ongoing study to test restoration of light sensitivity by retinal transplants (unpublished). The 12 timed-pregnant Long-Evans rats were used as donors for fetal tissue. All animals were treated according to the regulations in the ARVO and NIH guidelines. An overview of the experiments is shown in Table 1.

Table 1
Overview of immunohistochemistry experiments

	Light-perfusion	Dark-perfusion
Normal rats	$n=1$	$n=1$
α-Transducin	$n=1$	$n=1$
γ-Transducin	–	$n=1$
S-antigen	$n=1$	$n=1$
Retinal transplants	$n=10$ 14–298 days after surgery	$n=9$ 52–114 days after surgery
α-Transducin	$n=5$	$n=6$
γ-Transducin	$n=2$	$n=6$
S-antigen	$n=10$	$n=9$
Rhodopsin	$n=4$	$n=4$

Table 2
Immunoreactivity in normal rat retinas

	Light			Dark		
	Inner segments	Outer segments	n[a]	Inner segments	Outer segments	n
α-Transducin	+	−	1	−	+	1
γ-Transducin	n.i.[b]	n.i.	−	−	+	1
S-antigen	−	+	1	+	−	1

[a] n, number of retinas investigated.
[b] n.i., not investigated.

2.2. Light damage

Female albino Sprague-Dawley rats were exposed to blue light for 2–4 days in a custom-made acrylic plastic/wire cage surrounded by 48″ daylight fluorescent light bulbs covered with a blue plastic filter (Lee filter # 197, transmission peak at 420 nm). The light intensity inside the cage was 678–1291 lux (63–120 ft. cd.). After the light exposure, the rats were returned to normally lighted cages in the animal facilities (12 h light on/off; light intensity 8–320 lux, 0.5–30 ft. cd.). The light damage procedure has been described in an abstract (Seiler, Aramant, Petry, Callahan, Sandberg & Pawlyk, 1997) and in a manuscript (Seiler et al., submitted).

2.3. Preparation of donor tissue

The donor tissue was prepared as described (Seiler & Aramant, 1998), with some modifications. Rat embryos (embryonic days E17–E20) were removed by cesarean section and stored in ice-cold oxygenated hibernation medium (after Kawamoto & Barrett, 1986). Fetal retinas were carefully dissected free from surrounding tissues, and then embedded in a gel, either growth factor reduced matrigel (GRF-matrigel, Collaborative Biomedical products, Bedford, MA) or alginate (kindly provided by Drs T. Hadlock and J. Vacanti, Boston, MA; and obtained from Pronova Biomedical, Oslo, Norway). Embedded tissue, stored on ice in hibernation medium, was used within 1–5 h. Just before implantation, the tissue was cut into rectangular pieces (0.4–0.8 mm wide, and 0.8–1.5 mm long) to fit our custom-made implantation tool (patent pending).

2.4. Transplantation procedure

Retinal sheets were implanted as described (Seiler & Aramant, 1998). Light-damaged rats were anesthetized by intraperitoneal injections of sodium pentobarbital (38–40 mg/kg) and atropine (0.4 mg/kg), followed by xylazine (3–7 mg/kg) 10 min later. A small incision ($\sim$1 mm) was cut behind the pars plana, parallel to the limbus. Using a customized implantation tool, the tissue was inserted into the subretinal space. The incision was closed with 10–0 sutures and each eye was treated with gentamycin and artificial tears.

2.5. Tissue processing

After a survival time of 2–42 weeks (see Table 1), experimental animals were euthanized with an overdose of sodium pentobarbital (300 mg/kg). Rats were perfusion-fixed through the ascending aorta (with the descending aorta clamped off) with 4% paraformaldehyde containing 0.18% picric acid in 0.1 N Na-phosphate buffer (pH 7.2) under normal lighting conditions (light-adapted) or under dim red light with the rats' eyes covered (dark-adapted). Dark-perfused eyes were enucleated and stored in Bouin's fixative (Sigma, St. Louis) and protected from light for at least 6 h before further dissection. After overnight postfixation, the area containing the transplant was dissected out and embedded in low-melting paraffin. Eight-micron paraffin sections were mounted onto slides. Slides of selected transplants that showed good photoreceptor organization with outer segments in contact with host RPE were processed for immunocytochemistry (see below). The transplant experiments for this study were done over a period of 2 years, therefore varying survival times were used.

2.6. Immunocytochemistry

An overview of the experiments is shown in Table 1. Sections of many transplants and of the normal retinas were stained for the same antigen repeatedly in different experiments. Sections were deparaffinized, washed with phosphate-buffered saline (PBS) and incubated in 20% horse serum. The following monoclonal antibodies were used for staining: S-antigen (clone A9C6, Dr Donoso, Philadelphia PA; Donoso, Folberg & Arbizo, 1985) 1:20 000–1:40 000; rod α-transducin (clone TF 15) 1:20 000; and γ-transducin (clone TF 28) 1:250 (Dr Fung, Los Angeles, CA; Navon & Fung, 1988), rhodopsin (rho 1D4, 1:50; Dr Molday, Vancouver, BC; Molday, 1989). Most sections were incubated overnight at 4°C. After several washes with PBS, the binding of

Table 3
Immunoreactivity in retinal transplants

	Light				Dark			
	Inner segments	Outer segments	n[a]	Graft age, postnatal day	Inner segments	Outer segments	n	Graft age, postnatal day
α-Transducin	+	–	4	39–58	–	+	5	49–110
	?[b]	?	1	37	+	(+)	1	108
γ-Transducin	+	–	2	58	–	+	2	49, 108
					+	–	2	105, 108
					?	?	2	67, 110
S-antigen	–	+	10	13–296	+	–	5	87–110
					?	?	4	49–110
Rhodopsin	–	+	4	39–58	–	+	4	108–110

[a] n, number of transplants investigated.
[b] ?, staining weak or questionable (high background).

the primary antibody was detected using the Vector Elite ABC kit for mouse antibodies (Vector Laboratories, Burlingame CA). As a control, the primary antibody was omitted.

3. Results

An overview of the results is shown in Table 2 (normal retinas) and Table 3 (retinal transplants).

3.1. Immunoreactivity in normal rat retinas (see Table 2)

α-Transducin staining was in the inner segments in the light-adapted normal retina, and in the outer segments in the dark-adapted retina. For γ-transducin, only one dark-adapted retina was investigated. As expected, γ-transducin was found in the outer segments.

Contrary to transducin, the light-adapted retina showed S-antigen staining in the outer segments. The dark-adapted retina stained for S-antigen in the inner and not in the outer segments.

Rhodopsin-immunoreactivity was found mainly in the outer and not in the inner segments in both- light- and dark-adapted retina. In addition, there was fainter staining of the outer plexiform and outer nuclear layer.

3.2. Immunoreactivity in transplants (see Table 3)

α-Transducin: In four of the five light-adapted transplants, the main immunoreactivity was seen in the inner segments, somewhat fainter in the outer nuclear layer and the terminals of transplant photoreceptors, and very faint in the outer segments as shown in Fig. 1a, b. In the edges of some transplants where the photoreceptor layer had formed rosettes, α-transducin immunoreactivity was considerably weaker (Fig. 1a). In five of the six investigated dark-adapted transplants, α-transducin immunoreactivity was seen in the outer segments (Fig. 1c, d). One dark-perfused transplant showed clear but faint staining of outer segments; however, the main immunoreactivity was in the inner segments.

γ-Transducin: Fig. 2a shows two light-adapted transplants in which the strongest immunoreactivity for γ-transducin was seen in the inner segments, similar to α-transducin. In the six investigated dark-adapted transplants, two were clearly stained for γ-transducin in the outer segments (Fig. 2b). However, two others showed γ-transducin staining in the inner segments. Two transplants did not show any clear immunoreactivity (not shown).

S-antigen: Fig. 3a shows S-antigen immunoreactivity in rod outer segments as found in all ten light adapted transplants. In addition, the inner segments of some cones were stained (Fig. 3a). In five of the nine dark-adapted transplants, S-antigen immunoreactivity was seen in inner but not outer segments (Fig. 3b). In four other dark-adapted transplants, we were unable to determine the staining pattern due to high background and faint specific staining (not shown). Immunoreactivity with the γ-transducin antibody was also unclear in two of these.

Rhodopsin: Fig. 4 shows rhodopsin distribution in the outer segments of both light- and dark-adapted transplants. As in a normal retina, rhodopsin distribution in transplants did not show any variation with lighting conditions.

3.3. Immunoreactivity in light-damaged host retinas

Host animals light-damaged for 2 days ($n = 3$) still had

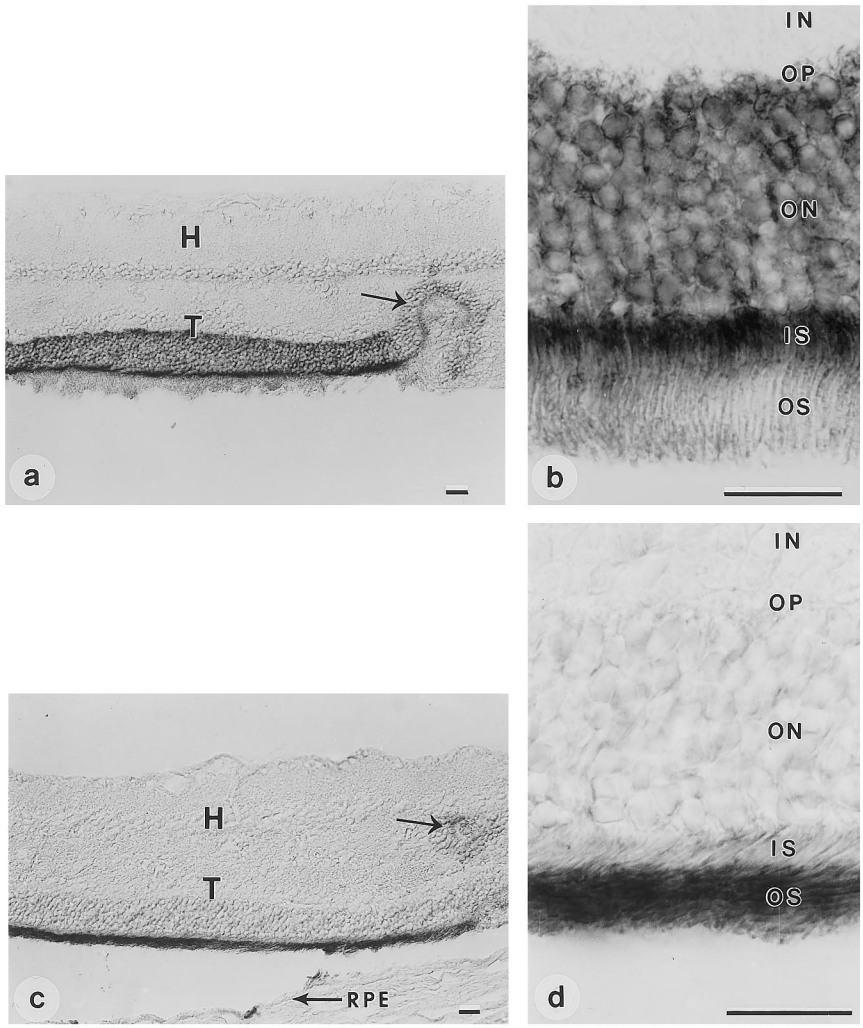

Fig. 1. α-Transducin (TF 15) in retinal transplants. (a) Transplant perfusion-fixed in light. The transplant has developed a parallel layer of photoreceptors which is clearly immunoreactive for α-transducin in the inner segments and in the outer nuclear layer. The curled up photoreceptor layer (rosette) in the edge of the transplant shows only weak transducin immunoreactivity. There is no α-transducin staining in the overlying light-damaged host retina. The host RPE are not seen in the frame of the picture because of a processing artefact. E17 donor, 147 days after surgery. (b) The same pattern can be seen in another light-perfused transplant. The main immunoreactivity is in the inner segments. The outer segments are only faintly stained, much less than the outer nuclear layer and the photoreceptor terminals in the outer plexiform layer. E19 donor, 61 days after surgery. (c) Transplant perfused at the end of dark cycle. The main immunoreactivity has now shifted to the outer segments of the parallel organized photoreceptors. The outer segments of photoreceptors in a rosette (arrow) do not show any staining. The outer nuclear layer is only very faintly stained. E18 donor, 71 days after surgery. (d) Enlargement of (c). All figures are oriented with the photoreceptor layer towards the bottom and the ganglion cell layer towards the top of the micrograph. All sections shown are 8-μm paraffin sections. Magnification bars = 20 μm. List of labels used in the micrographs. H, host; IN, inner nuclear layer; IS, inner segments of photoreceptors; ON, outer nuclear layer; OP, outer plexiform layer; OS, outer segments of photoreceptors; RPE, retinal pigment epithelium; T, transplant.

two to three rows of photoreceptors with outer segments in the retinal periphery. Outer segments had been completely destroyed in animals light-damaged for 3 days ($n = 11$) and 4 days ($n = 5$).

The immunoreactivity for α-transducin and S-antigen of photoreceptors with outer segments in the periphery showed the same light-dependent distribution as in the transplant. Remaining short outer segments also stained for rhodopsin. Most of the retina contained few photoreceptors with little or no transducin immunoreactivity. However, these photoreceptor cell bodies in the remaining outer nuclear layer still stained for S-antigen and rhodopsin in areas without outer segments (data not shown).

4. Discussion

We have shown normal distribution and redistribution of α-transducin, γ-transducin, S-antigen, and rhodopsin in photoreceptors derived from embryonic neural retina transplanted as freshly harvested intact sheets. These results indicate that photoreceptors of the transplant are responding to light.

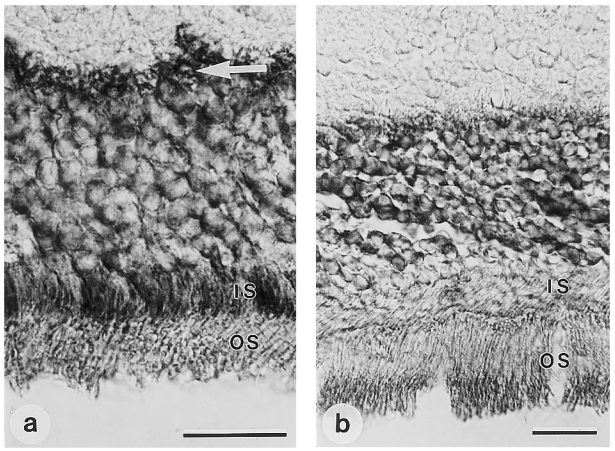

Fig. 2. γ-Transducin (TF 28). (a) Transplant fixed in light (same as in Fig. 1b). The main immunoreactivity is in the inner segments and in the photoreceptor terminals (arrow) in the outer plexiform layer. (b) Transplant fixed at the end of the dark cycle. The outer segments are clearly stained. No immunoreactivity in the inner segments. E 18 donor, 112 days after surgery.

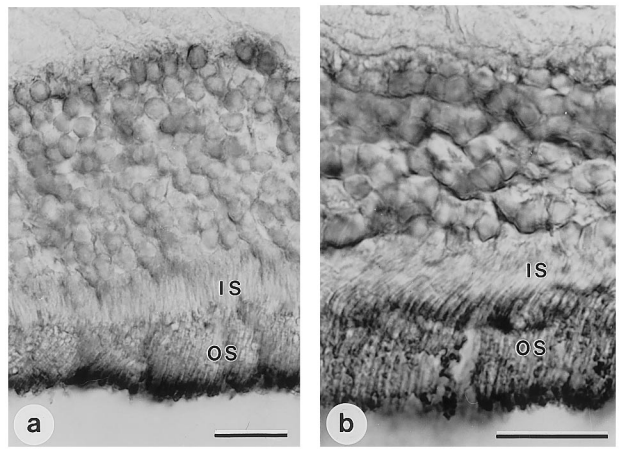

Fig. 4. Rhodopsin (rho1D4). There is no difference in the staining pattern between the light- and the dark-perfused transplant. (a) Transplant fixed in light (same as in Fig. 1b). The outer segments are clearly stained, the inner segments are unstained. (b) Transplant fixed at the end of the dark cycle (same as in Fig. 2b).

However, the light/dark shift in some transplants either was absent or could not be detected due to ambiguous staining. Due to the difficulties involved in working under dim lighting conditions, some dark-perfused animals were not as well fixed as animals perfused in the light. In addition, incompletely fixed retinas could have been affected by the red light during the process of enucleation. Thus, this may explain the high background and low immunoreactivity of some dark-perfused experiments. It has been shown that α-transducin immunoreactivity is sensitive to fixation (Roof & Heth, 1988).

On the other hand, the degree of photoreceptor viability may reflect the level of transplant organization and interaction with the host RPE. Thus, transplants with suboptimal organization might show a reduced immunoreactivity for phototransduction proteins. In agreement with this hypothesis, we found the level of immunoreactivity to transducin in photoreceptors forming parallel layers to be considerably higher than those forming rosettes. In the current study, we used an antibody concentration that was optimal for normal rat retina. In a previous study (Seiler & Aramant, 1994), we demonstrated α-transducin immunoreactivity in photoreceptors of aggregate transplants that were organized in rosettes using the same antibody as in the current study. However, the antibody concentration had to be increased 10–100 × to stain the transplant rosettes.

While the outer segments in contact with RPE in the dark-perfused transplants stained strongly for α-transducin, the outer segments in rosettes were not stained. This indicates that photoreceptors in rosettes have a much lower capability of responding to light than normally organized photoreceptors. This difference was not seen with rhodopsin or S-antigen staining. Addi-

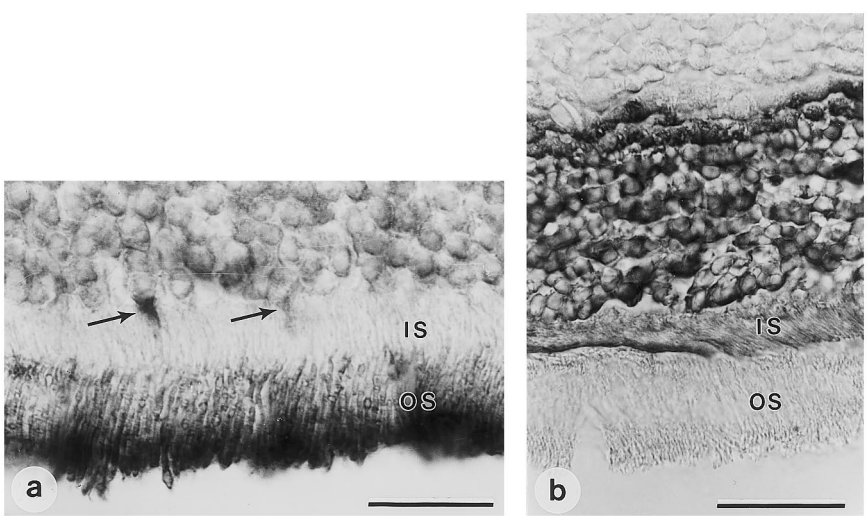

Fig. 3. S-antigen (A9C6). (a) Transplant fixed in light (same transplant as in Fig. 1b). The outer segments are clearly stained. Two labeled cone inner segments can also be seen (arrows). (b) Transplant fixed at the end of the dark cycle (same as in Fig. 2b). The inner segments, but not the outer segments are stained.

tionally, transducin immunoreactivity was absent in the light-damaged host retina (except for the periphery where some photoreceptors were spared). In contrast, S-antigen and rhodopsin immunoreactivity of residual damaged host photoreceptors without inner and outer segments was much higher.

Several other studies have found a correlation between the degree of dysfunction and the level of immunoreactivity of α-transducin and S-antigen (Jansen, Aguirre, van Veen & Sanyal, 1990; Mirshahi, de Kozak, Tarraf, Razaghi, Thillaye & Faure, 1991; Mirshahi, Thillaye, Tarraf, de Kozak & Faure, 1993; Gropp et al., 1997). After exposing albino rats to continuous light, transducin immunoreactivity disappeared 2–4 days after exposure whereas S-antigen and rhodopsin immunoreactivity persisted for 1–2 months (Mirshahi et al., 1991). Mutant rds mice develop outer segments that are short and abnormally shaped. Their inner segments showed more immunolabeling for S-antigen as compared to normal retinas (Jansen et al., 1990). In RCS rats, the light-dark shift of S-antigen is disturbed (Mirshahi et al., 1993). Gropp et al. (1997) used immunohistochemistry for S-antigen to correlate the distribution of photoreceptor proteins with the progression of disease in a dog model of rod-cone degeneration. S-antigen labeling in rod spherules decreased significantly with disease progression.

Furthermore, α-transducin could not be detected in photoreceptors of animals previously maintained under constant light although no morphological abnormalities were noted (Mirshahi et al., 1991). This suggests that the localization and behavior of transduction molecules can be indicative of photoreceptor health and integration.

Redistribution of cellular proteins is thought to be an important part of phototransduction in rod photoreceptors (Kuhn et al., 1984). These light evoked changes in distribution of proteins might help molecules move from a site of synthesis to one of function. For example, transducin appears to be synthesized in the inner segment and is transported to the outer segment under dark conditions where it may be involved in the increase in the sensitivity of photoreceptor rods at night. The timing of these positional changes in photoreceptor proteins is consistent with rod utilization. Changes were shown to be due to the light environment and not dependent on the time of day (McGinnis, Austin, Stepanik & Lerious, 1994). A disruption in the light evoked shift could lead to an accumulation of substances near their site of synthesis. Therefore, immunolocalization of these proteins may serve as an important tool for assessing the level of degeneration or regeneration in retinas.

In summary, we have demonstrated that histologically normal photoreceptors in intact-sheet fetal retinal transplants redistribute transducin and S-antigen according to lighting conditions, resembling normal photoreceptors with light responses. This encouraging result indicates that the transplanted photoreceptors can function normally. We plan to correlate these findings with electrophysiological testing. What now needs to be investigated is how the transplant connects with the host retina.

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