Recovery of Photoreceptor Outer Segment Length and Analysis of Membrane Assembly Rates in Regenerating Primate Photoreceptor Outer Segments

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Purpose. Photoreceptor outer segments are in a dynamic state of membrane addition and disposal. This study was undertaken to determine how a standardized period of retinal detachment and varying periods of reattachment affect the renewal process.

Methods. To investigate the effects that retinal detachment and reattachment may have on this process, the neural retina from 12 adult rhesus monkeys (*Macaca mulatta*) was detached from the overlying retinal pigment epithelium (RPE) by subretinal injection of a balanced salt solution. After a standardized detachment period of 7 days, the two tissue layers were reapposed. Animals were labeled with ^{3}H-fucose and killed at times ranging from 3–150 days after reattachment.

Results. During the 7 day detachment period, the majority of rod outer segments (ROS) and cone outer segments (COS) degenerated, but inner segments remained intact. During the first week after reattachment, a rapid increase in rod and cone outer segment length occurred in the absence of disc shedding. This was accompanied by re-establishment of a modified morphologic relationship between the apical processes of the RPE and the regenerating outer segments. ROS and COS regained approximately 40% of their control lengths after a 2 wk reattachment period. By 30 days of reattachment, ROS had regained 72% of their normal length and COS had regained approximately 48%. After 150 days of reattachment, photoreceptor outer segment mean length was not statistically different from control areas. Autoradiographic results confirmed that new disc membranes were synthesized after reattachment. The rate of ROS membrane assembly was subnormal at reattachment time points up to 30 days.

Conclusions. Retinal detachment leads to a reduction in photoreceptor outer segment absolute length and membrane assembly rates. Increasing time of retinal reattachment is positively correlated with an increase in outer segment absolute length and a corresponding increase in membrane assembly rates. This recovery pattern in eyes without underlying pathology and after a relatively brief detachment interval may represent the upper limit of the recovery process. Invest Ophthalmol Vis Sci. 1993;34:175–183.

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Most human patients who have suffered a retinal detachment experience progressive improvement in the vision of their affected eye after surgical reattachment of the retina.[1] Psychophysical measurements of individuals whose retinas had been successfully reattached indicate that a recovery in acuity, sensitivity, and color vision usually occurs during a period of 1 yr after reat-

tachment.[2] In some patients, a diminution of acuity or color vision defects can persist even after prolonged periods of recovery, particularly in patients in whom the macula is involved.[1] To optimize the recovery of vision after detachment, it would be desirable to establish the correlations between these specific cellular events and their functional consequences. Experimental studies of detached and reattached retinas in cats and primates have begun to reveal some of the cellular events that underlie the recovery process.[3–9]

Kroll and Machemer were the first to document that mammalian rod and cone photoreceptor outer segments degenerate shortly after retinal detachment but retain some capacity to regenerate if the retina is surgically reapposed to the apical surface of the retinal pigment epithelium (RPE).[10–11] Studies of amphibian retinas in vitro have shown that detached retinas decrease their rate of outer segment membrane assembly after 2 days in culture.[12] However, data from in vivo studies in the cat indicate that membrane assembly can occur even in retinas detached for prolonged periods.[13] The extent of outer segment degeneration after detachment and its subsequent regeneration after reattachment is almost certainly a crucial determinant in the recovery of visual function. We previously examined the extent of rod outer segment (ROS) and cone outer segment (COS) regeneration in cat retinas that had been detached for up to 6 wk and reattached for up to 7 mo.[5] More recently, we measured the recovery of absolute length of ROS and COS in monkeys whose maculas had been detached for a constant 1 wk period and then reattached for 3, 5, 7, or 14 days.[6]

In the present investigation, we extend those results to retinas reattached for 30 and 150 days, demonstrating that photoreceptor outer segments regain their normal length given a relatively short detachment period and a sufficiently long recovery interval.

Secondly, we report that the rate of ROS disc membrane assembly is substantially reduced (by 66%), when compared to control rates, during the first 2 wk after reattachment. Although this rate increases with increasing time of reattachment, it remains subnormal for up to 30 days after reattachment and possibly longer. The results support the hypothesis that the rate at which outer segment disc membranes are assembled after reattachment, as well as the re-established balance between assembly and disposal, may be controlling factors in determining the pace and extent of visual recovery.

MATERIALS AND METHODS

Monocular retinal detachments were produced in 12 adult rhesus monkeys whose average age was >10 yr. A standardized detachment period of 7 days was followed by a surgical reattachment procedure. The detachment and reattachment intervals, in days, are as follows, with the number of animals at each time-point given in parentheses: 7:3 (2), 7:7 (2), 7:14 (3), 7:30 (3) and 7:150 (2). Detachments were drawn on a standard ophthalmic detachment chart at the end of each surgery. Indirect ophthalmoscopy was performed from time to time. The animals were killed with an overdose of sodium pentobarbital, followed by intracardiac perfusion of fixative. All animals were cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with federal, state, and local regulations.

Surgery

Unilateral retinal detachments were created using the technique described in a previous publication.[6] In brief, animals were anesthetized, and an infusion cannula was inserted to maintain intraocular pressure during the vitrectomy procedure. A complete vitrectomy was performed. Using the superior incision, a micropipette (80–100 μm tip) was inserted into the vitreous cavity, and balanced salt solution (Alcon, Ft. Worth, TX) was slowly injected through the pipette as it advanced through the retina. A small retinal detachment formed that could be enlarged to the desired size by regulating the amount of fluid injected. Initially, the neural retina was elevated several hundred micrometers above the RPE surface. However, over the next 7 days, detachment height decreased spontaneously. Retinae were reattached by a simple fluid gas exchange with a mixture of 75% sulfur hexafluoride gas (Matheson, Newark, CA) and 25% room air passed through a 0.2 μm filter. All animals recovered without complications.

Light and Electron Microscopy

The fixation protocol and staining techniques employed in this study were published previously.[6] Briefly, animals were entrained to a 12:12 light-dark cycle and killed 4 hr after light onset. Eyes were fixed by intracardiac perfusion of buffered 1% glutaraldehyde and 1% paraformaldehyde. After the anterior segments were removed, eyecups were immersed in the same fixative overnight. This was followed by postfixation in 2% buffered osmium tetroxide, dehydration in graded ethanols, and embedment in Araldite 6005 resin (Ladd Research Industries, Burlington VT). One micrometer-thick sections were cut for light microscopy and stained with an aqueous mixture of methylene blue, azure II, and toluidine blue in borate buffer. Ultrathin sections for transmission electron microscopy were cut with a diamond knife, stained with uranyl acetate and lead citrate, and carbon-coated before viewing.

Outer segment lengths were measured using a Zeiss (Thornwood, NY) Universal Microscope equipped with a ×100 oil immersion objective and an ocular micrometer. The protocol and criteria essentially were the same as described previously.[4] Aligned rod and cone outer segments were measured in the macular region of normal control eyes and from the reattached region in experimental eyes. Measurements from control eyes and from experimental eyes with identical detachment/reattachment time-points were compared using a Student's t-test with the Statworks software package (Abacus Concepts, Berkeley, CA). In no case were there significant differences between means within a single condition. After this comparison, measurements from normal areas of experimental eyes and normal areas from the opposite control eyes were pooled and averaged together. Likewise, measurements taken from animals with identical detachment/reattachment intervals also were averaged together. Only outer segments that were well-oriented for their full length were measured. At many of the earlier reattachment time points, rudimentary outer segments were very disorganized or absent entirely; their lengths were not measured. Similarly, those photoreceptors with no apparent outer segment were not included in the measurements.

Autoradiography

Sections were processed for electron microscopic autoradiography using the flat substrate method of Young and Droz[14] as outlined in Anderson, Fisher, and Breding.[15] We previously used ^{3}H-L-fucose as a specific glycoprotein marker for membrane assembly.[15] Five hundred millicuries of ^{3}H-L-fucose (45–70 Ci/mmol/l; Amersham, Arlington Heights, IL) in 100 ml of balanced salt solution was injected directly into the vitreous cavity via the pars plana with a 30 G needle. Labeling intervals are described in Table 1. The 150 day animals were not labeled. Thin sections (60–70 nm) were dipped in Ilford (Mobberley, Cheshire, UK) L-4 emulsion under Na vapor illumination and exposed for 4–6 wk at 4°C. The autoradiograms were developed in complete darkness using phenidone developer (Lauder Chemicals, San Mateo, CA) for 1 min at 15°C or elon-ascorbic acid after gold latensification.[16] Measurements of labeled disc displacement were made directly from the screen of a Philips (Eindhoven, The Netherlands) CM10 electron microscope using built-in measuring software. Assembly rate was calculated by dividing the displacement of the leading edge of the cluster of developed silver grains (in micrometers) from the most basal outer segment disc by the number of days since injection. Only outer segments well aligned from base to tip were measured.

TABLE 1. ^{3}H-Fucose Injection Schedule

Detachment (days)	*Reattachment (days)*	*Labeling Interval (hr)*
7*	3	72
7	7	168 (7 days)
7	14	2
7*	14	96
7*	30	96

* Two animals were injected at these time points. Some of the data presented in this table were published previously[6] and are included here for completeness.

RESULTS

Figure 1 is a set of histograms that shows outer segment length measurements from macular rods and cones in animals with retinal reattachment times of 7, 14, 30, and 150 days. At the 7 and 14 day time points, mean ROS length was 8.7 and 9.9 μm, respectively, whereas mean COS lengths were 6.5 μm and 7.2 μm, respectively.[6] This increase in ROS and COS length during the first week was much greater than the relatively small 1–2 μm increase shown during the second week. In addition, the range of ROS and COS lengths was much broader at the 7, 14, and 30 day time points when compared to the 150 day or control groups. After 14 days reattachment, ROS were 33% and COS were 36% of their mean adult lengths, ($\bar{x}$ = 9.9 and 7.2 μm, respectively). By 30 days post-reattachment, the mean length of ROS had increased to 13.0 μm, or 44% of the length measured in control eyes, and COS had a mean length of 9.6 μm, or 48% of their normal adult length. By 150 days of reattachment, neither peripheral nor macular ROS or COS mean length were different statistically from the means of their corresponding controls.

Autoradiographic Studies

Rods and cones in the reattached retinas had radiolabeling patterns identical to those found in normal control eyes after the intravitreal injection of ^{3}H-fucose (Figs. 2A, B)-ie, COS had a diffuse distribution of labeled material, whereas ROS had their silver grains confined to a discrete band. Two hours after ^{3}H-fucose injection, a concentration of silver grains was apparent over the myoid region of photoreceptors from normal regions and regions reattached for 14 days (data not shown). In two experimental eyes with 3 day reattachments, ^{3}H-fucose was injected intravitreally at the time of reattachment. The electron microscopic autoradiograms of retinal sections obtained from them indicated the small amount of outer segment material was heavily labeled,[6] although no discrete band of radiolabeled protein could be identified because of the outer segment disorganization that still occurs after only 3 days of reattachment. No radiolabeled phagosomes were identified in the RPE.

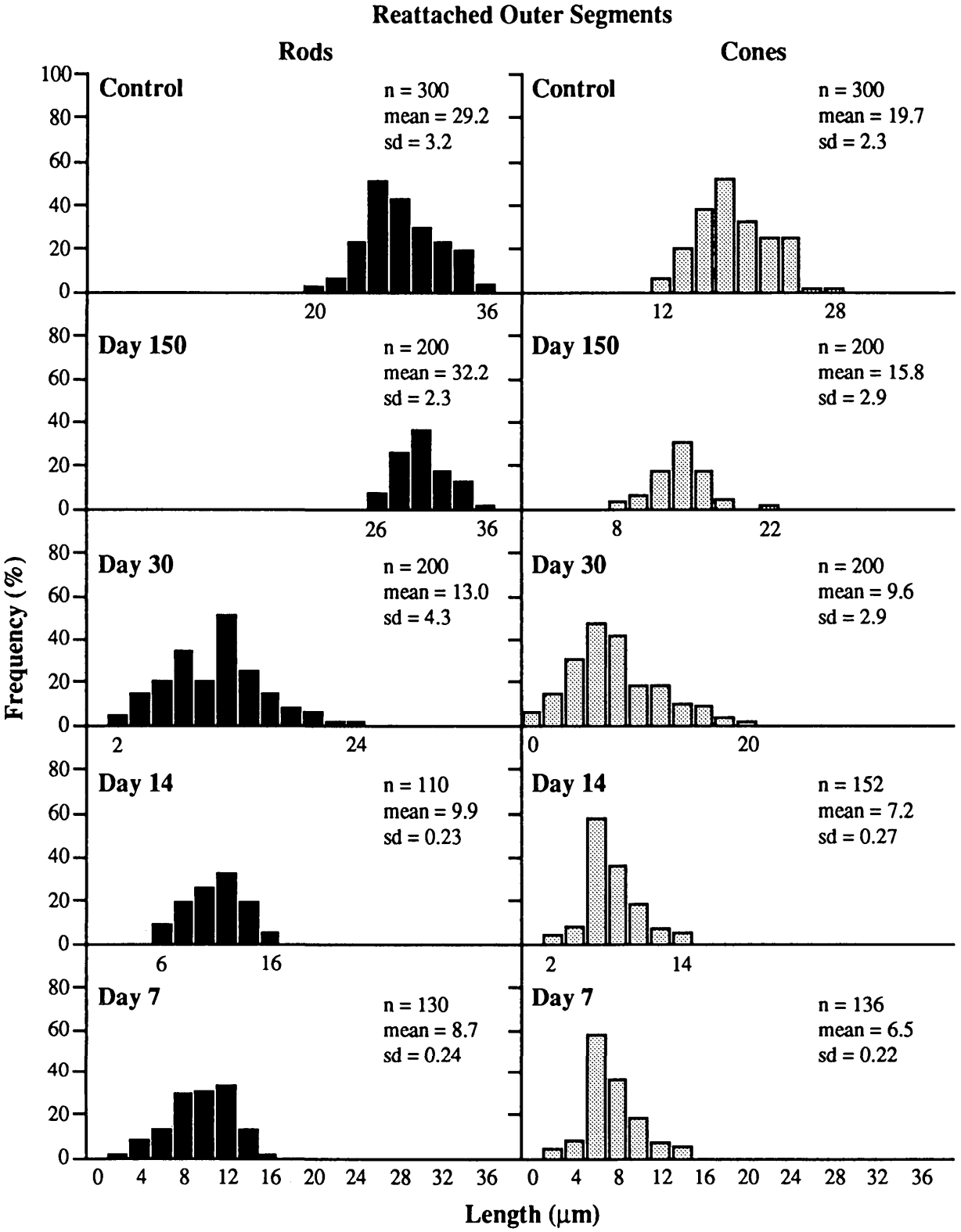

FIGURE 1. Frequency histogram of ROS and COS length as a function of reattachment interval. ROS and COS lengths were measured in areas of reattachment using the protocol described in the text. Lengths were measured in normal control eyes and at 7, 14, 30, and 150 days after reattachment. Measurements were grouped into 2 μm bins and plotted as a percentage of overall frequency. Inner segments with no apparent outer segment were not counted. In general, both ROS and COS tended to elongate, and their distributions widened considerably with increasing time of reattachment. Between 0 and 7 days of reattachment, macular rods regained almost 30% of their normal mean length. At the 2 wk time point, mean length of reattached macular ROS increased to 33% of normal, and reattached macular COS were about 35% of normal. By 30 days, lengths of ROS and COS had increased to 44 and 48% of their control values, respectively. By 150 days, there was no statistically significant difference between controls and experimental values (t-test: $P < 0.01$). Portions of these data were published previously.[6]

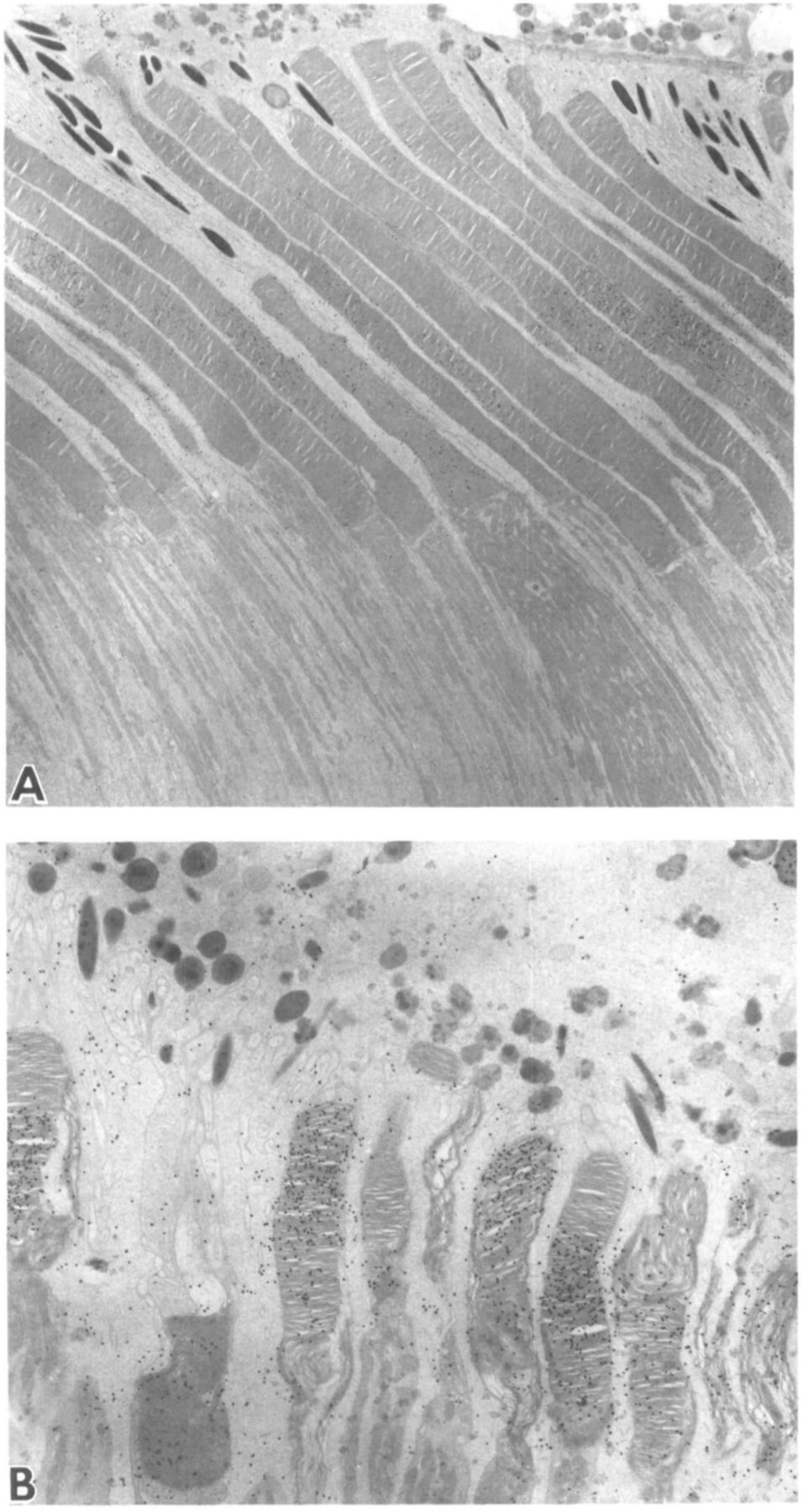

FIGURE 2. Electron microscopic autoradiograms of control and reattached OS. (**A**) Control area. In the rods, the radiolabeling is restricted to a discrete area, whereas the cones show a diffuse pattern of silver grains. The pattern of radiolabeling after ^{3}H-fucose injection in the rod or cone outer segments does not change in the reattached retinas. (**B**) Seven day detachment followed by a 14 day reattachment. Band displacement is somewhat variable as is the length of ROS at this time point. This indicates the variability in renewal rate at early reattachment time points. (**A**, ×4200; **B**, ×5600.)

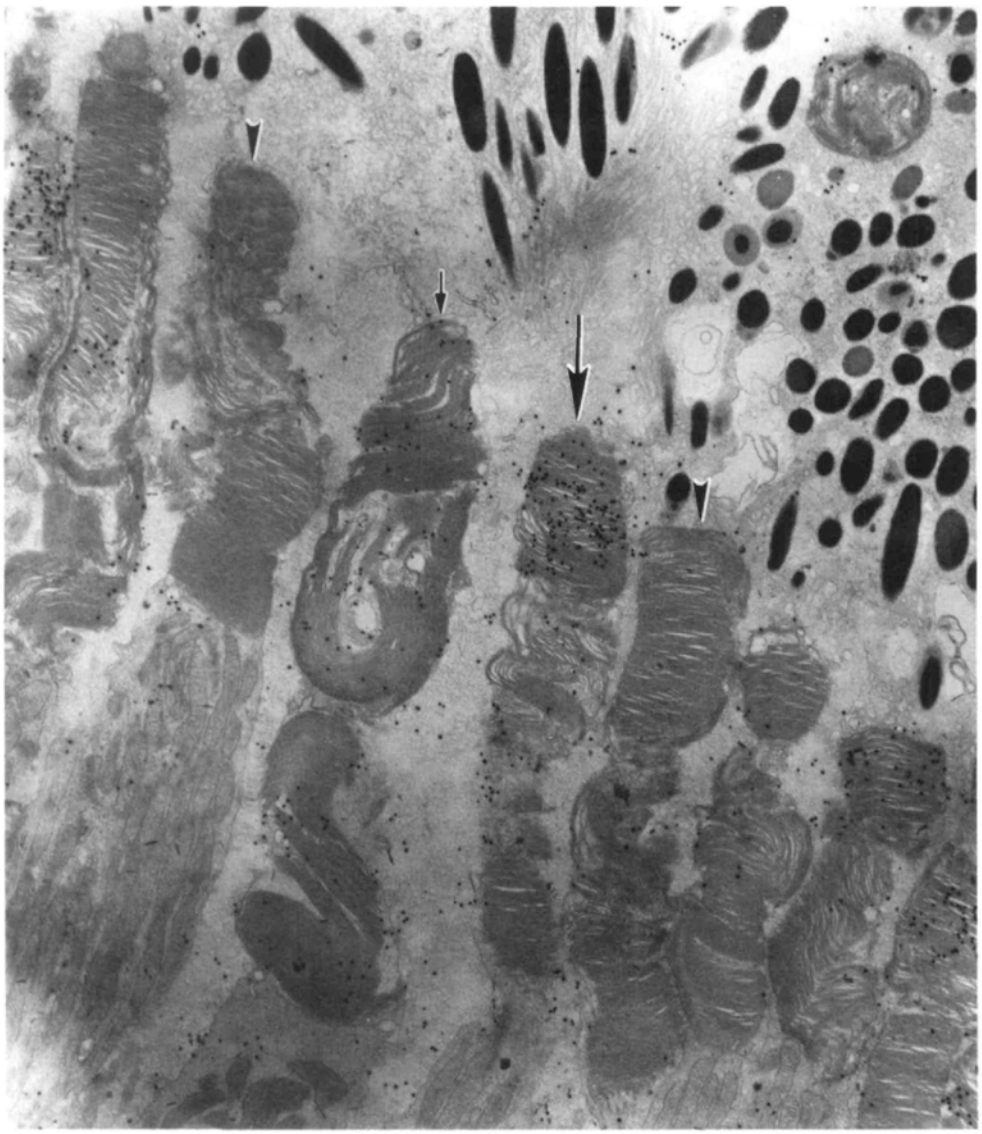

FIGURE 3. Electron microscopic autoradiogram of a retina detached for 7 days and reattached for the same period. ^{3}H-fucose was injected at the time of retinal reattachment. One ROS has a concentration of radiolabeling at its distal tip (large arrow), whereas several others appear already to have shed their labeled discs (arrowheads). The regenerating COS (small arrow) show the typical diffuse pattern of incorporation. (×5250.)

In the animal labeled at the time of reattachment and killed 7 days later, a cluster of radiolabeled silver grains was clearly visible in the shortened ROS (Fig. 3). In this animal, the distance from the base of the outer segment to the leading edge of the advancing front of silver grains was measured in 27 ROS, yielding a mean assembly rate of 1.15 μm/day (Fig. 4). In those cases where there was more than one animal per condition, the assembly rate data were pooled, because there were no statistically significant differences noted between animals. In the two 14 day reattachments, which had been labeled at reattachment day 10, the mean ROS assembly rate in 110 rods was 1.25 μm/day (Fig. 4). The increased outer segment length and organization at the longer reattachment time points allowed for greater numbers of outer segments to be acceptably aligned for measurement. The two animals with 30 day reattachments were labeled on day 26 and killed 4 days later. The mean assembly rate in the ROS of these animals was 2.44 μm/day (Fig. 4). Measurement of 836 ROS from the control areas of six eyes yielded a mean assembly rate of 3.23 μm/day. When assembly rates from all reattachment time points were pooled and compared to the pooled controls, the difference between the means proved to be statistically significant at the 0.001 level.

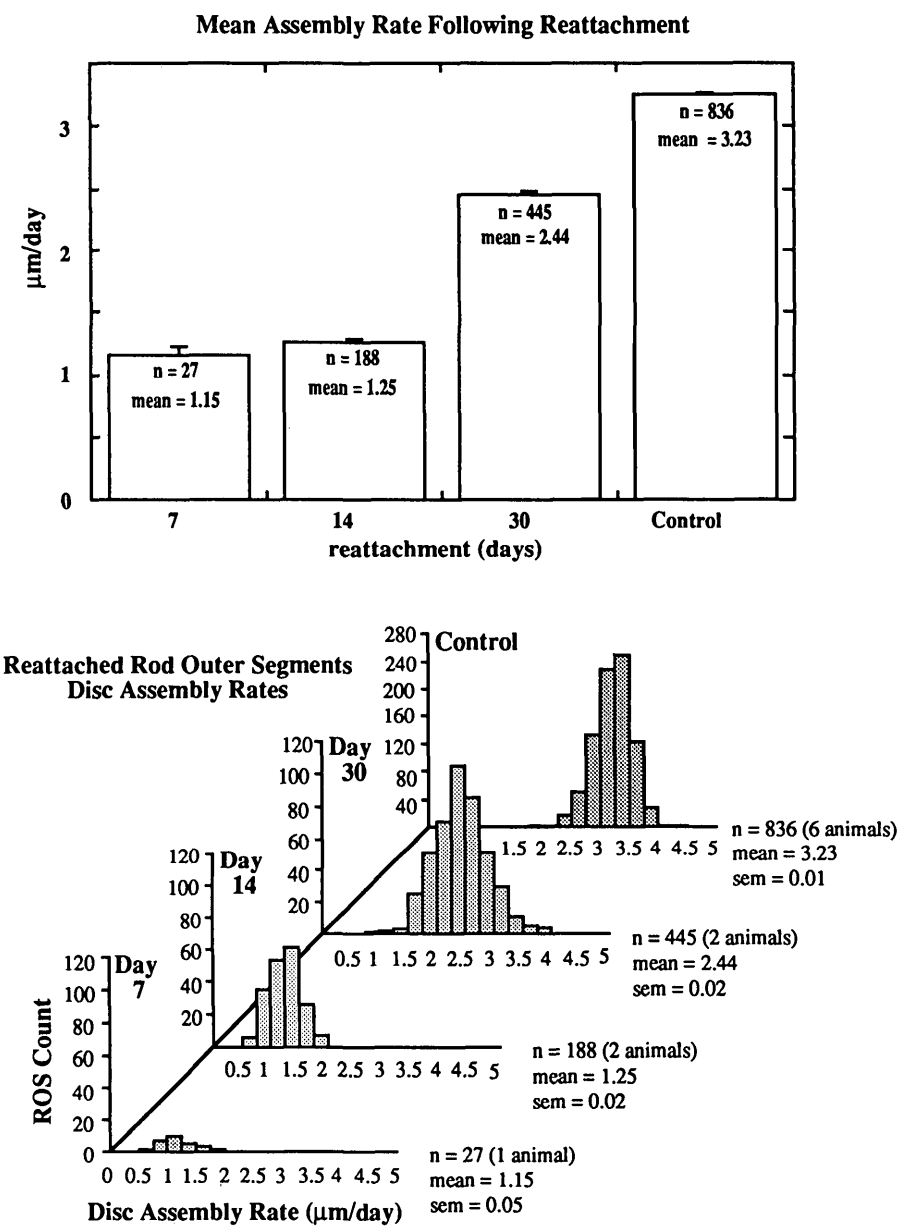

FIGURE 4. Rates of disc membrane assembly calculated from the electron microscopic autoradiograms. (**A**) Mean assembly rate of ROS after reattachment was calculated as the displacement of the labeled band from the base of the outer segment, in microns, divided by the number of days of labeling. After 7 and 14 days of reattachment, the rate was approximately one third of the control value. After 30 days of reattachment, the rate increased to 75% of normal. 'N' on each bar represents the number of outer segments counted. Error bars represent one standard deviation from the mean. Control measurements were pooled after calculations indicated there were no statistically significant differences between the means of the individual animals. In normal rhesus monkey ROS, the mean rate of disc displacement had been reported to be approximately 2.5 μm/day. The mean rate obtained from electron microscopic autoradiograms of normal control ROS in the present study indicate a somewhat higher value (3.23 μm/day). This could be a result of a difference in resolution, because the estimates obtained in the previous study were gathered by measuring light microscopic autoradiograms; the present study used values measured directly from the screen of the electron microscope. (**B**) Histograms illustrating the distribution of assembly rates at the various time points after reattachment. Variability in assembly rate is greater in the reattached retinas than in the six pooled controls.

DISCUSSION

Reapposition of the detached retina to the apical surface of the RPE initiates a process of cellular repair and functional recovery in photoreceptors.[3–8] The early morphologic changes are characterized by the clearance of subretinal debris, the beginning of regrowth of rod and cone outer segments, and the restoration of the morphologic relationship between the photoreceptor outer segments and the apical processes of the RPE.[3,5,6] An integral part of this recovery process is the assembly of newly synthesized disc membrane into appropriately organized outer segments. A 7 day retinal detachment produced by balanced salt injection into the subretinal space results in virtually complete degeneration of primate photoreceptor outer segments.[6] During the first 7 days of recovery after retinal reattachment, the regenerating outer segments elongate. Evidence of disc shedding from the outer segment tips is first observed at reattachment day 7.[6] These observations, together with the autoradiographic data presented here, indicate clearly that during the first week of reattachment, the regenerating photoreceptors re-establish the two processes that define the outer segment renewal cycle—assembly of new disc membranes at the base of the outer segment

and disposal of old disc membrane from the tip.[17] This process of self repair is almost certainly one of the first and most vital steps in restoring visual function and visual acuity, because in its absence there can be little or no functional recovery.

There is a strong positive correlation between outer segment length and duration of reattachment. Even brief periods of reattachment are sufficient to allow for substantial recovery of outer segment length (30% in the first 7 days). Previous autoradiographic data obtained in the cat retina suggest it is unlikely that photoreceptors, even after a lengthy period of detachment from the RPE, ever completely stop assembling outer segment membrane.[13] However, their ability to configure this membrane into a normal outer segment appears to be severely impaired in the absence of an apposed RPE. Our data indicate that once reapposition of these two tissue layers has taken place, assembly of outer segment material into organized disc stacks occurs rapidly. The appearance of a front of radiolabeled glycoprotein in regenerating ROS, with data that demonstrates its displacement with time, clearly indicates that disc morphogenesis is proceeding after short-term reattachment. Similarly, the radiolabeling pattern identified in regenerating COS is identical to the diffuse pattern of incorporation that is characteristic of normal COS,[18] also suggesting that vectorial transport and incorporation of nascent protein into COS was not permanently impaired by detachment.

Although earlier results from studies of experimental retinal reattachment in the owl monkey,[3,10] rhesus monkey,[6,11] and cat[5] showed that the photoreceptors retain a significant capacity for recovery after short-term retinal detachment, the present study provides the first quantitative data on outer segment regeneration. In addition, the results provide the first indication that subnormal rates of disc membrane assembly could be a factor in the rate of visual recovery after reattachment. The results from an earlier study suggested that a supranormal episode of disc production occurs immediately after retinal reattachment.[2] Our autoradiographic results provide no evidence for such an event. Rather, they indicate that outer segment regrowth occurs steadily, but that the rate of disc production is less than one-third of the control values during the first 14 days and only two-thirds that of the control rate at 30 days after reattachment. The rate of disc production we calculated from the control eyes predicts that regenerating ROS could reach their normal length within 7–9 days of reattachment. However, we found that at 30 days, rods and cones were about two-thirds normal length in the control eyes and that they attained their normal lengths sometime before 150 days post- reattachment. The ability of the retina to recover normal outer segment length within 150 days of reattachment is consistent with psychophysical studies that demonstrate in most the cases examined there was a progressive improvement in visual function during that same period.[2]

As judged by the absence of phagosomes in the RPE cytoplasm, normal disc shedding does not appear to be a significant factor in regulating outer segment length at the earliest reattachment time-points (3 and 5 days).[6] These results are similar to those reported during outer segment development when shedding begins only after an initial period of outer segment formation.[19,20] In the absence of shedding, ROS could —even at the reduced renewal rate of 1.25 μm/day— attain normal length in 23 days. Data obtained from the animals at the 30 day reattachment time point indicate that the mean assembly rate remains subnormal, as does ROS absolute length. Whether normal rates of assembly are ever fully restored in the reattached retina remains to be determined.

Although numerous variables may contribute to final visual outcome in human patients, the extent of photoreceptor outer segment degeneration after retinal detachment and their regeneration after reattachment generally are acknowledged to be two of the important variables in visual recovery. Indeed, in the absence of outer segment regeneration there can be no significant visual recovery. However, the relationship between the extent and time course of outer segment regeneration and the ensuing recovery after retinal reattachment remains poorly defined. The results from this study are the first to show that the return of vision, at least in the first few weeks after reattachment, takes place against the background of an abnormally low rate of photoreceptor outer segment disc membrane turnover. Future studies, aimed at identifying biologically active molecules that promote photoreceptor cell survival, maintain normal rates of outer segment morphogenesis, and improve the ability of photoreceptors to assemble outer segment components could lead to improvements in the rapidity or extent of visual recovery after retinal detachment.

Key Words

membrane assembly, photoreceptor regeneration, photoreceptors, quantitative autoradiography, retinal reattachment.

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