Correction. In the article “The structure of Marek disease virus DNA: The presence of unique expansion in nonpathogenic viral DNA” by K. Fukuchi, A. Tanaka, L. W. Schierman, R. L. Witter, and M. Nonoyama, which appeared in number 3, February 1985, of Proc. Natl. Acad. Sci. USA (82, 751–754), the authors request that the following changes be noted. On p. 754, the last sentence of the Note Added in Proof should read “A similar expansion also has been recently reported by Hirai et al. (25).” Ref. 25 should read as follows: Hirai, K., Honma, H., Ikuta, K. & Kato, S. (1984) Arch. Virol. 79, 293–298.

Correction. In the article “Specificity analysis of mouse monoclonal antibodies defining cell surface antigens of human renal cancer” by Connie L. Finstad, Carlos Cordon-Cardo, Neil H. Bander, Willet F. Whitmore, Myron R. Melamed, and Lloyd J. Old, which appeared in number 9, May 1985, of Proc. Natl. Acad. Sci. USA (82, 2955–2959), there is a printer’s error in Table 3 on p. 2958. Specifically, one of the two breast fibroadenomas should be F23∗ showing strong fluorescence (∗).

Correction. In the article “Exon-shuffling” maps control of antibody- and T-cell-recognition sites to the NH2-terminal domain of the class II major histocompatibility polypeptide Aβ by Ronald N. Germain, Jonathan D. Ashwell, Robert I. Lechler, David H. Margulies, Kathleen M. Nickerson, Gen Suzuki, and Jenny Y. L. Tou, which appeared in number 9, May 1985, of Proc. Natl. Acad. Sci. USA (82, 2940–2944), the authors request that the following correction be noted. On p. 2943, line 6 in the right-hand column of text should read “(which affects amino acids 67, 70, and 71 of Aβ1; see ref. 40).”

Correction. In the article “Substances originating from optic nerve of neonatal rabbit induce regeneration-associated response in the injured optic nerve of adult rabbit” by H. Hadani, A. Harel, A. Solomon, M. Belkin, V. Lavie, and M. Schwartz, which appeared in number 24, December 1984, of Proc. Natl. Acad. Sci. USA (81, 7965–7969), the authors request that the following correction be noted. In the legend to Table 1, the P value in footnote ‡ should be < 0.005 rather than < 0.05.
Substances originating from the optic nerve of neonatal rabbit induce regeneration-associated response in the injured optic nerve of adult rabbit

(central nervous system/ regeneration/ grafts/ visual system/ mammals)

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Communicated by Michael Sela, August 16, 1984

ABSTRACT We have recently shown that cell bodies of an injured optic nerve of adult rabbit can be induced to express regeneration-associated response by external signals derived from nonneuronal cells of regenerating nerves of lower vertebrates. In this study it is shown that even substances derived from a nonregenerating mammalian system also can trigger such a regenerative response. Thus, substances derived from intact nerves of neonatal rabbits and of adult rabbits, to a lesser extent, were active in triggering a regeneration-associated response, whereas substances derived from injured nerves of adult rabbit were not. However, if subsequent to the injury the nerve was implanted with a silicone tube containing medium conditioned by neonatal optic nerves, the substances derived from the implanted injured nerve were active. Thus, it appears that the ability of a periaxial environment to provide triggering substances correlates with axonal growth. Therefore, we name these substances growth-associated triggering factors (GATFs). It is suggested that mammalian cells are unable to express a regenerative response after an injury due to the failure of their nonneuronal cells to produce regeneration-triggering substances. This disability may be circumvented by an appropriate implantation procedure.

The central nervous system (CNS) of mammals exhibits a limited ability to regenerate after axonal injury in contrast to their lower vertebrate counterparts (1–3). In systems that are endowed with a high posttraumatic regeneration capacity, the cell body response involves transition from a mature, fully differentiated state into a growing state. This shift presumably requires activation or derepression of genes conserved in a repressed form in the mature adult state. The failure of mammalian CNS to recover from axonal injury is manifested in part by the inability of the cell body to undergo such a shift in its genetic program and, thus, to exhibit characteristic changes of regeneration (4–6).

Several studies have clearly demonstrated that the environment (and more specifically, the glial cells) play an important role in the regeneration of axons after injury (7–16). We have been using the visual system of adult rabbits as a model for investigating the contribution of the environment to the regeneration-associated response of the neuronal cell body. In this system, the neuronal cell bodies in the retina are accessible. The accessibility enables the evaluation of the cell body response subsequent to the environmental modifications, which are induced by transplantation of nerve segments or by implantation of substances derived from xenogeneic and homogenetic systems (17).

Using this system, we recently have shown that it is possible to induce regeneration-associated changes in retinal of severed optic nerves of adult rabbits by external substances. These substances were obtained from the regenerating optic nerve of fish (17). The goal of the present work was to find out if injured optic nerves of adult rabbit (representing nonregenerative system) have the potential to produce these triggering substances, if not to elucidate when the loss of the environmental ability to provide substances that trigger regenerative response occurs. To this end we supplied by implantation substances originating from optic nerves of neonatal and adult rabbits into injured optic nerves of adult rabbits. Subsequent to the implantation of medium conditioned by the neonatal nerve, the corresponding retinæ demonstrated sprouting activity in vitro and changes in protein synthesis. Furthermore, substances derived from intact optic nerves of adult rabbit also exhibited activity, though to a lesser extent. Thus, it appears that the ability of nerves to provide diffusible substances that affect, directly or indirectly, the triggering of regenerative response correlates with the growth state of the axon, independently of whether the growth is of regenerating nerves or of developing nerves.

MATERIALS AND METHODS

Surgical Approach. The rabbit is anesthetized by xylazine at 5 mg/kg and ketamine at 35 mg/kg, intramuscularly. Under sterile conditions, lateral canthotomy 15 mm in length is performed, and the conjunctiva is then opened along the limbus laterally from 12 to 6 o'clock. Six extraocular muscles, the superior and later rectus, superior oblique, and inferior, lateral and retractor bulbi, are then each engaged by a muscle hook, carefully isolated towards their origins and disinserted near their scleral insertion. The disinsertion of the superior rectus is performed 5 mm away from the sclera. This insertion is then grasped with a fixation forceps and used for retracting the globe medially. Tenon's capsule, fat, and other orbital contents are then cleaned carefully away from the globe and pushed towards the orbital bones. Thus, the nerve can be exposed to the desired extent, which for our purpose was about 1 cm. During the exposure process, care must be taken not to injure the ciliary arteries that lie on both sides of the nerve. The exposed nerve is then either crushed by forceps for 30 sec or transected 6–7 mm away from the globe. When transplantation of a nerve segment is performed, the grafted neonatal nerve is sutured end-to-end to the transected adult rabbit optic nerve with 10-0 monofilament sutures. In order to supply the diffusible substances, we devised a procedure whereby we introduce to the crushed optic nerve of rabbit a "wrap-around" implant made of a silicone tube.

Preparation of Neonatal Optic Nerve Graft. Neonatal rabbit (1–7 days old) is injected with a lethal dose of sodium

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Abbreviation: CNS, central nervous system.

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pentobarbital. The cranium is then opened, the cerebrum is excised, and the optic chiasm and cranial part of the optic nerves is exposed. Under the operating microscope, the optic chiasm is separated from the tectum and the optic nerve is cut at the level of the optic foramen. A 10-0 suture is then applied in situ to each proximal optic nerve end of the neonatal rabbit. Such manipulations are used for the handling and suturing of the neonatal nerve to the transplanted adult rabbit optic nerve.

Preparation of Conditioned Medium and Silicone Tube for a Wrap-Around Implantation. Optic nerve segments of neonatal or adult rabbits are dissected out from the eye to the optic chiasm and are incubated for 1.5 hr in Dulbecco's modified Eagle's medium (DME medium) (GIBCO) free of serum. At the end of the incubation, the medium, now defined as a conditioned medium, is collected. For the preparation of the implant, collagen type V is applied into a 6-mm-long silicone tube (Burkle, Federal Republic of Germany), which has a 2-mm inner diameter and 4-mm external diameter. The silicone tube coated with collagen is then incubated for 24 hr at 10-14°C in a tissue culture containing conditioned medium or any of the other substances as described below. All wrap-around implantations were carried out while the protein concentration (400 μg/ml) in the conditioned media was kept constant.

Pulse-Labeling of Rabbit Retinal Proteins. Retinæ of adult rabbit are dissected out 6-7 days after the surgical manipulation. Retinæ contralateral to the injured side also are dissected out separately; each retina is incubated (for 2 hr at room temperature) in DME medium free of methionine, which is supplemented with [35S]methionine (40 μCi per retina; 1230 Ci/mmol; Amersham; 1 Ci = 37 GBq). At the end of the incubation, the retinæ are collected, homogenized, and centrifuged. Supernatants obtained with high-speed centrifugation for 10 min at 100,000 x g (Airfuge, 25 psi; 1 psi = 6895 Pa) are then collected and their specific activities are determined. This soluble fraction is assumed to contain cytoplasmic proteins including those derived from the ganglion cells and are destined to be axonally transported. The ratio between specific activities of the labeled proteins in the left (operated) versus the right (unoperated, control) sides was used as the test parameter for protein synthesis.

Gel Analysis of Radiolabeled Retinal Proteins. Radiolabeled retinal proteins (50-100 x 10^3 cpm) are applied to NaDodSO4/PAGE (7-20% gradient). After electrophoresis the gel is fixed and then is soaked in water for 30 min, followed by 30 min in 1 M sodium salicylate (Merck). The gel is then exposed to x-ray film (Agfa). Radiolabeled Proteins. Rabbit's retinal explantation is carried out basically as described for cultures of retinæ of lower vertebrates (18-21). The rabbits' retinæ are dissected out 7 days after the surgical manipulation of the corresponding optic nerve. They are then chopped into squares 350 μm wide and placed in poly(l-lysine) (50 μg/ml, Sigma)-coated dishes in DME medium supplemented with fetal calf serum (6%), HEPES (20 mM), and gentamycin sulfate (10 μg/ml). Cultures are incubated at 37°C with 5% CO2. Growth is scored, starting 48 hr after the explantation.

RESULTS

An adult rabbit optic nerve was injured and grafted with substances derived from the optic nerve of neonatal rabbit (1-7 days old). The retinæ of various experimental groups were dissected out and pulse-labeled in vitro with [35S]methionine. In a test tube containing conditioned proteins in the left (operated) versus the right (unoperated) sides in each animal (defined as L/R) was used as the test parameter. As shown in Table 1, transplantation of a segment of an optic nerve of the neonatal rabbit into the severed optic nerve of the adult rabbit resulted in an increased incorporation of [35S]methionine into the retinal protein (a L/R ratio > 1 was observed). Furthermore, wrap-around implants of silicone tube coated inside with collagen and soaked with medium conditioned by optic nerves of neonatal rabbits caused a significant increase of the L/R ratio. A significant reduction in the metabolic activity of the retina was observed in nongrafted nerves that either were cut or crushed. This resulted in an L/R ratio <1. Medium conditioned by intact nerves of an adult rabbit, when implanted into an injured optic nerve of adult rabbit, caused increased incorporation of [35S]methionine by the retina of the injured nerve. This incorporation was to a lesser degree than that caused by implantation of medium conditioned by a neonatal nerve (Table 1).

The radiolabeled retinal proteins were further analyzed by gel electrophoresis. The increased labeling induced by implantation of substances originating from the optic nerve of neonatal rabbit was accompanied by a selective increase of polyopeptides of the following sizes: 130, 110, 74, 64, and 26 kDa (Fig. 1A). In contrast, the profile of the proteins derived from retinæ of nerves that had been crushed without subsequent implantation did not exhibit alterations in these particular polyopeptides (Fig. 1B). A summary of the effects of the various implanted substances on the selective expression of these polyopeptides is included in Table 2. In all of the tested preparations, the increased incorporation of [35S]methionine always was manifested in the same polyopeptides.

To verify whether such alterations are associated with any regeneration activity, we examined the sprouting ability of these retinæ in culture. Retinæ of injured optic nerves that had been implanted with silicone tubes containing bovine serum albumin or with medium conditioned by the optic nerve of the neonatal rabbit were cultured 1 wk after the injury. Fig. 2 shows the sprouting activity from the retina of an injured optic nerve that was implanted with medium conditioned by the neonatal optic nerve (Fig. 2A). No such activity could be detected in retina of injured optic nerves implanted with a silicone tube containing bovine serum albumin (Fig. 2B). The neuritic nature of the growing fibers is further emphasized by the growth cone structure visualized by using scanning electron microscope (Fig. 2C).

<table>
<thead>
<tr>
<th>Experimental groups*</th>
<th>L/R</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crushed or transplanted or crushed implanted with albumin</td>
<td>0.71 ± 0.1^2</td>
<td>8</td>
</tr>
<tr>
<td>Implanted or transplanted (neonatal rabbit optic nerve)</td>
<td>1.87 ± 0.2</td>
<td>7</td>
</tr>
<tr>
<td>Implanted (adult rabbit optic nerve)</td>
<td>1.15 ± 0.2</td>
<td>3</td>
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^1Unilateral (left side) transaction or crush injury of the optic nerve of an adult rabbit was performed. Immediately after the injury, the nerve was transplanted with a segment of neonatal optic nerve or implanted with a silicone tube coated with collagen onto which medium conditioned by neonatal/adult rabbit optic nerve was adsorbed. No significant differences were observed (i) between crushed only and crushed and implanted with bovine serum albumin and transplanted or (ii) between implanted and transplanted with the same neuronal source; therefore, these two groups were combined respectively. One week after the injury, retinæ on both sides of the various experimental groups were excised separately and pulse-labeled with [35S]methionine.

^2Results are expressed by the ratio L/R (mean ± SEM) of the specific activity of the radiolabeled proteins in the operated side (L) versus the contralateral unoperated side (R). The number of animals examined in each experimental group is shown in parentheses.

^3The value is significantly different from control, with P < 0.05 by Student's two-tailed t test.

Table 1. Increased [35S]methionine incorporation by rabbit retinal proteins induced by substances originating from the optic nerve of neonatal and adult rabbits.
DISCUSSION

This study shows that when substances originating from an optic nerve of a neonatal rabbit are implanted into a severed optic nerve of an adult rabbit, they induce regenerative responses in the corresponding retina. This regenerative response is manifested by the growth capacity of the adult retina in culture and by alterations in the pattern of the retinal proteins. It is suggestive of a few of these polypeptides may function as "growth-associated proteins" (4-6).

The failure of mammalian CNS neurons to regenerate has been correlated with the inability of their cell bodies to undergo changes in the pattern of their protein synthesis and their lack of sprouting activity in culture (4-6). Indeed, in systems that regenerate, such as the visual system of lower vertebrates, these two characteristic phenomena do occur after optic nerve injury (22-29). Our previous results and those reported in this work strongly indicate that such changes can be induced in mammalian CNS provided that the environment is suitably modified. Furthermore, it appeared from these works that a combination of neuronal properties and environmental conditions ultimately determine the extent of the regenerative responses (15, 17, 30).

The present study demonstrates that substances originating from intact optic nerve of a neonatal rabbit, when implanted into a severed optic nerve of an adult rabbit, cause a retinal response characteristic of regeneration (Table 1; Figs. 1 and 2). Such substances do exist in the intact nerve of adult rabbit as well, but to an extent that was found to be lower than in the neonatal nerve and was hardly noticeable in a preparation derived from an adult injured nerve.

Retinae of intact optic nerves of an adult rabbit do not express synthesis of the regeneration-associated polypeptides or sprouting activity. Nevertheless, the intact nerve itself gives rise to soluble substances that can trigger such a response in the retina of an injured optic nerve (Table 1). This would imply the existence of an inhibitory mechanism that regulates the response machinery in the intact nerve and that is probably disrupted after injury. Such an inhibitory mechanism might be mediated directly or indirectly by the neuronal target. In contrast to the rabbit visual system, in the fish optic nerve (a regenerative system), axonal injury was accompanied by an increase in the ability of the substances derived from it to trigger a regenerative response when implanted into a rabbit optic nerve (17). This difference may give a further clue as to the reasons for failure of mammalian CNS to regenerate. Based on the observed correlation between the ability of nonneuronal cells to provide substances that trigger regenerative response and axonal growth, we propose that these diffusable substances function as "growth-associated triggering factors" or "GATFs." This would be in analogy with the neuron-derived materials required for axonal growth, which were designated growth-associated proteins or "GAPs." Regeneration competence may be determined by the balance between expression of GAs by the neurons and GATFs by the surrounding nonneuronal cells.

The implanted substances (GATFs) might represent the signal that triggers the regenerative response. Alternatively, the implanted substances might be responsible for providing signals for preceding events, such as the activation of glial cells within the injured adult nerve, and thereby for making the producers of substances that trigger regenerative response.

Preliminary results indicate that medium conditioned by injured nerve that was previously implanted with medium conditioned by neonatal rabbit optic nerve is active in terms of its ability to provide substances that can trigger a cell body response. This still does not allow the elimination of any of the other mechanisms suggested above. Therefore, it
Fig. 2. Sprouting activity in a culture of rabbit retina dissected out 7 days after optic nerve injury and implantation of media conditioned by an optic nerve of neonatal rabbit. (A and C) Phase (A) and scanning (C) electron micrographs of cultured retina corresponding to injured optic nerve of adult rabbit that was implanted with medium conditioned by an optic nerve of neonatal rabbit. Note the structure of a growth cone in C. (B) Phase micrograph of cultured retina corresponding to injured optic nerve of adult rabbit that was implanted with bovine serum albumin. Three retinæ of implanted nerves and three retinæ of control nerves were examined separately. (A and B, ×60; C, ×7870.)

is possible that the implanted substances directly trigger the cell body response. As a result, the response machinery is turned on, and fibers grow, and further induce the differentiation of the nonneuronal cells towards signal production. Such a bidirectional relationship between axon and supportive cells has been proposed in other studies (31–33).

In the present work, the changes in protein synthesis and sprouting activity were measured in the retina. It is suggested that the induction of regeneration from the retina ganglion cells in the rabbit. Further studies are required to verify this issue and to establish if the induction of the cell body response would lead to a complete functional recovery. It is still possible that further regeneration is prevented because of additional deficiencies or impedances that take place in the course of the regeneration process subsequent to the cell body response.

This work was supported by grants from the Israel Ministry of Health and United States–Israel Binational Foundation to M.S., who is an incumbent of the Helena Rubinstein Career Development Chair.