Peptides Released by Ameboid Microglia Regulate Astroglial Proliferation

DANA GIULIAN and TIMOTHY J. BAKER
Department of Neurology, Program of Neurosciences, Baylor College of Medicine, Houston, Texas 77030

ABSTRACT Peptides that stimulate astroglial proliferation are produced in traumatized adult rat brain by 10 d after injury. These same peptides are released by ameboid microglia activated in vitro. Our findings suggest that astroglial scarring is regulated in part by the release of factors from ameboid microglia near the site of brain injury.

More than 50 years ago, Rio Hortega noted that injury to mammalian brain caused migration of ameboid microglia to the wound site (1). As these microglia (1, 2) engulfed cellular debris, astroglia proliferated and formed a scar. Although such glial responses might help to determine the success or failure of axonal regeneration (3–5), the mechanisms that regulate glial activity after brain injury remain unknown. Recent work on gliogenesis in the goldfish visual system and the mammalian brain showed the presence of peptides, referred to as glia-promoting factors (GPFs),[1] that selectively stimulated astroglial or oligodendroglial proliferation in vitro (6–9). Moreover, the specific activities of the GPFs increased during periods of gliogenesis, suggesting that these factors regulated glial cellular responses in vivo. Our preliminary study showed that the injured brain of the adult rat also contained elevated levels of GPFs. The search for cells which secrete GPFs during brain injury formed the basis for this report.

MATERIALS AND METHODS

Cell Cultures: Cerebral cortices isolated from newborn albino rats were stripped of the meninges, minced in a defined culture medium (10), and dissociated by trituration in 0.25% trypsin in phosphate-buffered saline (PBS), pH 7.4. 100,000 cells were grown on poly-L-lysine–coated 22-mm^2 glass coverslips in 35-mm plastic dishes at 37°C (8) with a humidified 95% air/5% CO_2 atmosphere in defined medium containing 10% fetal bovine serum. After 48 h, the cells were washed three times with defined medium. 10–100-μl aliquots of GPFs in PBS were added to 1.5 ml defined culture medium for 48–72-h incubations. The final protein concentrations ranged from 0.05 to 5.00 μg/ml culture. Buffer control cultures received matching aliquots of PBS alone. Under these conditions, there was a 150–250% increase in total cell number found in control cultures 7 d after cell plating. Enriched preparations of oligodendroglia or astroglia were prepared with modifications of the method described by McCarthy and de Vellis (11).

Indirect immunofluorescence techniques were used to identify astroglia containing glial fibrillary acidic protein (GFAP) or oligodendroglia with galactocerebroside (8). Cells adhering to coverslips were washed with Dulbecco's minimal essential medium containing 20% heat-inactivated goat serum (GS) and fixed for 30 min at −20°C with 90% acetone/10% acetic acid (vol/vol). The coverlips were dipped sequentially in 100% acetone, 70% ethanol, PBS, and Dulbecco's minimal essential medium with 1% GS. Rabbit anti-human GFAP-antibody (whole serum; Accurate Chemical & Scientific Corp., Westbury, NY) was diluted 1:200 with Dulbecco's minimal essential medium with 1% GS and was applied directly to the coverslips. Coverslips were incubated for 45 min at 37°C in 95% air/5% CO_2 with high humidity and were washed with Dulbecco's minimal essential medium. A goat anti-rabbit IgG conjugate of rhodamine isothiocyanate (Accurate Chemicals & Scientific Corp.) was diluted to 1:100 with Dulbecco's minimal essential medium–1% GS was next applied for 45 min at 37°C. Coverslips were washed five times with Dulbecco's minimal essential medium–1% GS and distilled H_2O and were mounted in glycerol containing 1 mg/ml of *p*-phenylenediamine and 10% PBS. Fluorescent-labeled cells were viewed at × 200 with a Nikon Diaphot microscope using epifluorescence. Identification of galactocerebroside-positive oligodendroglia was carried out in a similar fashion with cell fixation after antibody binding. Rabbit anti-galactocerebroside serum, produced in our laboratory, was used with a dilution of 1:100.

Ameboid microglia (12) were isolated from mixed glial cultures grown in 75-cm^2 plastic culture flasks containing 10 ml defined medium with 10% fetal bovine serum at a density of 85,000 cells/ml. After 7 d, confluent cultures were vigorously agitated on a rotary shaker for 15 h (37°C; 180 rpm). The resulting suspension, containing process-bearing cells and microglia, was transferred to a new 75-cm^2 culture flask. After a 3-h adhering interval, process-bearing cells were suspended by gently shaking flasks at room temperature. This cell suspension was discarded. Each flask was then treated with 0.2% trypsin in defined medium and vigorously shaken by hand to release adhering microglia. Once the majority of the microglia had been resuspended, fetal bovine serum was added to the defined medium (15% final volume) and the cell suspension added to new plastic flasks. After a second 3-h interval to allow adhesion, the medium was removed, and adhering microglia were resuspended using trypsin. Final preparations showed a nearly homogeneous population with 95% nonspecific esterase-positive microglia (Giulian, D., and T. J. Baker, unpublished data). There was a 10% recovery of all esterase-positive microglia found in the starting cultures. These ameboid microglia showed the MAC-1 and MAC-3 antigens, an ability to engulf 5-μm latex beads, and the presence of the acetylated low density lipoprotein receptor (Giulian, D., and T. J. Baker, unpublished data; see also references 2 and 12). Fixed *Staphylococcus aureus* in suspension (Pansorbin, Calbiochem-Behring Corp., La Jolla, CA) was used to stimulate microglial secretions (20 μl added per 1.5 ml culture medium). Unlike peripheral blood monocytes, microglia were peroxidase negative and in contrast to

[1] *Abbreviations used in this paper:* GFAP, glial fibrillary acidic protein; GPFs, glia-promoting factors; GS, goat serum.

tissue macrophage, microglia proliferated in vitro (Giulian, D., and T. J. Baker, unpublished data; see also references 2 and 12). Microglia could be selectively destroyed using L-leucine methyl ester, a lysosomotrophic agent effective against phagocytic cells (14).

Isolation of GPFs: Four GPFs have been isolated from goldfish brain (6–9). Two of these peptides, GPF_1 (estimated molecular mass 15 kD) and GPF_3 (6 kD), acted upon oligodendroglia while GPF_2 (9 kD) and GPF_4 (3 kD) served as mitogens for astroglia (8). In this study, we examined GPFs isolated from the brain of rat. Anesthetized adult female rats (Holtzman Co., Madison, WI) received from 10 to 15 puncture wounds in the cerebral cortex to a depth of 3 mm using a 26-gauge needle. The damaged cortex was isolated 10 d after surgery with the contralateral hemisphere providing intact tissue control. Brain samples were dispersed by sonication in ice-chilled PBS and centrifuged at 15,000 *g* for 30 min. The supernatants were filtered through 0.45-μm filters and applied to a standardized gel filtration column (Bio-Rad Laboratories, Richmond, CA; P-10, 100 × 0.9 cm). Defined culture media conditioned for 24 h by enriched populations of ameboid microglia were concentrated using ultrafiltration (YM-2, Amicon Corp., Danvers, MA) and fractionated by gel filtration as described for brain material. Each fraction was tested for its mitogenic effect upon of oligodendroglia or astroglia. We found that the apparent molecular masses and specificity of biologic activities were the same for GPFs isolated from fish and rat brain (8, 9). Furthermore, fish and rat GPFs co-purified using ion exchange chromatography and reverse-phase high performance liquid chromatography, which suggests that these factors were structurally similar (Giulan, D., and T. J. Baker, unpublished data; see also references 8 and 9). GPFs in brain supernatants from injured and intact adult rats or conditioned media from activated and control microglia were separated by consecutive column elutions. Pooled fractions containing biologic activity were matched for the experimental and control groups and used to determine dose responses.

Mean cell numbers were calculated for each culture by scoring the number of fluorescently labeled astroglia or oligodendroglia found in 10 randomly selected microscopic fields (0.314 mm^2). Data were expressed as fold increase in cell number when compared to PBS-treated control cultures (8). Protein concentrations were estimated using the fluorescamine method with bovine serum albumin standards (15). Gel filtration provided a 250- to 500-fold purification for brain GPFs and a 100- to 300-fold purification for microglial GPFs.

RESULTS AND DISCUSSION

The events which lead to astroglial proliferation in the injured mammalian brain are not known (1, 2). Our laboratory has identified four brain-derived peptides, glia-promoting factors (GPFs), that stimulate the proliferation of specific glial populations in culture (6–9). Increasing levels of GPFs during periods of gliogenesis in the regenerating goldfish visual system and in the developing rat brain suggested that these peptides regulated glial growth in vivo (6–9). We questioned whether GPFs also played a role in mediating cellular responses in traumatized adult mammalian brain.

The damaged brain extract was fractionated using gel filtration and assayed for the presence of GPFs by incubation with mixed glial cultures grown in defined medium (8). The supernatant contained factors that stimulated the appearance of astroglia in culture with peaks of biologic activity corresponding to GPF_2 (apparent molecular mass of 9 kD) and GPF_4 (3 kD) (8).[2] Adult rat cortex 10 d after stab wound injury did not contain detectable levels of the oligodendroglia-stimulating factors, GPF_1 or GPF_3 (Figs. 1 and 2). Dose-response curves showed a three- to fourfold increase in the specific activities of GPF_2 and GPF_4 recovered from damaged brain when compared to those obtained from intact control tissue (Fig. 2).

To determine the cellular sources of astroglia-promoting peptides, we assayed for GPFs in medium conditioned by rat

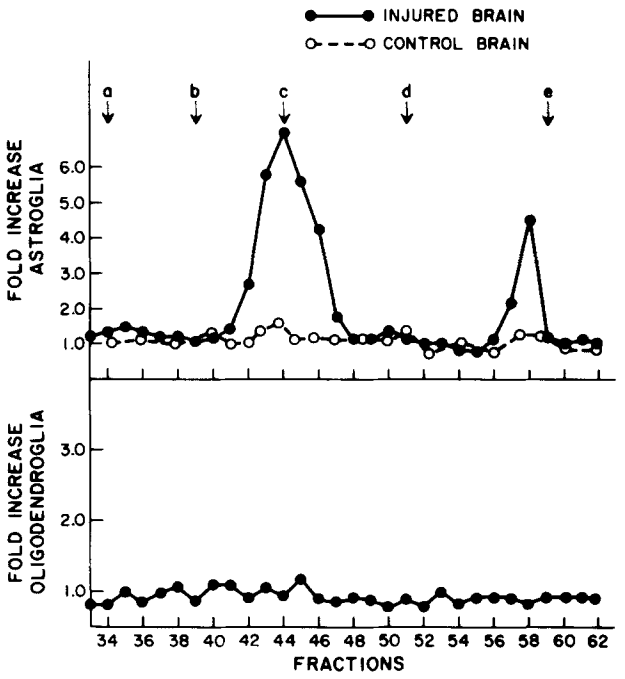

FIGURE 1 Recovery of GPFs from injured mammalian brain. Soluble protein was applied to a P-10 column (100 × 0.9 cm) and eluted with sterile PBS, pH 7.4. 100-μl aliquots of each fraction were assayed for the ability to increase the number of astroglia (*upper panel*) or oligodendroglia (*lower panel*) grown on poly-L-lysine-coated coverslips in 35-mm dishes containing 1.5 ml of defined medium. Mean numbers of cells per mm^2 were calculated for each culture, using indirect immunofluorescence techniques with galactocerebroside as a marker for oligodendroglia and GFAP for astroglia. Data were expressed as the degree of increase in cell number when compared with a control culture incubated with 100 μl of PBS. Each data point represented a mean value calculated from three cultures. 50 mg of soluble protein from a supernatant of injured rat cerebral cortices (injured) showed two peaks of astroglia-stimulating activity. These peaks contain the peptides, GPF_2 and GPF_4, which are also recovered from regenerating goldfish visual system and the developing rat brain (6–9). Supernatant from contralateral intact hemispheres (control) processed in identical fashion did not show glia-stimulating activity. Molecular mass markers: (*a*) 17 kD; (*b*) 14.5 kD; (*c*) 9.2 kD; (*d*) 6.4 kD; (*e*) 2.5 kD.

brain cultures. Initial study showed that neuron-free cultures containing mixed populations of cells produced astroglia-stimulating GPFs. We next examined specific glial populations found in these cultures (oligodendroglia, astroglia, or microglia) for the production of GPF_2 and GPF_4 (Fig. 3). Medium conditioned for 24 h by astroglia, oligodendroglia, or microglia did not stimulate the proliferation of astroglia (Fig. 3). As noted earlier, glia engulf cellular debris found in injured brain tissue (1, 2). Since phagocytosis is known to activate monocyte secretion of growth factors (17), we attempted to activate the release of GPFs by incubating glia with a potent phagocytic signal, fixed *S. aureus* (14). We found that only "stimulated" microglia (Fig. 3) released significant amounts of the astroglia-promoting factors.

The microglial secretion products were further characterized by gel filtration as described for injured brain supernatants. Defined medium conditioned by activated microglia for 24 h contained astroglia-stimulating activity which corresponded to GPF_2 (9 kD) and GPF_4 (3 kD, Fig. 4). Microglia did not release detectable amounts of oligodendroglia-stimu-

[2] The injured brain also contained a third growth factor (18 kD), which has been identified as interleukin-1 (data not shown; see also reference 16).

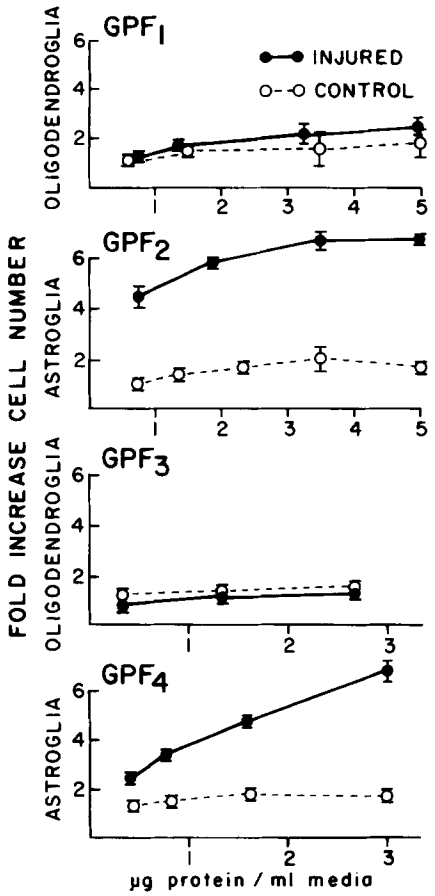

FIGURE 2 Dose-response for GPFs found in supernatants of injured or intact rat cerebral cortex. Fractions from tissue supernatants recovered by gel filtration were screened for biologic activity as described in legend to Fig. 1. Pooled fractions containing specific GPFs were compared to pooled matching fractions obtained from intact tissue controls. Data were expressed as degree increase in the number of astroglia or oligodendroglia. Each value represented a mean score calculated from at least five cultures. The protein concentration in pooled fractions was estimated by the fluorescamine method using bovine serum albumin standards. Increases in the specific activity of GPF_2 and GPF_4 were observed after injury to the rat brain.

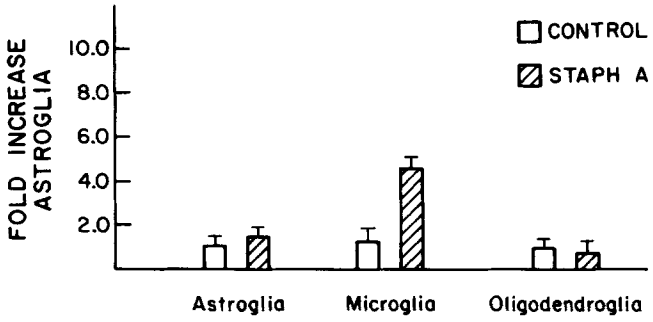

FIGURE 3 Proliferation of astroglia in the presence of medium conditioned by enriched glial cell cultures. Approximately 20,000 cells of isolated microglia (95% nonspecific esterase-positive cells) astroglia (98% $GFAP^{(+)}$ cells) or oligodendroglia (92% galactocerebroside-positive cells) were grown in 35-mm culture dishes containing 1.5 ml defined medium in the presence or absence of fixed *S. aureus* (20-µl suspension). After 24 h, 150 µl of these conditioned media were assayed for astroglia-stimulating activity as described in Materials and Methods. Only microglia which were activated by *S. aureus* released factors that stimulated proliferation of astroglia.

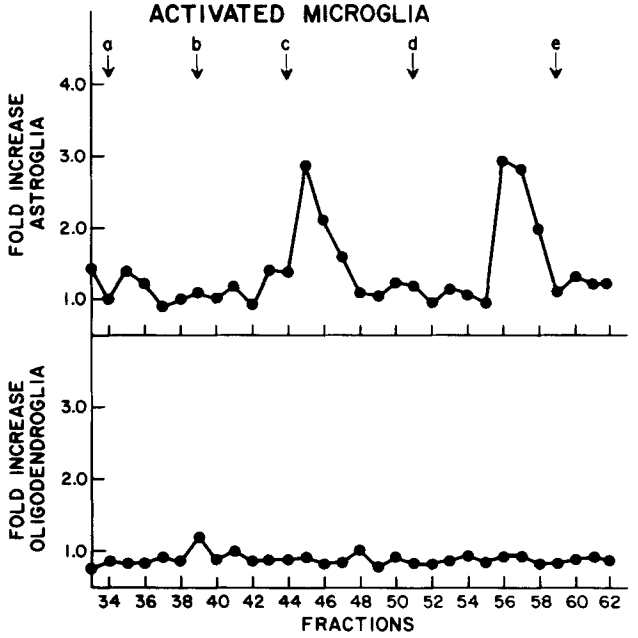

FIGURE 4 Medium conditioned by activated microglia contained GPF_2 and GPF_4. 100,000 ameboid microglia were grown in a 75-mm^2 culture flask containing 7 ml of defined medium. Microglia were activated by addition of a suspension of 150 µl fixed *S. aureus* for 24 h. Conditioned medium was concentrated to 1 ml by ultrafiltration, applied to a standardized gel filtration column, and assayed as described in legend to Fig. 1. Molecular mass markers: (*a*) 17 kD; (*b*) 14.5 kD; (*c*) 9.2 kD; (*d*) 6.4 kD; (*e*) 2.5 kD.

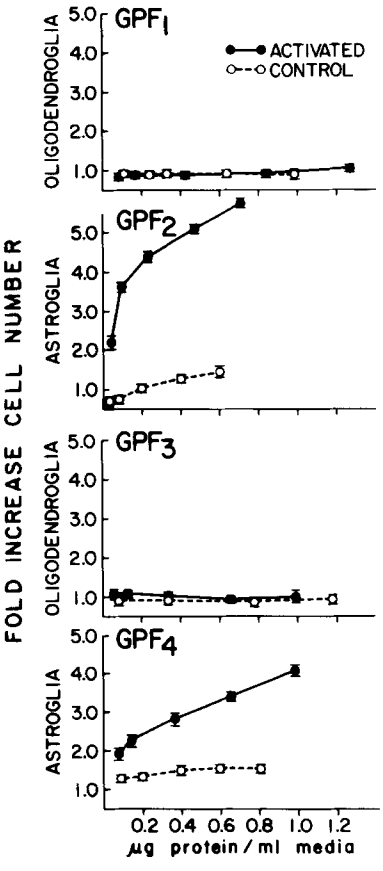

FIGURE 5 Dose-response curves for GPFs isolated from activated or control populations of ameboid microglia. GPFs found in media of microglia incubated with or without *S. aureus* were isolated by gel filtration as described in legend to Fig. 4. Activated microglia showed a marked increase in the secretion of GPF_2 and GPF_4 when compared with medium from matching control preparations.

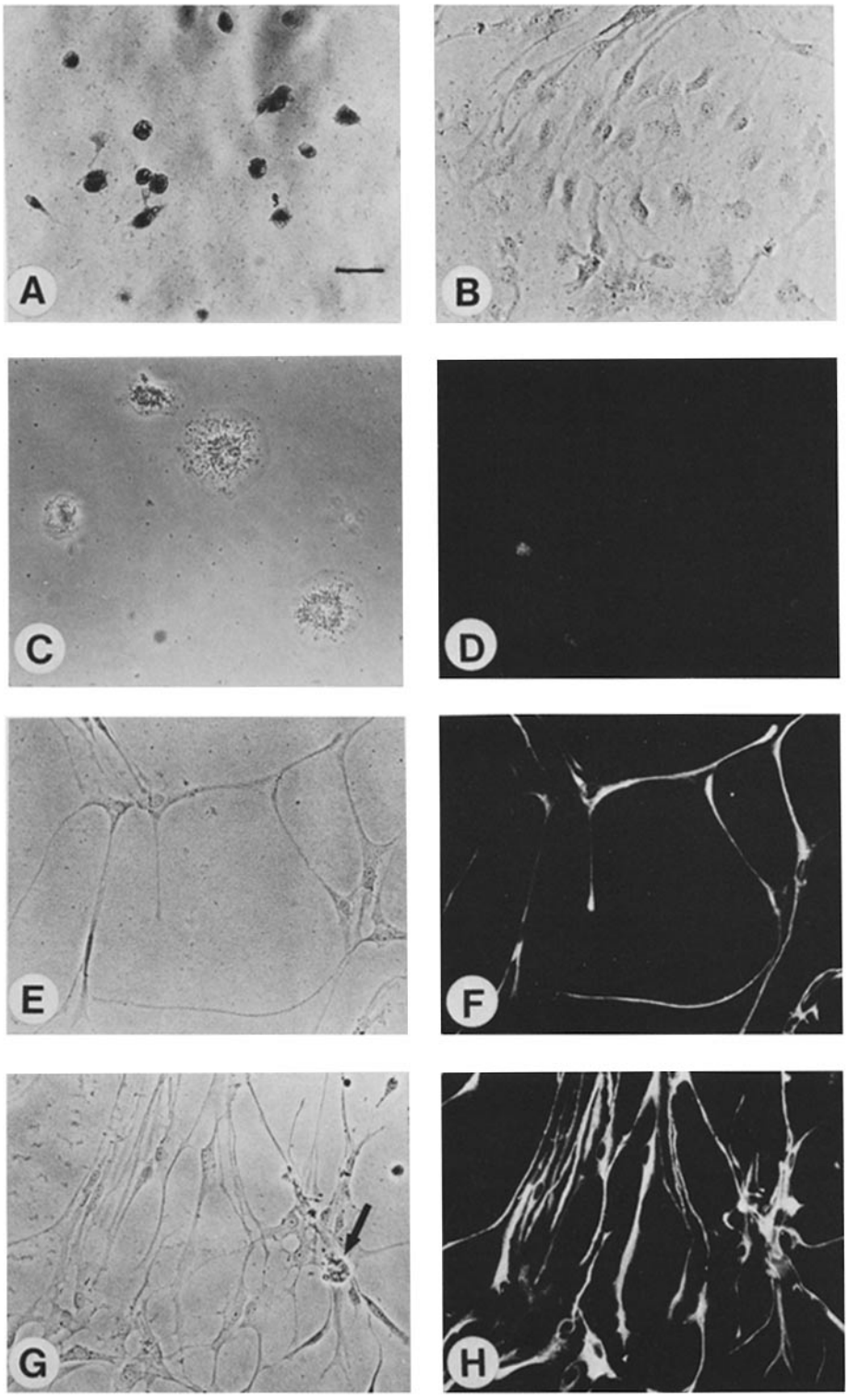

FIGURE 6 (*A*) A phase-contrast photomicrograph shows the enriched population of nonspecific esterase-positive microglia that were isolated by adhesion techniques. (*B*) A phase-contrast photomicrograph of enriched astroglial culture stained for nonspecific esterase after treatment with 5 mM L-leucine methyl ester. This treatment eliminated nonspecific esterase-positive microglia. (*C*, *E*, and *G*) Phase-contrast micrographs of enriched glia preparations that were incubated for 2 h with 20 μl of suspended fixed *S. aureus*, washed with fresh defined medium, and after 48 h in culture were stained for GFAP (*D*, *F*, and *H*). Cultures of enriched microglia (*C* and *D*) activated by phagocytosis of fixed *S. aureus*. Faint, nonspecific fluorescence staining (*D*) for GFAP was associated with the engulfed bacteria. Cultures of astroglia incubated with fixed bacteria (*E* and *F*) grow at a rate similar to that of untreated control cultures (see Table I). Astroglia proliferated rapidly, however, when grown together with activated microglia (*G* and *H*). Arrow (*G*) indicates a microglial cell atop astrocytes. Bar, 25 μm.

lating factors. Dose-response curves demonstrated that *S. aureus* increased the secretion of GPF_2 and GPF_4 by three- to fivefold when compared to unstimulated microglial cultures (Fig. 5). Further analysis of brain and microglial GPFs showed the astroglia-stimulating factors co-purified by anion exchange chromatography (DEAE-5PW, Beckman Instruments, Inc., Palo Alto, CA; 0.5 M NaCl gradient) and by reverse-phase high performance liquid chromatography (C3 column, BioRad Laboratories; 20–70% acetonitrile gradient with 10 mM trifluoroacetic acid; Giulian, D., and R. Allen, unpublished data). Based upon purification profiles, the specificity of biologic activities, and the stimulated release of growth factors, we concluded that activated microglia were a likely source of astroglia-stimulating peptides recovered from injured brain.

To test for a direct stimulatory action of microglia upon astroglia, we next examined glia–glia interactions by co-culture of enriched cell populations. We monitored the proliferation of the $GFAP^{(+)}$ astroglia that were grown in defined medium for 48 h in the presence or absence of microglia (Fig.

TABLE I. *Effects of Co-culture upon Astroglial Proliferation*

Cell culture conditions	Number GFAP(+) astroglia per mm²	Fold increase over control
Astroglia only (control)	11.5 ± 3.0	1.0
Microglia only	<0.1	—
Astroglia + *S. aureus*	8.8 ± 1.3	0.8
Astroglia + microglia	21.6 ± 1.5	1.9
Astroglia + microglia + *S. aureus*	65.9 ± 8.2	5.7

Cultures of enriched astroglia (11) or microglia were grown on poly-L-lysine-coated coverslips in 35-mm plastic dishes containing 1.5 ml of defined medium. Cells were incubated with or without 20 μl of suspended fixed *Staphylococcus aureus* (*S. aureus*, Pansorbin, obtained from Calbiochem-Behring Corp.) for 2 h in 10% fetal bovine serum. Cultures were then washed with fresh defined medium and 48 h later were stained by indirect immunofluorescence for glial fibrillary acidic protein (GFAP) found in astroglia. Data were expressed as mean number cells per mm² ± SEM. Cell numbers were computed from 10 randomly selected fields from each of five cultures per experimental group. Only those cultures containing microglia activated by *S. aureus* showed a significant increase in number of astroglia.

6). There was a five- to sixfold increase in astroglial number when these cells were grown with microglia that had been stimulated with *S. aureus* (Table I). Since *S. aureus* did not alter astroglia proliferation in the absence of microglia, we concluded that microglia acted as effector cells to promote the growth of astroglia in culture.

The implications of our in vitro data need to be considered cautiously. First, it is likely that GPFs represent only one of many regulatory mechanisms that control glial cell growth in vivo. Secondly, the ability of secreted products to influence cell growth depends not only upon the quantity and potency of the growth factor released but also the number of secretory cells and the availability of targets. Although different brain cell populations may produce GPFs in vivo, we believe ameboid microglia, which infiltrate wound sites soon after central nervous system injury, are a principal source of astroglia-stimulating peptides.

Two peptides which served as mitogens for astroglia were recovered from injured brain tissue and were released in vitro by stimulated ameboid microglia. We suggest that activated microglia release factors that regulate astroglial growth and proliferation at sites of brain injury. Therapies to control microglial responses might therefore reduce astroglial scar formation associated with brain injury.

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