Microglial Mitogens Are Produced in the Developing and Injured Mammalian Brain

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Abstract. The central nervous system produces growth factors that stimulate proliferation of ameboid microglia during embryogenesis and after traumatic injury. Two microglial mitogens (MMs) are recovered from the brain of newborn rat. MM1 has an approximate molecular mass of 50 kD and a pI of ~6.8; MM2 has a molecular mass of 22 kD and a pI of ~5.2. These trypsin-sensitive proteins show specificity of action upon glia in vitro serving as growth factors for ameboid microglia but not astroglia or oligodendroglia. Although the MMs did not stimulate proliferation of blood monocytes or resident peritoneal macrophage, MM1 shows granulocyte macrophage colony-stimulating activity when tested upon bone marrow progenitor cells.

Microglial mitogens may help to control brain mononuclear phagocytes in vivo. The MMs first appear in the cerebral cortex of rat during early development with peak levels around embryonic day E-20, a period of microglial proliferation. Microglial mitogens are also produced by traumatized brain of adult rats within 2 d after injury. When infused into the cerebral cortex, MM1 and MM2 elicit large numbers of mononuclear phagocytes at the site of injection. In vitro study shows that astroglia from newborn brain secrete MM2. These observations point to the existence of a regulatory system whereby secretion of proteins from brain glia helps to control neighboring inflammatory responses.

Materials and Methods

Cell Cultures

Mixed glial cell cultures were prepared as described earlier (Giulian et al., 1986a) from dissociated cerebral cortex of newborn albino rats (Holtzman, Madison, WI). Cells were placed in 35-mm plastic dishes upon poly-L-lysine-coated glass coverslips in chemically defined culture medium (Bottenstein and Sato, 1979) supplemented with 10% fetal bovine serum; after 48 h, cells were grown in chemically defined medium without serum. Ameboid microglia were isolated from the brains of newborn rat using the method of Giulian and Baker (1986).

Astroglia isolated by the method of McCarthy and De Vellis (1980) were plated at 5 × 10^4 on 100-mm plastic dishes and freed of microglia by incubation with 10 mM L-leucine methyl ester (Sigma Chemical Co., St. Louis, MO) (Giulian and Baker, 1986) plus 500 µg/dish of carbonyl iron (Sigma Chemical Co.) (Wong and Varesio, 1984). 24 h later, iron particle-engulfing microglia were removed by a magnet. Enriched glial preparations were grown on glass coverslips (without poly-L-lysine coating) for 24 h in chemi-
Figure 1. Fluorescence photomicrographs showing the effects of brain extracts upon growth of ameboid microglia. Brain extracts were obtained from animals of different ages and incubated with mixed cell cultures for 72 h at a concentration of 200 μg protein per ml of chemically defined medium. Ameboid microglia were identified as Dil-ac-LDL(+) cells. (A) Control culture; (B) extract from embryonic stage E-18; (C) extract from adult rat. As shown, the developing rat brain contained factors that stimulated the appearance of Dil-ac-LDL(+) microglia. These stimulated cultures often contained dense clusters of ameboid cells (B). Bar, 20 μm.
A 125 i oo 200 300 ~ug PROTEIN/ml MEDIUM

Figure 2. Ameboid microglial proliferative activity found in brains of different ages. (A) 200 µg of brain extract were added for 72 h to glial cultures grown in chemically defined medium. Data, expressed as mean number of Dil-ac-LDL(+) ameboid microglia per microscopic field ± SEM, were obtained from at least five cultures per group. As shown, the greatest microglial growth activity was found in cerebral cortex obtained from embryonic stage E-16 to the time of birth (PN-1). (B) Dose response curves for mitogenic activity recovered from extracts of developing and mature brains. Data, expressed as mean fold increase in microglial number ± SEM when compared to control cultures, were obtained from at least five cultures per time point. Greater biologic activity per microgram brain protein was noted in cerebral cortex of animals from the perinatal period. (o) E20; (●) PN1; (△) adult.

Figure 3. Presence of microglial mitogens in extracts from cerebral cortex. Soluble protein recovered from injured adult 3 d after trauma, normal adult, and newborn rat were fractionated by gel filtration (BioGel P-10, 95 × 0.8 cm) in 150 mM PBS. Data are expressed as mean fold increase in microglial number ± SEM when compared to control cultures. Three peaks of microglial proliferative activity were observed in some tissues. The two microgenic factors with larger molecular masses, designated MM1 and MM2, were found only in injured adult and newborn brain. The third peak of biologic activity with a low molecular mass was common to all brain extracts and not further studied. 2 mg of soluble brain protein were applied to each column. Each fraction contained 670 µl of which 25 µl were used in triplicate cultures to test biologic activity.

Identification of Glial Cells

Ameboid microglia were identified by fluorescence microscopy using acetylated low density lipoprotein (ac-LDL) bound to the fluorescent probe 1,1'-dioctadecyl-3,3',3''-tetramethyl-indocarbocyanine perchlorate (DiI; Pitas et al., 1981; Giulian and Baker, 1986). In vitro labeling was carried out in chemically defined medium with 1 µg/ml of Dil-ac-LDL for 12 h at 37°C. The Dil-ac-LDL was obtained from Biomedical Technologies, Inc. (Cambridge, MA). Cultured ameboid cells were also visualized by monitoring phagocytosis of fluorescently labeled polystyrene microspheres (0.7 microns; Covaspheres Particles, FX Green, Duke Scientific Corp., Palo Alto, CA) and by histochemistry for non-specific esterase (Koski et al., 1976). Standard indirect immunofluorescence techniques were used to identify astroglia containing glial fibrillary acidic protein (GFAP) and oligodendroglia containing galactocerebroside (GC) (Giulian et al., 1986a).

Bioassays

To test for mitogenic effects, cells grown in 1.5 ml chemically defined culture medium were incubated with increasing concentrations of growth factors for 72 h. Proliferation of ameboid microglia, astroglia, oligoden- droglia, peritoneal macrophage, blood monocytes, and adherent bone marrow cells were monitored by scoring the number of specifically labeled cells in nine randomly selected fields (0.314 mm²) from each of three or more coverslips. The data were expressed as fold increase over control cultures incubated with matching aliquots of PBS ranging from 1 to 50 µl/ml. 1 U of MM activity represents the amount of factor needed to double the number ameboid microglia in newborn rat brain cultures after a 72-h incubation in 1.0 ml of chemically defined medium. Specific activities were expressed as units per µg protein as determined by the fluorescamine assay (Bohlen et al., 1973).

To measure [3H]thymidine incorporation, isolated ameboid microglia were placed in 96-well plates (50,000 cells per well) and incubated for 72 h in 200 µl of chemically defined medium containing various concentrations of MMs. During the last 15 h of the incubation period, 0.5 µCi of [3H]thy- midine (26 Ci/mmol; Amersham Corp., Arlington Heights, IL) were added...
Molecular masses of microglial mitogens as estimated by HPLC gel filtration. Protein was separated by two TSK 125 columns in series (300 x 7.5 mm) with an elution buffer of 200 mM PBS at a flow rate of 0.5 ml/min. MM1 was found to have an apparent mass of 50 kD and MM2 to have a mass of ~20 kD. Data are expressed as mean fold increase in microglial number ± SEM when compared to control cultures. Each fraction contained 500 µl of which 25 µl were used in triplicate cultures to test biological activity. Molecular mass markers (zx) are shown in the upper panel.

Isoelectric Focusing Cell (Bio-Rad Laboratories). Fractions were separated by electrophoresis at room temperature using 21 cm gels. Low molecular weight toxins found in the concentrated eluate were removed by gel filtration using a 0.4-µm filter. MM2 were similar to those found using the Rotofor Isoelectric Cell. Eluted samples from an IEF control gel (zx, bottom) showed that small amounts of recovered ampholytes did not affect numbers of microglia in culture.

Isolation of Microglial Mitogens

Cerebral cortices from albino rats of different ages were isolated, stripped of meninges, and dispersed by mild sonication in 150 mM PBS (1:10 [wt/vol]). The brain sonicate was centrifuged at 15,000 g for 15 min and the supernatant passed through a 0.4-µm filter. MM2s were isolated by gel filtration using a P10 column (95 x 0.8 cm; Bio-Rad Laboratories, Richmond, CA) with 150 mM PBS (pH 7.4) as the eluting buffer (Giulian et al., 1985) and concentrated by ultrafiltration through YM 10 filters (Amicon Corp., Danvers, MA). Molecular masses of the MM2s were estimated by gel filtration HPLC using two TSK-125 columns (300 x 7.5 mm; Waters Associates, Milford, MA) in series that had been standardized with proteins of known molecular masses (range 5-90 kD).

Two methods for IEF were used. An analytic method used slab gels of acrylamide (5 x 10 x 0.1 cm; LKB Instruments, Inc., Gaithersburg, MD) containing 0.08 % trifluoroacetic acid. The pH gradient was measured directly from the IEF gel with an Ingold pH surface electrode (Fisher Scientific Co.) MM2s were eluted from 0.5 x 4 cm columns (300 x 7.5 mm; Waters Associates, Milford, MA) with 150 mM PBS (pH 7.4) as the eluting buffer (Giulian et al., 1985) and centrifuged for 2 min at 15,000 g. The samples were then separated by electrophoresis at room temperature using 21 cm gels and centrifuged for 5 min at 15,000 g. The samples were then separated by electrophoresis at room temperature using 21 cm gels, and the supernatant was passed through a 0.2-µm filter. MM2s were isolated by gel filtration HPLC using two TSK-125 columns (300 x 7.5 mm; Waters Associates, Milford, MA) in series that had been standardized with proteins of known molecular masses (range 5-90 kD).

Two methods for IEF were used. An analytic method used slab gels of acrylamide (5 x 10 x 0.1 cm; LKB Instruments, Inc., Gaithersburg, MD) with 0.08 % trifluoroacetic acid. The pH gradient was measured directly from the IEF gel with an Ingold pH surface electrode (Fisher Scientific Co.) MM2s were eluted from 0.5 x 4 cm columns of gels into sterile deionized water by vigorous shaking for 12 h at 4°C. The eluate was concentrated with a SpeedVac Concentrator (Savant, Farmingdale, NY), filtered through 0.2-µm filter, and assayed for mitogenic activity. Alternatively, preparative IEF was performed using the Rotofor Preparative Isoelectric Focusing Cell (Bio-Rad Laboratories). Fractions recovered from gel filtration chromatography were pooled and dialyzed against deionized water overnight. After dialysis, 2.5 ml of Servalyt pH 3-10 iso-dalt grade amylase solution (40 % amylase wt/vol; Serva Fine Biochemicals Inc., Garden City Park, NY) was added to the sample, and the volume brought up to 50 ml with deionized H2O to give a final 2% amylase concentration. The sample was separated by isoelectric focusing for 4-5 h at 12 W constant power at 4°C into 20 fractions. After determining pH, each fraction was sterilized by filtration and tested for biologic activity. (Control experiments showed no effect of eluted amylases upon cultured microglia.) This material was also fractionated by reverse phase high performance liquid chromatography (RP-HPLC; 75 x 4.6 mm Ultrapore C8 column; Beckman Instruments, Inc., Palo Alto, CA) using an acetonitrile gradient (0-55% over 52 min at 1 ml per min) with deionized water containing 0.08% trifluoroacetic acid.

Surgical Procedures

A penetrating injury to adult rat brain was inflicted with a flame-heated 25 gauge needle mounted on a stereotaxic device (David Kopf Instruments, Tujunga, CA) and inserted through a burr hole to a depth of 1.0 mm in the cerebral cortex (Giulian et al., 1988a). At different intervals after surgery,
tissue samples (~2 mg wet weight) taken from the sites of cortical injury were incubated in 200 μl of culture medium for 24 h. Production of microglial mitogenic activity was then measured by the standard microglial cell assay.

To study the effects of MMs in vivo, we injected small volumes of isolated growth factors into rat cerebral cortex. Adult rats (250–300 g) were deeply anesthetized by intraperitoneal injection (a mixture of 8.5 mg/ml xylazine, 42 mg/ml ketamine hydrochloride, and 1.4 mg/ml acepromazine maleate) and placed in a stereotaxic device (David Kopf Instruments). After the scalp was reflected, burr holes were positioned over the cerebral cortex at 4.5 mm caudal to bregma and 4.0 mm lateral to the sagittal suture. Using a 50-μl syringe (Hamilton Company, Reno, NE) mounted in the stereotaxic device, we infused 0.5 μl of PBS containing a MM at a depth of 1.5 mm from the surface of the brain over a 2-min interval. Control injections of PBS or PBS containing 200 ng of cytochrome C were made in an identical location on the contralateral side. All solutions contained fluorescent microspheres (1:500 [wt/vol]; Covaspheres Particles, FX Green, Duke Scientific Corp.) in order to mark the site of infusion. The syringe needle was slowly withdrawn and the scalp closed with wound clips. 48 h later, the animals were deeply anesthetized and cylindrical biopsies (2.0 × 1.0 mm) were removed rapidly from the injection sites after cardiac perfusion with heparin-phosphate buffered saline solution (500 USP U/liter). The tissue samples were then incubated for 12 h at 37°C in 0.5 ml of chemically defined culture medium containing 2 μg/ml Dil-ac-LDL. The tissues were fixed with 3% formaldehyde in PBS for 1 h and viewed at 200 × using fluorescence microscopy and photographed immediately. Comparisons were made between the mean number of Dil-ac-LDL(+) cells per field in MM-injected, PBS-injected, and cytochrome C-injected sites as delineated by the presence of coinjected microspheres.

**Results**

Many investigators have noted microglial proliferation during early brain development (Rio-Hortega, 1932; Ling, 1981; Matsumoto and Ikata, 1985; Giulian and Baker, 1986). The rapid increase in numbers of microglia found in embryonic cerebral cortex (Ling, 1981; Matsumoto and Ikata, 1985; Giulian et al., 1988b) suggest the presence of mitogenic factors driving cell proliferation. Extracts obtained from the cerebral cortices of rats of different ages were added to mixed glial cultures for 72 h. There was a significant increase in the number of Dil-ac-LDL(+) ameboid microglia in cultures grown in the presence of embryonic brain extracts (Fig. 1). The developmental pattern showed that rat cerebral cortex contained microglial growth factors between embryonic day E-16 and PN-1 (Fig. 2 A). Dose response curves (Fig. 2 B) demonstrated the greatest brain concentrations of microglial mitogenic activity near the time of birth. Study of tissue biopsies indicated that 1 mg of cerebral cortex of newborn rat released 1.3 ± 0.3 U of microglial mitogenic activity within a 24-h period; in contrast no significant biologic activity was associated with crude extracts from normal adult brain. When compared with other glia-promoting factors, the peak of microglial mitogenic activity was similar to that of astroglial growth factors in developing rat cerebral cortex but before the postnatal burst seen for oligodendroglial growth factors (Giulian et al., 1986a; Giulian and Young, 1986).

**Microglial Mitogens Found in Developing Brain**

Extracts from newborn brain separated by gel filtration showed three peaks of mitogenic activity (Fig. 3). Two of these peaks, designated MM₁ and MM₂, were not detected in normal adult brain. The third peak of biologic activity, common to all extracts from cerebral cortex, was not studied further. Gel filtration HPLC indicated MM₁, has an apparent molecular mass of ~50 kD (Fig. 4) while slab gel IEF showed an approximate isoelectric point of 6.8 ± 0.2 (n = 4; see Fig. 5). MM₂ was found to have an apparent molecular mass of 20 kD (Fig. 4) and an isoelectric point of 5.2 ± 0.2 (n = 6; Fig. 5). These factors could also be separated by RP-HPLC with a single peak of MM₁ eluting at 45–48% acetonitrile and a peak of MM₂ at 27–30% acetonitrile (Fig. 6). A purification scheme combining gel filtration, IEF, and RP-HPLC yielded between 10,000- and 20,000-fold increases in specific biologic activities for MM₁ and MM₂ when compared to extracts of newborn brain (Table 1). These partially purified MMs showed a direct action upon microglia both in cell proliferation and in [³H]thymidine incorporation bioassays (Fig. 7) using highly enriched cultures of ameboid cells (>98% homogeneous population).

**Table I. Isolation of Microglial Mitogens from Newborn Rat Brain**

| Specific activity | MM₁ | MM₂ |
|------------------|-----|-----|
| **Preparation**  |     |     |
| Extract          | —   | 0.002 |
| Gel filtration   | 0.06| 0.04 |
| IEF              | 6.2 | 5.0  |
| RP-HPLC          | 40.0| 25.0 |

Extracts of newborn brain were prepared by sonication and centrifugation at 15,000 g. Gel filtration with BioGel P10 separated two distinct microglial mitogens, MM₁ and MM₂. IEF separation used the Rotofor Isoelectric Cell; RP-HPLC used a C8 column with an acetonitrile gradient. Specific activities obtained from representative preparations are expressed as units of activity per μg of protein. 1 U of MM activity represents the amount of factor needed to double the number ameboid microglia in newborn rat brain cultures after a 72-h incubation in 1.0 ml of chemically defined medium. Using a combination of gel filtration, IEF, and RP-HPLC we estimate at least a 20,000-fold purification of MM₁ and 12,500-fold for MM₂ from extracts of newborn rat cerebral cortex.

Giulian et al. Microglial Mitogens Found in Brain
Figure 7. MM₁ and MM₂ stimulated ameboid microglia to incorporate [³H]thymidine. Mitogens were isolated from newborn brain extract by a procedure involving gel filtration, IEF, and RP-HPLC. Each value, expressed as mean number cpm ± SEM, was obtained from at least five cultures of isolated ameboid cells (50,000 cells per well; >98% homogeneous population) incubated with growth factors for 72 h.

The effects of trypsin upon MMs were monitored by incubating partially purified factors with trypsin (1 μg trypsin per 100 μg protein). The enzymic activity was blocked by the addition of soy bean trypsin inhibitor (2 μg inhibitor for 1 μg trypsin) at the end of a 60-min incubation period at 37°C. Enzyme-treated controls received both trypsin and trypsin inhibitor before incubation. Trypsin-digested factors were applied to mixed glial cultures and the number of DiI-ac-LDL(+) microglia were determined after 72 h. Data, expressed as fold increase in cell number above PBS-treated

Figure 8. Production of microglial mitogens by injured brain. Three biopsies of adult brain were taken from penetrating wound sites at increasing intervals after injury and incubated for 24 h in chemically defined medium. The conditioned medium assayed for microglial mitogenic activity showed the highest levels of activity between days 2 and 5 after penetrating brain injury. Data are expressed as mean fold increase in microglial number ± SEM when compared to control cultures. Extracts of injured brain tissue fractionated by gel filtration showed the presence of both MM₁ and MM₂ (see Fig. 3).

Figure 9. Microglial mitogens are specific in their action upon brain glia. Dose response curves show that MM₁ and MM₂ increase the number of DiI-ac-LDL(+) ameboid microglia but not GC(+) oligodendroglia or GFAP(+) astrogia in culture. Data are expressed as mean fold increase in microglial number ± SEM when compared to control cultures. Mitogens were isolated from newborn brain extract by a procedure involving gel filtration, IEF, and RP-HPLC.

Figure 10. MMs effects upon mononuclear phagocytes isolated from outside the CNS. At between 1 and 5 U, MMs had no effect upon resident peritoneal macrophage (top) or blood monocytes (center). MM₁, did, however, stimulate proliferation of bone marrow progenitor cells (bottom). Data are expressed as mean fold increase in cell number ± SEM when compared to control cultures. (●) MM₁; (▲) MM₂.
cultures, showed that trypsin-treated MM₁ (2.7 ± 0.2-fold increase for enzyme control vs. 1.4 ± 0.1 trypsin-treated; \(t = 6.05, df = 13, P < 0.001\)) and trypsin-treated MM₂ (2.8 ± 0.2-fold increase for enzyme control vs. 1.1 ± 0.2 trypsin-treated; \(t = 6.01, df = 14, P < 0.001\)) had significant reductions in biologic activity. Thus, the developing brain contains two distinct trypsin-sensitive proteins which are mitogens for ameboid microglia in culture.

**Microglial Mitogens after Brain Injury**

Since brain mononuclear phagocytes proliferated in response to tissue injury (Rio-Hortega, 1932), we examined CNS at sites of trauma for the presence of microglial growth factors. Biopsies of injured and intact control tissues were incubated in chemically-defined culture medium for 15 h at 37°C. Traumatized brain tissue released the greatest amounts of microglial mitogenic activity between 2 and 5 d after injury (Fig. 8). 1 mg of adult rat cerebral cortex recovered at 2 d after a penetrating injury released \(\sim 1.2 \pm 0.2\) U of MM activity (from four samples each using three pooled of 6 mg each) compared with <0.1 U found in normal adult animals (from four pooled samples). Gel filtration chromatography showed that extracts from injured brain contained two microglial mitogens not found in uninjured normal adult CNS (Fig. 3). These two peaks of biologic activity corresponded to MM₁ and MM₂ with the same apparent molecular masses (Fig. 3) and isoelectric points as found for those growth factors recovered from newborn brain (data not shown). We concluded that identical microglial mitogenic factors were found in developing and injured brain.

**Cell Specificity of Microglial Mitogens**

The specificities of biologic activity for MM₁ and MM₂ were tested against brain glia in culture. MMs were potent mitogens for ameboid microglia but had no effect upon proliferation of GFAP(+) astroglia or GC(+) oligodendroglia in vitro (Fig. 9). These results clearly demonstrated that a distinct class of growth factors was recovered from the CNS. Coupled with earlier work, these observations reveal that cell specific mitogens exist for each of the brain glial cell types. Such selective biologic activities further support the notion that MMs serve as immunoregulators of brain mononuclear phagocytes.

Ameboid microglia are a class of mononuclear phagocytes which can be distinguished from resident peritoneal macrophage and blood monocytes by morphology, proliferative capacity, and response to growth factors (Giulian and Baker, 1986; Giulian, 1987). In testing for mitogenic effects upon

![Figure 11. Brain-derived MMs and colony-stimulating activity. To compare MMs with colony-stimulating factors, MM₁ and MM₂ were tested for effects upon bone marrow cells grown in culture. Fluorescence photomicrographs showing colonies of cells found in control (A), or MM₁-stimulated (B), or MM₂-stimulated (C) cultures. Dose response curve (D) shows that only MM₁ promoted formation of Dil-ac-LDL(+) colonies (defined as >50 cells per cluster). (○) MM₁; (▲) MM₂.](image-url)
Astroglia and Production of Microglial Mitogens

In Vivo Biologic Effects of Microglial Mitogens

Astroglia and Production of Microglial Mitogens

Table II. Colony-stimulating Activity of MM

| MM1 activity | Total number | Gran | Mac | Gran-Mac | Other |
|--------------|--------------|------|-----|----------|-------|
|              | colonies scored |      |     |          |       |
| 1 U          | 42           | 2    | 57  | 40       | 0     |
| 2 U          | 47           | 4    | 40  | 57       | 0     |
| 5 U          | 117          | 21   | 27  | 48       | 4     |

Bioassays for colony-stimulating activity used the basic techniques of Metcalf (1984) applied to adult rat bone marrow. 2 x 10^6 cells were placed in 35-mm dishes containing 0.3% agar with DMEM supplemented with 20% FBS. MM1 was added at the time of plating. After 7 d, cultures were fixed, dried, and stained with luxol fast blue and hematoxylin (Metcalf, 1984). A colony consists of >50 cells per cluster. As shown, MM1 stimulates formation of cell clusters containing predominantly granulocytes (by convention assigned to neutrophils) or macrophages, suggesting granulocyte-macrophage colony-stimulating activity. Increasing concentrations of MM1 stimulate more granulocyte colonies, a pattern noted by Metcalf (1984) for GM-CSF. 1 U of MM1 activity represents the amount of factor needed to double the number ameboid microglia in newborn rat brain cultures after a 72-h incubation in 1.0 ml of chemically defined medium. Gran, neutrophilic granulocytes; Mac, macrophage; Gran-Mac, mixed colonies with neutrophils and macrophages; Other, mixed colonies with small unstained cells, eosinophils, neutrophils, and/or macrophages.

As described in previous work, two classes of colony-stimulating factors induce proliferation of cultured peritoneal macrophage (Fig. 10, top) or blood monocytes (Fig. 10, center). We did note, however, that MM1, but not MM2, stimulated proliferation of mononuclear phagocytes from bone marrow (Fig. 10, bottom).

As described in previous work, two classes of colony-stimulating factors induce proliferation of cultured ameboid microglia (Giulian and Ingeman, 1988). To investigate the relationship of the brain-derived MMs and hematopoietic growth factors, we tested the MMs for colony-stimulating activity. As shown in Fig. 11, MM1, but not MM2, elicited a striking growth of colonies containing DiI-ac-LDL(+) mononuclear phagocytes. Examination of cell types in the bone marrow cultures stimulated with growth factor showed MM1 had granulocyte-macrophage colony-stimulating factor activity (Table II).

Astroglia and Production of Microglial Mitogens

As noted earlier, there is a close relationship between proliferating ameboid microglia and astroglia in vitro. We examined this relationship by monitoring MM activity in medium conditioned by cultures of highly purified astroglia (>99% homogeneous population). As shown in Fig. 12, cultured astroglia secrete a single factor with a pI of ~5.2 and a molecular mass of ~20 kD; this biologic activity corresponds to MM2. Astroglia-secreted MM2 was further purified using gel chromatography, preparative Rotofor IEF, and SDS-PAGE. We found a major protein band with a molecular mass of ~22 kD that retained biologic activity when eluted from SDS-PAGE (Fig. 13). Our observations link the growth of microglia with secretory products from astroglia. Astroglia did not secrete detectable levels of MM1, suggesting different cellular sources for MM1 and MM2 in vivo.

In Vivo Biologic Effects of Microglial Mitogens

To investigate the biologic effects of microglial growth factors in vivo, we injected highly purified MM1 and MM2 (1–2 U each) into the cerebral cortex of adult rats. At 48 h after the

Figure 12. Astroglial production of microglial mitogen. Highly enriched preparations of astroglia were incubated in chemically defined medium. This medium conditioned for 48 h was fractionated and tested for microglial mitogen activity. (Top) As shown by IEF separation, astroglial conditioned medium contained only one peak of biologic activity with a pI of ~5.2. (Bottom) This biologic activity was fractionated further by gel filtration using HPLC. The apparent molecular mass of ~20 kD identifies this factor as MM2. No MM1-like activity was noted in astroglial conditioned medium.

Figure 13. MM2 isolated from astroglial conditioned medium. 500 ml of medium were concentrated by ultrafiltration (YM-2). The biologic activity corresponding to MM2 was next fractionated by two consecutive runs of preparative IEF. MM2 showing a pI of ~5.2–5.3 appeared as a major silver-stained band of protein with a molecular mass of 22 kD (lane B). Gel slices were eluted overnight with deionized water, concentrated by centrifugation under vacuum, and fractionated by slab gel electrophoresis (BioGel P2, 210 x 0.8 cm). Biologic activity (far right) recovered from gel slices corresponded to the 22-kD band. Data are expressed as mean fold increase in microglial number ± SEM when compared to control cultures. Lane A, molecular mass markers (~200 ng each) which include ovalbumin (43 kD), carbonic anhydrase (29 kD), β-lactoglobulin (18 kD), lysozyme (14 kD), bovine trypsin inhibitor (6 kD), and insulin (3 kD).
injection of 0.5 μl containing MM₁, MM₂, or 200 ng cytochrome C we found that only MMs increase Dil-ac-LDL(+) cells at the site (Fig. 14). The amount of MM activity infused into the brain was estimated to be in the range of MM activity released over a 48-h interval by tissue at the site of penetrating injury in adult cerebral cortex. We concluded that physiologic concentrations of MMs were capable of activating brain mononuclear phagocytes in vivo (Fig. 15).

Discussion
Soluble proteins found in the CNS help to regulate the
Figure 15. Quantitation of mononuclear phagocytes appearing after 48 h at MM injection sites. 200 ng of MM1 (1.5 U) (●), MM2 (1.0 U) (●), or 200 ng cytchrome C (in 0.5 μl of PBS) (●) were injected into the cerebral cortex of adult rats. Data are expressed as mean fold increase in the number of Dil-ac-LDL(+) cells per field ± SEM when compared to control sites injected with 0.5 μl PBS. The number of Dil-ac-LDL(+) mononuclear phagocytes were determined for at least five biopsies for each data point.

growth and differentiation of astroglia (Pruss et al., 1981; Leutz and Schachner, 1981; Giulian et al., 1988b; Richardson et al., 1988; Lim et al., 1989; Loret et al., 1989) or oligodendroglia (Giulian and Young, 1986; McMorris and Dubois-Dalcq, 1988). In this report we establish the presence of another class of brain-derived growth factors, microglial mitogens. In contrast to oligodendroglia growth factors, the MMs appeared early in development and at sites of injury. Biochemical properties including apparent molecular mass, pl, elution profile by RP-HPLC as well as specificities of biologic actions, clearly separated astroglia-promoting factors from the MMs. Recombinant forms of interleukin 1 (IL-1), one of the astroglial factors, do not stimulate microglial proliferation.

Ameboid microglia are the principal immunoeffector cells within the brain (Giulian, 1987) involved in such functions as secretion of cytokines, antigen presentation (Frei et al., 1986), and production of cytotoxins (Giulian, 1990). Recent observations indicate that microglia also mediate wound healing in neural tissues (Giulian et al., 1989). This report describes the existence of brain-derived mitogens that stimulate the proliferation of ameboid microglia. These mitogens, designated MM₁ and MM₂, may be distinguished from one another by their apparent molecular masses, their isoelectric points, and their elution profiles using RP-HPLC. MM₁, but not MM₂, stimulates growth of bone marrow-derived mononuclear phagocytes.

We propose that MM₁ and MM₂ help to expand ameboid microglia populations during specific periods of brain development or at sites of CNS injury. Several lines of evidence support this hypothesis. First, concentrations of MMs are increased during that period of brain development when microglia undergo rapid proliferation. As these cells lose their proliferative capacity (Giulian and Baker, 1986), the brain levels of the MMs decline. Although normal adult brain has only low levels of MMs, these mitogens increase dramatically by 2 d after penetrating brain injury. It is during this time that large numbers of Dil-ac-LDL(+) cells appear at the wound site (Giulian et al., 1989). In vitro experiments show that both MM₁ and MM₂ have specificity of action for brain mononuclear phagocytes. In vivo experiments show that MMs infused into the cerebral cortex elicit local inflammatory responses. Further study will be needed to determine if the MMs induce proliferation, chemotaxis, or recruitment of quiescent brain cells in vivo. The presence of MMs within sites of injury and their ability to elicit an inflammatory response in vivo argues that these growth factors are physiologically important regulators of CNS inflammation.

Are the brain-derived MMs similar to other known growth factors? A recent study of growth factors and immunomodulators show that only certain classes of colony-stimulating factors (CSFs) induce proliferation of ameboid microglia (Giulian and Ingeman, 1988). In particular, granulocyte-macrophage CSF (GM-CSF) and multiple potential-CSF (multi-CSF; also referred to as interleukin 3) are potent microglial mitogens. These colony-stimulating factors were originally identified as growth factors for hematopoietic cells (Metcalfe, 1985; Clark and Kamens, 1987). Although the origin of microglia is uncertain, in vitro studies suggest that the ameboid cell may be similar to progenitor mononuclear phagocytes found in bone marrow (Giulian, 1987; Giulian and Ingeman, 1988). Importantly, we find that MM₁ has colony-stimulating factor-like activity. Definitive work comparing rat CSFs with brain MMs awaits further biochemical study. Two other reports suggest the presence of colony-stimulating factor-like activity within the CNS. In the mouse, Nicola et al. (1979) find the brain contains GM-CSF-like biologic activity while Frei et al. (1986) described multi-CSF-like activity from cultured brain glia. Our observations are in agreement with Nicola et al. (1979) in that MM₁ has GM-CSF activity but neither MM₂ or MM₃ show multi-CSF-like effects. The molecular mass of 50 kD we have assigned to rat MM₁ does not match human GM-CSF (18-22 kD) or mouse GM-CSF (23 kD), but, as noted by Metcalfe (1984), size estimates of this glycosylated hematopoietic growth factor have varied greatly perhaps due to species differences and tissue-specific glycosylation. Sequence analysis will be needed to determine the structural relationships of MMs to other growth factors.

There is growing evidence that immune system secretion products regulate cellular events in damaged neural tissue. Neurotoxins released by mononuclear phagocytes may mediate a variety of neuropathic conditions including loss of neurons after stroke (Giulian and Robertson, 1990; Giulian, 1990). Moreover, the cytokine IL-1 produced by microglia stimulates astrogliosis at sites of trauma (Giulian et al., 1988a). We believe that microglial mitogens represent yet another class of immunoregulators that influence specific cell functions within the brain. As described here, astrogliosis stimulate microglial proliferation by secretion of a mitogen. Thus, we have a reciprocal relationship between two classes of brain glia. Understanding the mechanisms that control microglial populations may lead to new strategies to limit the extent and nature of CNS tissue damage.

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