INTERLEUKIN 1 OF THE CENTRAL NERVOUS SYSTEM IS PRODUCED BY AMEBOID MICROGLIA

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Several lines of evidence indicate that glia play an important role in supporting the growth of neurons (1–7). Using peripheral nerve grafts, Aguayo et al. (1) have shown that neurons of the mammalian central nervous system (CNS)[1] are capable of dramatic axonal sprouting when they are in a suitable glial environment. By altering glial cell populations in developing and regenerating neural systems in vivo, other investigators (3, 4) confirmed that glia were necessary for successful neuronal migration or axonal growth. In vitro studies extend these observations by suggesting that glia secrete growth regulating factors (4, 5) or produce extracellular matrix proteins that influence neuronal growth (1, 6–8). Thus, an understanding of events that mediate glial cell growth may help to elucidate the mechanisms that control neuronal development and recovery from CNS injury (8–9).

Recently, lymphokines and monokines found in the CNS have been implicated as regulators of glial cell growth and proliferation (10–12). IL-1 has been isolated from the brain of rats following injury (10), and cultured brain cells have been found to secrete factors in vitro similar to IL-1 (12). IL-1 is a peptide growth factor released by many cell types, but particularly by activated mononuclear phagocytes in response to endotoxin or following phagocytosis of bacteria or inert particles such as silica (13). IL-1 is thought to mediate tissue damage during inflammation by stimulating T cell function (14), by inducing prostaglandin and protease release (15), and by stimulating fibroblast proliferation (16). Since IL-1 has been isolated from traumatized mammalian brain and shown to be a potent astroglial growth factor, we have proposed (10) that IL-1 may regulate astroglial proliferation at the site of CNS injury.

The cellular sources of brain IL-1 remain uncertain. Although blood monocytes enter the brain after certain types of trauma (17–19), cells intrinsic to the brain have also been implicated as a source of the IL-1. Fontana et al. (12), for example, report that enriched populations of astroglia release IL-1 in culture. Alternatively, ameboid microglia, mononuclear phagocytic cells of the brain,

[1] Abbreviations used in this paper: CNS, central nervous system; FGF, fibroblast growth factor; GFAP, glial fibrillary acidic protein; LDL, low-density lipoprotein.

have also been shown (20) to release peptide factors that stimulate glial growth. Using newly developed methods to separate astroglia from ameboid microglia (21), we examined specific glial populations for their abilities to secrete IL-1. We report here that ameboid microglia, when activated, release significant quantities of IL-1.

Materials and Methods

Enriched Astroglial Cell Cultures.   Astroglia prepared by the method of McCarthy and de Vellis (22, 23) were grown on glass coverslips in 35-mm culture dishes (10). Indirect immunofluorescence techniques were used to identify astroglia as those cells bearing glial fibrillary acidic protein (GFAP), and oligodendroglia as those cells bearing galactocerebroside (GC) (21–24). Enriched astroglial cultures, treated with 5 mM L-leucine methyl ester (25) for 3 h at 37°C to eliminate contaminating microglia (20, 26) contained >98% fibroblast-like astroglia. These cultures, grown in 1.5 ml defined medium (27), were incubated with partially purified IL-1 from rat microglia (1.5% by vol) for 72 h. The proliferation of astroglia was determined by counting cells in 10 randomly selected fields (0.314 mm^2) in at least three cultures for each experimental group. Astroglial number in cultures incubated for 72 h with IL-1 or column fractions was expressed as fold increase over that in control cultures that received PBS in volumes equivalent to those of the factors added to treated cultures. Under these conditions, control astroglial cell number increased by 150–250% over a 5-d period (22). Astroglial proliferation was also measured by incorporation of [^{3}H]thymidine into cells grown in 96-well microtiter plates (30,000 cells/well) containing 200 μl of defined culture medium and IL-1. After incubation for 60 h, 1 μCi of [^{3}H]thymidine (sp. act., 2.0 mCi/mmole) (Amersham Corp., Arlington Heights, IL) was added to each well for an additional 12 h. Quantitation of incorporated radioactivity was determined by scintillation counting of well contents harvested onto glass filter strips.

Enriched Microglial Cell Cultures.   We have recently (21) described the isolation of ameboid microglia from the brain of newborn animals. Ameboid microglia were obtained (20, 21) from mixed glial cell cultures grown for 1 wk in plastic flasks containing defined medium with 10% FCS. The flasks of 1-wk-old mixed glial cells were agitated for 15 h on a rotary shaker (180 rpm) at 37°C, and the cells suspended by agitation were transferred to plastic flasks. Ameboid microglia selectively adhered to plastic within 2 h at 37°C in defined medium containing 10% FCS (23). Adherent cells were resuspended in a Ca^{2+}/Mg^{2+}-free PBS with 5 mM EDTA, and transferred to a plastic flask containing defined medium with 10% FCS. The cells adhering after a second 2-h period consisted of 95 ± 3% nonspecific esterase–positive (26) ameboid microglia and contained the acetylated low-density lipoprotein receptor. The probe for acetylated low-density lipoprotein (LDL), fluorescent diocytadecyl-3,3,3′,3′-tetramethyl carbinocyanine (DiI-ac-LDL) (28) was a gift from Dr. David Via of the Department of Medicine, Baylor College of Medicine.

We have, in a recent report (21), compared the properties of microglia with those of monocytes and macrophage of newborn rat. All three cell types were phagocytic cells that expressed the acetylated low-density lipoprotein receptor, as well as the macrophage antigens MAC-1 and MAC-3 (19–21, 26). Ameboid microglia could be distinguished from monocytes and macrophages by a lack of peroxidase activity at the time of initial plating of dissociated brain cells (21). Moreover, ameboid microglia proliferated in vitro and incorporated [^{3}H]thymidine, while under identical culture conditions, monocytes and macrophages did not increase in cell number or show significant thymidine incorporation. And finally, ameboid microglia, when incubated with retinoic acid, grew out processes several hundred microns in length (21). Based upon such observations, we believe that ameboid microglia are a class of phagocytic cell that may be distinguished from blood monocytes and spleen macrophage (21).

Identification and Isolation of IL-1.   IL-1 activity was assayed as incorporation of [^{3}H]thymidine by mouse thymocytes (29) and by the D-10 cell line assay (30). IL-1 activity was expressed as U/ml according to the procedure of Schmitt and Ballet (31). Antibody

neutralization of microglial IL-1 was performed with anti–murine IL-1 by Dr. Mitchell Dukovich in the laboratory of Dr. Steven Mizel at Bowman Gray School of Medicine (32). This IgG fraction isolated from goat serum was produced against purified murine IL-1, and has been extensively characterized. The fraction specifically neutralizes murine IL-1, has been used for affinity purification of IL-1 (32), and has been used to recover recombinant murine IL-1 (36).

Suspensions of fixed *Staphylococcus aureus*, 20 μl of washed bacteria per 1.5 ml of culture medium (Calbiochem-Behring, San Diego, CA); LPS, 5 μg/ml (from *Escherichia coli*; Sigma Chemical Co., St. Louis, MO); or recombinant murine IFN-γ 100 U/ml (provided by Dr. Isaiah J. Fidler, M. D. Anderson Hospital), were tested for their abilities to stimulate IL-1 release from microglia and astroglia.

The isolation of brain IL-1 was performed by previously described methods (10, 29). Gel filtration chromatography was performed using a calibrated 100 × 0.9-cm P10 (Bio-Rad Laboratories, Richmond, CA) column in PBS (10). Bioassays were carried out on fractions ranging from 3 kD to those found in the void peak, with an exclusion size of ~20 kD. 50 ml of macrophage- or microglia-conditioned defined medium were concentrated by ultrafiltration using a YM-2 membrane (Amicon Corp., Danvers, MA), and the sample was clarified by filtration through a 0.45-μm membrane filter (Acrodisc; Gelman Sciences, Ann Arbor, MI). A 1.0-ml sample containing 10 mg of protein was applied to the P10 column and 0.7-ml fractions were collected. Anion-exchange chromatography was performed using a Waters Model 720 HPLC apparatus and a TSK-5PW (0.75 × 7.5-mm) DEAE column (Bio-Rad Laboratories) (29). The chromatography was performed in 2.0 mM Tris buffer (pH 8.0), and the IL-1 activity was eluted by a gradient of 0.00–0.5 M sodium acetate, in 2.0 mM Tris (pH 8.0).

Results

Cellular Sources of Brain IL-1.   We wished to determine whether IL-1 was released into the culture medium conditioned by specific populations of glial cells. Initial studies using mixed cultures of astroglia and microglia, in serum-free medium, showed that small quantities of IL-1-like activity could be detected in the medium by 48 h. To monitor the capacity of astroglia and microglia to secrete IL-1, it was first necessary to isolate these cells from the brain of newborn rats. Ameboid microglial preparations (Fig. 1 *A*), obtained as described in Materials and Methods, were a homogeneous population with nearly all cells showing receptors for acetylated LDL (Fig. 1 *B*). Ameboid cells were readily distinguished from astroglia by phase-contrast microscopy and a lack of GFAP (Fig. 1). Using the methods of McCarthy and de Vellis (23), we obtained astroglial preparations with >90% GFAP$^+$ cells, which also contained ~5% ameboid microglia. These ameboid cells could be eliminated from astroglial preparations by exposure to the lysosomotrophic agent L-leucine methyl ester (Fig. 1, *E* and *F*) (20).

To test the ability of ameboid microglia and astroglia to release IL-1, culture dishes containing 150,000 ameboid microglia or astrocytes were stimulated with either LPS, fixed *S. aureus*, IFN-γ, or PBS. As shown in Fig. 2, microglia responded to 5 μg/ml of LPS or 20 μl of suspended fixed *S. aureus* with the release of 11.0 ± 1.0 and 13.3 ± 0.8 U of IL-1 activity per 1,000 microglial cells, respectively. Similar doses of LPS and *S. aureus* resulted in 1.5 ± 0.5 and 1.4 ± 0.2 U, respectively, of IL-1 per 1,000 astroglia. IFN-γ failed to stimulate IL-1 release from either cell type.

Biochemical and Immunological Characterization of Microglial IL-1.   To confirm that the thymocyte-stimulating activity released by microglia was in fact IL-1, several different experiments were performed on serum-free medium conditioned by cultured ameboid microglia. 50-ml samples of IL-1 activity prepared
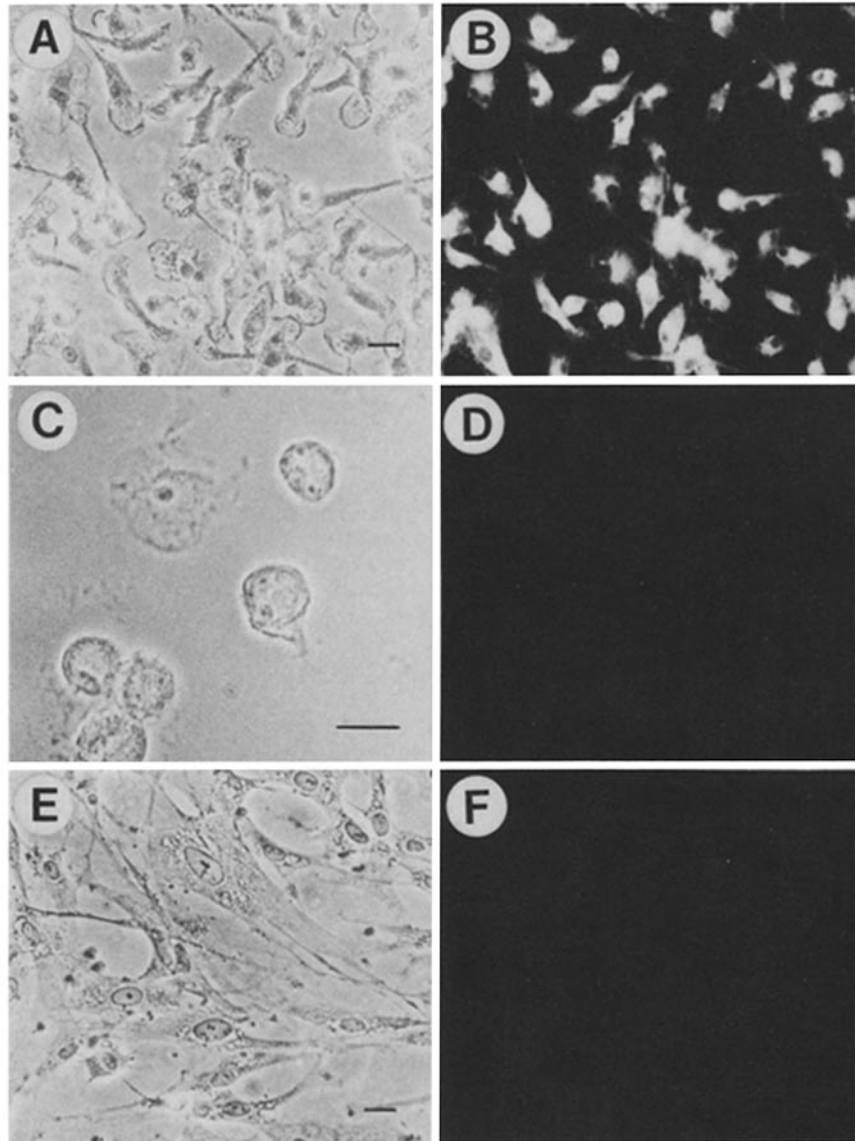
FIGURE 1. Photomicrographs of enriched preparations of ameboid microglia and astroglia isolated from newborn rat brains. A: Phase-contrast micrograph of ameboid microglia isolated from mixed glial cultures by selective adhesion (see Materials and Methods). B: Nearly all of the isolated ameboid microglia express the acetylated LDL receptor, as shown by the fluorescent probe diocytadecyl-3,3,3′,3′-tetramethyl carbinocyanine (Dil-ac-LDL). C: Phase-contrast photomicrograph of ameboid microglia after staining with GFAP. D: Fluorescence photomicrograph shows that ameboid microglia from C lack the astroglia-specific antigen, GFAP. E: Phase-contrast photomicrograph of enriched astroglia. F: Fluorescence photomicrograph shows that the astroglial culture from E lacks Dil-ac-LDL–positive microglia. Bar = 20 μm.

by culturing 7.5×10^6 microglia or 10×10^6 adherent peritoneal macrophages with 0.5 ml of a S. aureus suspension for 48 h were concentrated by ultrafiltration and fractionated by gel filtration chromatography using a P-10 column. As shown in Fig. 3, the activated microglia released an 18 kD factor that stimulated [^{3}H]-
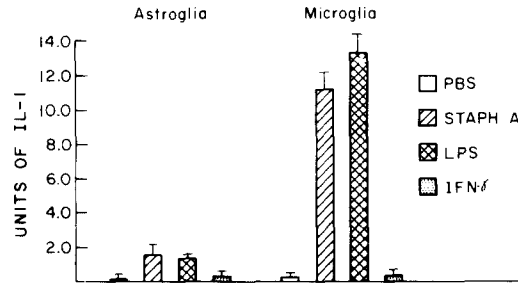
FIGURE 2. Release of IL-1 by enriched glial cultures. Astroglia and microglia (~150,000 cells/culture) were incubated for 48 h in the presence of PBS (20 μl/culture), fixed *S. aureus*, LPS, or IFN-γ to induce IL-1 release (see Materials and Methods). The conditioned medium was examined for IL-1 activity by the mouse thymocyte assay. IL-1 activity represents mean values ± SE obtained from at least five cultures per experimental group, and are expressed as U/10³ cells.
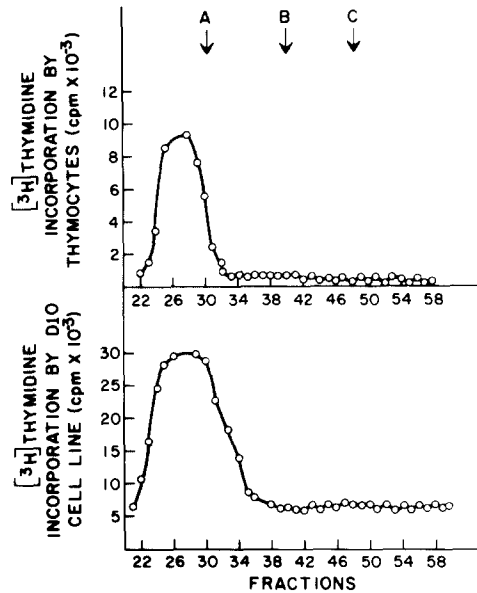

FIGURE 3. Gel filtration chromatography of culture medium from activated microglia. IL-1 activity was determined in triplicate for each fraction in the murine thymocyte assay (*top*) and the D-10 cell line assay for IL-1 (*bottom*). A single peak of activity of ~18 kD was found to stimulate [³H]thymidine incorporation. Molecular mass markers: A, 16.9 kD; B, 8.5 kD; C, 6.2 kD. The void peak did not contain biologic activity.

thymidine incorporation in murine thymocytes (Fig. 3, *top*) and in the IL-1-sensitive D10 cell line (Fig. 3, *bottom*). Medium conditioned by rat peritoneal macrophage produced IL-1 activity that eluted in an identical fashion (data not shown). The 18–20 kD fractions secreted by microglia and macrophages were further characterized by DEAE–anion-exchange HPLC. The IL-1 activity in both samples was found to elute at the same sodium acetate molarity (Fig. 4).

In addition to copurification, we examined microglial IL-1 by antibody neutralization. Antisera to murine IL-1 and human IL-1 were tested for the ability to inhibit rat microglial IL-1 stimulation of thymocyte incorporation of [³H]thymidine. An antibody to human IL-1-β was unable to neutralize the rat
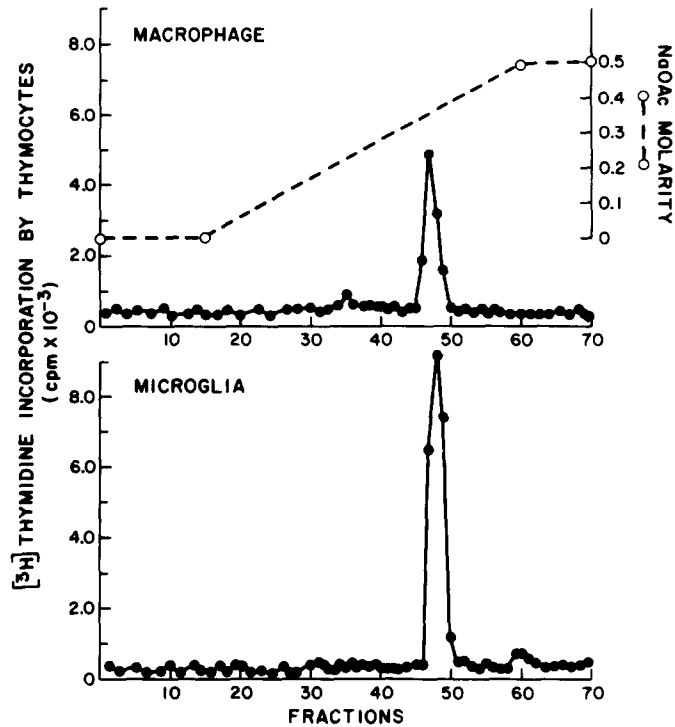
FIGURE 4. Anion-exchange chromatography of the 18 kD IL-1 activities released by activated microglia and macrophages. 2-ml samples from the peaks of IL-1 activity (Fig. 3) were fractionated by HPLC using a DEAE column and an increasing sodium acetate gradient (see Materials and Methods). Each fraction was assayed in triplicate for IL-1 activity in the murine thymocyte assay. Coelution was found for IL-1 obtained from both cellular sources.
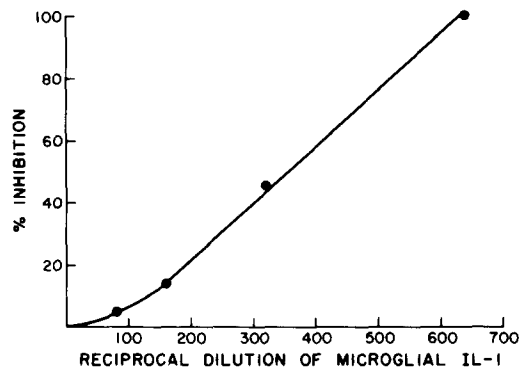

FIGURE 5. Inhibition of microglial IL-1 by antiserum to murine IL-1. Microglial IL-1 containing 90 U/ml was diluted over a range from 1:80 to 1:640. To each dilution, goat anti-murine IL-1 serum was added, and the cultures were incubated at 37°C for 18 h. IL-1-responsive murine thymocytes were added to each dilution, and the inhibition of IL-1 activity was calculated as a percentage of control culture without antibody (31).

microglial IL-1 (data not shown), nor did it inactivate rat macrophage IL-1, an observation previously reported (30). As shown in Fig. 5, antibody to murine IL-1 was able to neutralize the biological activity of rat microglial IL-1 and of rat
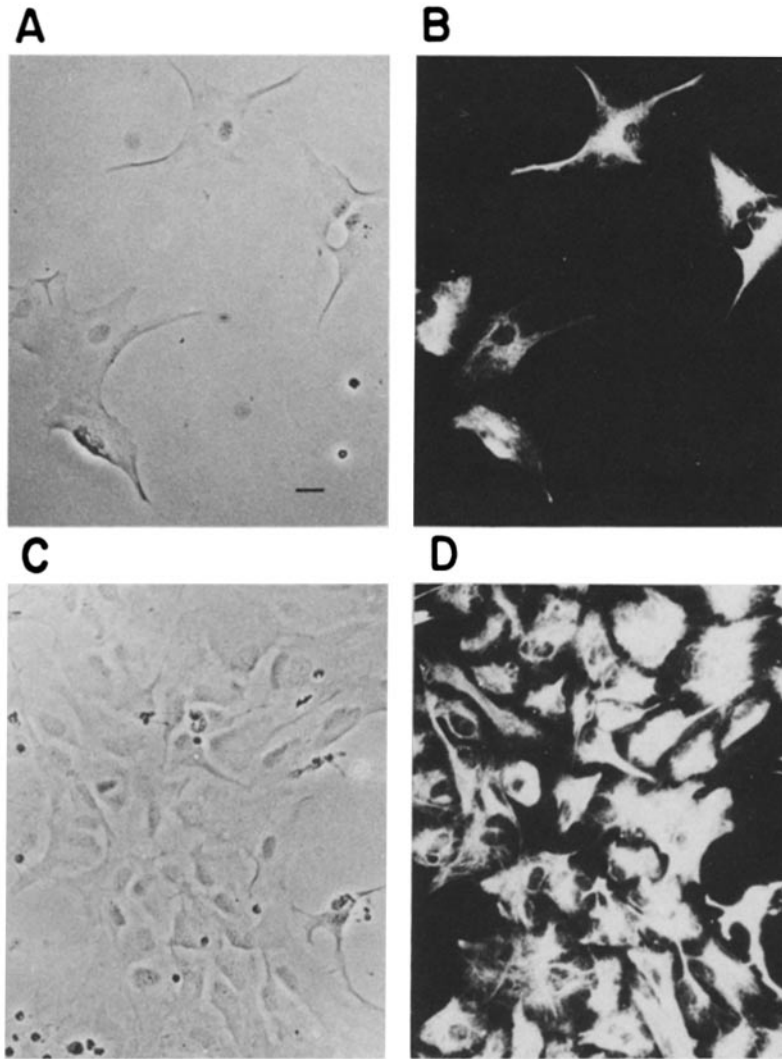
FIGURE 6.   Photomicrographs of enriched astroglial cultures after treatment with rat microglial IL-1. Cultures were treated for 3 d with either PBS (*A* and *B*) or 5% (by vol) of the 18 kD gel chromatography–purified IL-1 (*C* and *D*). As shown by immunofluorescence staining for GFAP, IL-1 markedly increased the number of astroglia (*D*).

macrophage IL-1 (30), suggesting that microglial IL-1 shares a common antigenic site with IL-1 released from rat peritoneal macrophage.

IL-1 and Astroglial Growth.   We have previously shown the ability of human IL-1 to stimulate the growth of rat astrocytes in mixed glial cell cultures (10). To confirm that rat microglial IL-1 could also stimulate the growth of rat astrocytes, rat microglial IL-1 purified by gel filtration chromatography was added to enriched astroglial populations. As shown in Fig. 6, there was a five- to sevenfold increase in the number of GFAP$^+$ astroglia after a 72-h incubation with microglial IL-1. We also observed a three- to fivefold increase in [^{3}H]-thymidine incorporation by enriched astroglial cultures (Fig. 7). Microglial IL-1
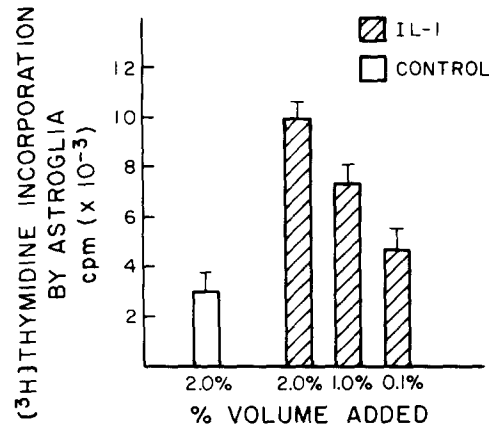
FIGURE 7.   Incorporation of [³H]thymidine by astroglia stimulated with gel chromatography–purified rat microglial IL-1. 3.0 × 10⁴ cells were grown in the presence of IL-1 for 72 h and incubated with 1 μCi [³H]thymidine for the final 12 h before harvesting.

therefore increases the number of astroglia by accelerating the proliferation of mature GFAP⁺ astroglia in vitro.

Discussion

Astroglia proliferate in the brain during embryonic development and after injury. Specific peptide growth factors have been isolated from the brain during these periods (10, 22), and have been implicated as regulators of gliogenesis (33). In this report, we show that one factor released by ameboid microglia is an 18 kD peptide that stimulates the growth of astroglia. By using copurification techniques, antibody neutralization, and two different types of IL-1 assays, we have shown that this factor is IL-1. Secondly, we have shown microglial IL-1 stimulates astroglial proliferation in vitro. The effect of IL-1 upon astroglia is, moreover, further supported by recent work in our laboratory showing that recombinant IL-1 is a mitogen for both brain astroglia and the astrocytoma cell line, U373 (unpublished data).

Microglia are probably not the only source of IL-1 within the CNS. For example, blood monocytes entering the brain after penetration injuries might also secrete IL-1 (18). Moreover, Fontana et al. (12) have shown that enriched cultures of astrocytes, as well as astroglial cell lines, are able to produce IL-1. We find that astroglial cultures can be stimulated to release IL-1, albeit in small quantities (Fig. 2). Our cultures of astroglia were treated with leucine methyl ester to eliminate microglia as a possible source of IL-1. In this way, by determining more accurately the level of IL-1 production by astroglia, we concluded that ameboid microglia are the major producers of brain IL-1 in vitro.

Although both astroglia and ameboid microglia might release IL-1 in vivo, we propose that ameboid cells are the more likely source of IL-1 during acute phases of brain injury. In general, astroglial scar formation is a later event in brain injury, one that follows microglial invasion of damaged tissue (17, 18). Since microglia are the first brain cells to appear in increased numbers at sites of trauma or infections, we suggest that IL-1 released from these ameboid cells stimulates the proliferation of neighboring astroglia (20). In a similar way, other

investigators have suggested that macrophages control wound repair by release of IL-1 to promote fibroblast proliferation (16).

Caution should, of course, be observed when attempting to relate in vitro findings to the in vivo actions of brain IL-1. The ability of any growth factor to influence cell proliferation in vivo depends upon not only the density of secretory cells, but also the availability of factor-sensitive target cells. Further work is needed to delineate IL-1 sensitivities of the various cell populations found within intact brain. The control of IL-1 secretion is also important when examining the role of IL-1 in vivo. Although microorganisms might stimulate IL-1 release from microglia during infection, the actual regulators of IL-1 secretion within the brain are unknown. Identification of such microglial activators will help to elucidate initial events regulating inflammation within the CNS.

The brain contains growth factors that stimulate astroglial proliferation during development and in response to injury (9, 20, 33). It is conceivable that developmental growth factors (33) and inflammatory factors found in the brain (21) are structurally similar. For example, fibroblast growth factor (FGF) isolated from brain has recently (34) been found to have a significant sequence homology with IL-1. Although FGF does not stimulate proliferation of mouse thymocytes or D10 cells (34), both FGF and IL-1 stimulate the growth of astrocytes (33, 35). Further study to determine the cellular source of brain FGF, and in particular its relationship to ameboid microglia, will be of interest.

Finally, if glia do, in fact, mediate neuronal development and axonal growth, then factors that control glial outgrowth or reduce glial scarring might be revealed from IL-1-related research. As a potent immunomodulator, IL-1 now represents another link between the immune system and the nervous system. Furthermore, since IL-1 release is a convenient and sensitive marker of microglial activation, measurement of IL-1 levels may prove useful in monitoring degenerative or inflammatory diseases of the CNS.

Summary

By screening specific populations of rat brain cells, we found that ameboid microglia secrete an 18 kD peptide with IL-1 biological activity. The IL-1 activity released by microglia was found to be identical to rat macrophage IL-1 on fractionation by gel filtration and high pressure liquid anion-exchange chromatography, and it was neutralized by an antiserum specific for murine IL-1. When added to astroglia grown in culture, microglial IL-1 increased the cell number of five- to sevenfold, and increased astroglial incorporation of [^{3}H]thymidine by three- to fivefold. We propose that the proliferation of astroglia in specific brain regions may be regulated by the signaled release of IL-1 from activated microglial cells.

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