

# Interleukin-1 Is an Astroglial Growth Factor in the Developing Brain

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**The immunomodulator interleukin-1 (IL-1) is found to be an astroglial growth factor during development of the mammalian brain. *In vitro* studies indicate that ameboid microglia, a class of brain mononuclear phagocytes, are the likely source of IL-1. Examination of different brain regions during development shows IL-1 production only after the appearance of ameboid microglia. These observations suggest that brain mononuclear phagocytes secrete growth factors that regulate normal growth and development of the CNS.**

Astroglia play an important role in determining the structural organization of the brain through diverse actions upon neurons (Rakic, 1972; Aguayo et al., 1981; Silver et al., 1982). During embryogenesis, primitive astroglia found in radial patterns provide routes along which neurons migrate from germinal zones to cortical regions of the CNS (Bignami and Dahl, 1974; Schmehel and Rakic, 1979; Levitt and Rakic, 1980). At later periods in brain development, astroglia secrete extracellular matrix constituents, including laminin, which are thought to promote selective axonal growth (Letourneau, 1975; Lander et al., 1982; Hopkins et al., 1985). In adult brain, clusters of "reactive" astroglia collect at sites of injury and appear to block the regrowth of axons and regeneration of damaged neural tissue (Bignami and Dahl, 1974; Latov et al., 1979; Pares and Levine, 1982; Miller et al., 1986). The mechanisms that direct these various glial responses are doubtlessly complex. Recently, much attention has been given to growth factors isolated from developing or injured CNS (Lim, 1980; Pruss et al., 1981; Lemke and Brookes, 1984; Giulian and Baker, 1985; Giulian et al., 1985; Giulian and Young, 1986) as possible regulators of astroglial function. We report here that developing mammalian brain contains the immunomodulator interleukin-1 (IL-1), a secreted protein that serves as an astroglial growth factor. Our observations suggest that immunomodulators released by brain mononuclear phagocytes help to regulate growth of the CNS during embryogenesis.

## Materials and Methods

**Cell cultures.** Albino rats (Holtzman, Madison, WI), timed for pregnancy, provided embryos and neonates used in these studies. After removal of the meninges, isolated cerebral cortices or cerebella were dissected in sterile PBS and dispersed by trituration in chemically defined medium (Bottenstein and Sato, 1979) containing DNase (1 mg/

ml; Sigma Chemical Co., St. Louis, MO) and trypsin (0.2% wt/vol; Sigma). Mixed populations of glia were grown on poly-L-lysine-coated 22 mm glass coverslips in 35 mm dishes (10<sup>6</sup> cells/dish) containing 1.5 ml chemically defined medium with 10% bovine calf serum. Cultures were placed in 1.5 ml of chemically defined medium when tested for secretion of IL-1.

**Interleukin-1 isolation and bioassays.** The IL-1 assay was based on the incorporation of <sup>3</sup>H-thymidine by murine thymocytes, as described elsewhere (Lachman et al., 1985). Briefly, thymuses from 8–12-week-old CD-1 mice (Charles River Breeding Farms, Wilmington, MA) were homogenized with a glass-Teflon hand homogenizer and thymocytes were cultured in Minimal Essential Medium containing 5% human serum at a cell density of 1 × 10<sup>7</sup> cells/ml in 96-well test plates. Serial dilutions of IL-1 were added to the thymocyte cultures and incubated for 48 hr at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. 6-<sup>3</sup>H-thymidine (0.2 μCi, 2 mCi/mmol; New England Nuclear) was added to each culture well for an additional 24 hr. After a total of 72 hr, the cultures were harvested onto glass fiber strips for scintillation counting.

To quantitate the number of units of IL-1, serial dilutions of samples were used to determine a response equal to 50% of a positive control. Units of IL-1 activity for brain fractions were calculated by the method of Schmitt and Ballet (1983).

As described earlier (Giulian et al., 1986a), we identified the brain-derived 18 kDa growth factor as IL-1 by coelution with rat macrophage IL-1 using gel filtration (Protein Pak 125; Waters) and anion-exchange chromatography (TSK-DEAE-5PW; Bio-Rad Laboratories). The partially purified brain-derived IL-1 was active in the D10 cell-line assay (Kaye et al., 1984) and could be neutralized by rabbit anti-murine IL-1 serum (assay performed by Dr. Mitchell Dukovitch in the laboratory of Dr. Steven Mizel).

Recombinant murine IL-1 (in the form of a precursor with *M<sub>r</sub>* 31 kDa) was kindly provided by Dr. Peter Lomedico (Hoffmann-LaRoche, Nutley, NJ), and recombinant human IL-1α (rhIL-1α; Gubler et al., 1986) was purchased from Genzyme (Boston, MA). All recombinant samples showed less than 0.05 ng/ml of endotoxin, as detected by the limulus amebocyte lysate assay (Associates of Cape Cod).

**Glial cell identifications and bioassays.** Ameboid microglia containing acetylated low-density lipoprotein (ac-LDL) receptors were identified using ac-LDL bound to the fluorescent probe 1,1'-dioctadecyl-1,3,3',3'-tetramethylcarbindolocyanate (DiI) (Pitas et al., 1981). Ameboid microglia were selectively destroyed by treatment for 6–12 hr in chemically defined medium containing 5 mM L-leucine methyl ester (Sigma; 12, 16).

Indirect immunofluorescence techniques were used to identify astroglia containing glial fibrillary acidic protein (GFAP; Eng and DeArmond, 1982) or oligodendroglia containing galactocerebroside (Raff et al., 1979), as described earlier (Giulian et al., 1985). Proliferation of GFAP(+) astroglia was monitored in mixed glial cultures isolated from embryonic rat cerebral cortex after 72 hr incubation with IL-1. Mean counts of cells were obtained by determining cell numbers in 10 randomly selected fields (0.314 mm<sup>2</sup>) for each culture, as viewed by epifluorescence microscopy at 220×. Prior to incubation with IL-1, about 10% of all cells were GFAP(+) astroglia in E-14 cultures and about 30% were GFAP(+) cells in E-20 cultures.

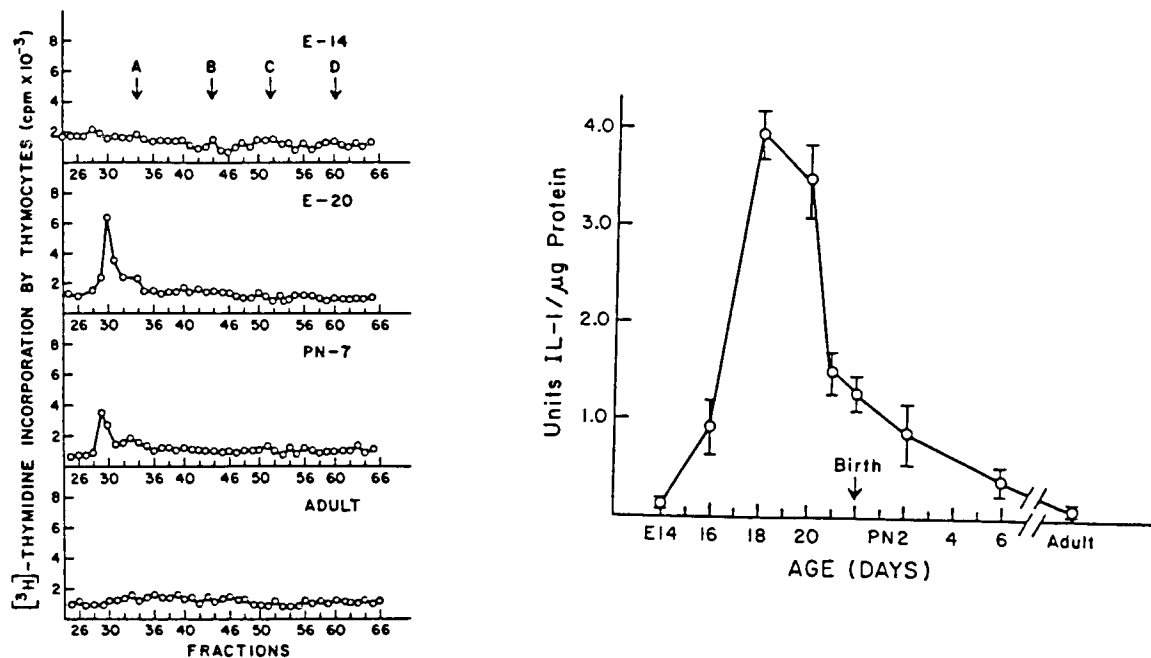
Light-microscopic autoradiography was performed on cultures of dissociated E-20 brain that had been grown in medium containing 10 μCi/ml of 6-<sup>3</sup>H-thymidine (sp act, 22 Ci/mmol; Amersham) for 15 hr prior to fixation. Coverslips mounted to glass slides were dipped in Kodak NTB2 emulsion diluted 1:1 with dH<sub>2</sub>O and developed with Kodak

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**Figure 1.** The appearance of IL-1 during development of the cerebral cortex. *Left*, Pooled sonicates of cerebral cortices from embryos (E-14 and E-20), postnatal (PN-7), or adult rats were prepared for column chromatography by sonication in PBS, followed by 0.45  $\mu$ m membrane filtration and centrifugation of 15,000  $\times$  g for 30 min. Gel filtration was performed on a 100  $\times$  0.9 cm column (P-10; BioRad) on samples containing 5–10 mg of protein. Fractions of 700  $\mu$ l were collected and assayed for IL-1 activity in the murine thymocyte <sup>3</sup>H-thymidine-incorporation assay. IL-1 activity, detected in the 18 kDa region, was recovered from the brain homogenates of late fetal period animals (embryonic E-20) and early postnatal period animals (PN-7). Gel-filtration fractions of sonicates from animals of the early embryonic stage (E-14) and adult brain did not contain IL-1 activity. Myoglobin peptides were used to calibrate the column (in kDa): A, 17; B, 8.5; C, 6.2; D, 2.5. *Right*, IL-1 activity, expressed as units/ $\mu$ g brain protein, was measured in supernatants of rat cerebral cortex that were fractionated by gel filtration, as for the left panel. All samples were frozen and assayed simultaneously in the murine thymocyte assay. Peak activity was found around stage E-18, or that period when amoeboid microglia were numerous and actively phagocytic.

Dektol Developer after a 4 week exposure. GFAP(+) astroglia viewed at 1200 $\times$  that showed greater than 10 grains/nucleus were scored as incorporating significant amounts of <sup>3</sup>H-thymidine. The percentage of such labeled cells was determined by viewing 50 randomly selected GFAP(+) cells/coverslip. More than 70% of GFAP(+) cells in cultures treated with 10 units recombinant human IL-1 $\alpha$  (rhIL-1 $\alpha$ ) for 72 hr were labeled, whereas less than 20% of cells were labeled in control cultures. Coverslips used for autoradiography, shown in Figure 4, were exposed for 5 months.

## Results

### Detection of interleukin-1 in developing brain

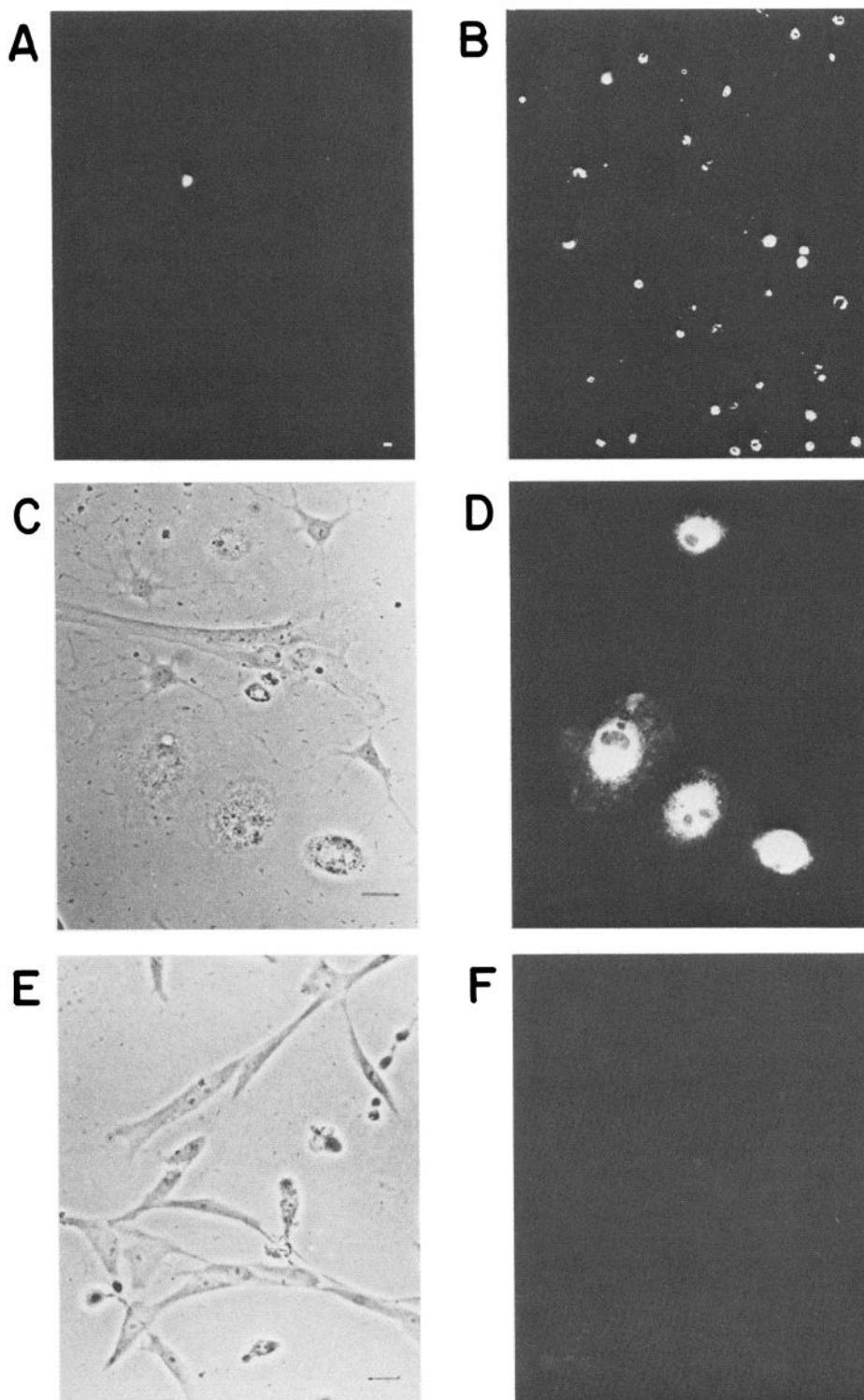
Astroglia containing GFAP proliferate in the cerebral cortex and cerebellum of perinatal rat (Rakic, 1972; Lim, 1980; Giulian and Baker, 1985; Miller et al., 1986). We sought to characterize astroglial growth factors found in such developing tissues (Giulian and Baker, 1985; Giulian et al., 1985). Initial studies of brain extracts suggested the presence of the immunomodulator IL-1, an 18 kDa peptide growth factor that serves as an activator of T cells and as a promotor of the inflammatory response (Dinarello, 1984). Cerebral cortices obtained from rats of different ages were dispersed by sonication in PBS, centrifuged for 30 min at 15,000  $\times$  g, and the supernatant passed through a 0.45  $\mu$ m filter. A total of 5–10 mg of soluble protein were eluted with PBS from a standardized gel-filtration column (Giulian et al., 1986b). Aliquots were tested for IL-1 activity using the murine thymocyte proliferation assay. As shown in Figure 1, a thymocyte-stimulating activity of about 18 kDa was detected in brain supernatants at embryonic stage E-20 and at postnatal day 7. Identification of this 18 kDa activity as IL-1 was estab-

lished, as described earlier, by coelution with rat macrophage IL-1 using anion-exchange chromatography, by neutralization with specific murine IL-1 antiserum, and by measurement of bioactivity in the D10 cell-line assay (Giulian et al., 1986a).

In order to quantitate the amount of brain IL-1 produced during development, soluble brain protein obtained from animals of different ages was fractionated by gel filtration. The 18 kDa fractions from each age group were pooled and concentrated by ultrafiltration (YM-2; Amicon). Using dose-response curves from the thymocyte assay, we determined the units of IL-1 activity/ $\mu$ g brain protein (Lachman et al., 1985). The highest concentrations of IL-1 in the normally developing cerebral cortex of rat appeared during the perinatal period from embryonic stages E-18 to the time of birth (Fig. 1, right panel). Normal adult brain had no measurable IL-1 activity, suggesting a decline in production of the immunomodulator with age.

### Appearance of amoeboid microglia during embryogenesis

Although a number of different cell types are reported to release IL-1, mononuclear phagocytes are among the most potent secretory cells (Dinarello, 1984). Recently, amoeboid microglia phagocytic cells found in the CNS have been identified as one source of brain IL-1 (Giulian and Baker, 1986; Giulian et al., 1986a). Since amoeboid microglia are quite active as phagocytes during development (Ling, 1981; Innocenti et al., 1983a), they are also likely producers of IL-1 in embryonic brain. Using a fluorescent probe for ac-LDL receptors—1,1'-dioctadecyl-1-3,3,3',3'-tetramethylindocarbocyanine bound to ac-LDL (DiI-ac-LDL)—we determined the numbers of amoeboid microglia in

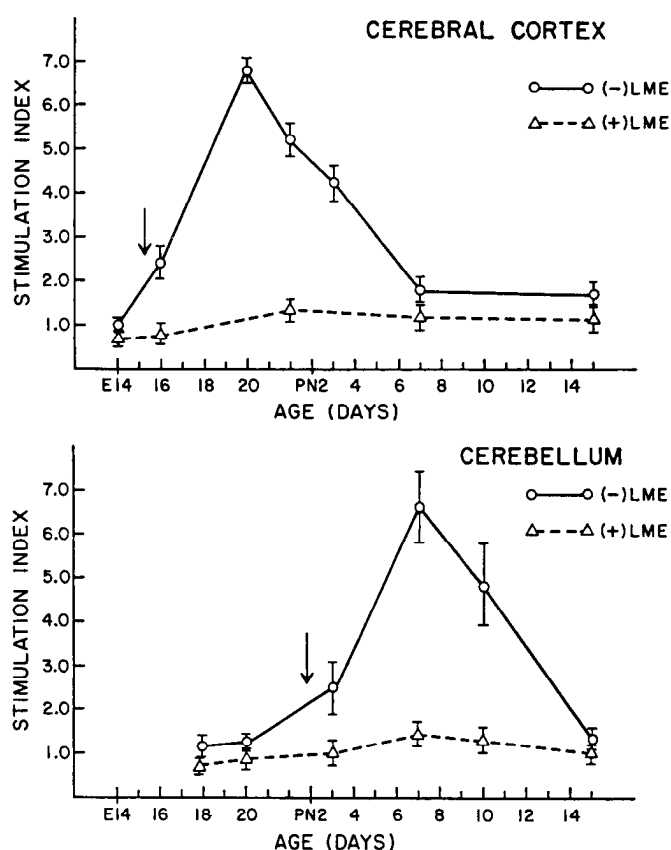


**Figure 2.** Microglia found in mixed brain cell cultures obtained from developing cerebral cortex of rat. Ameboid microglia containing the acetylated low-density lipoprotein receptor were identified by the fluorescent probe Dil-ac-LDL. Although few microglia were found in cultures obtained from the early embryonic period E-14 (*A*), many were present by the late embryonic period E-20 (*B*). Cultures obtained from postnatal day 1 rats (*C*, phase-contrast; *D*, fluorescence) also contained Dil-ac-LDL(+) microglia. Incubation of cultures with 5 mM L-leucine methyl ester for 12 hr selectively destroyed the microglia (*E* and *F*) (see also Table 1). Bar, 20  $\mu$ m.

freshly dissociated cerebral cortex taken from animals of different ages in the perinatal period. Although few Dil-ac-LDL(+) cells are present at stages E-14–15 (Fig. 2*A*), large numbers of Dil-ac-LDL(+) ameboid cells appear by stage E-20 (Fig. 2*B*). Quantitation of Dil-ac-LDL(+) cells (Table 1) indicates that the largest ameboid microglial population is found in the cerebral cortex near the time of birth (E-18 to PN-1), with a substantial drop in number by the second postnatal week.

#### *Ameboid microglia secrete interleukin-1*

In order to induce IL-1 secretion *in vitro*, embryonic glial cell cultures were stimulated by incubation with a macrophage activator, fixed *Staphylococcus aureus* (Dinarelli, 1984; Giulian et al., 1986a). A stimulation index (IL-1 activity in a medium of cultures incubated with *S. aureus*/IL-1 activity in a medium of control cultures) was used to compare IL-1 release from glia



**Figure 3.** Stimulation index showing IL-1 release from cells of the developing brain. Glial cultures obtained from cerebral cortices of rats of different ages were incubated for 24 hr with fixed *S. aureus* in order to elicit IL-1 secretion. The IL-1 activity in the conditioned medium was quantitated by  $^3\text{H}$ -thymidine incorporation using mouse thymocytes. The stimulation index for IL-1 release is given as a ratio of IL-1 activities for *S. aureus*-treated cultures/untreated control cultures. To confirm that ameboid microglia was the secretory cell, cultures were preincubated with L-leucine methyl ester (LME) in order to destroy Dil-ac-LDL(+) cells. *Top*, Cerebral cortex. *Bottom*, Cerebellum. *Arrows* indicate the age at which ameboid microglia are first detected in these tissues.

obtained at different stages in development. We found that the peak of stimulated IL-1 secretion (E-18 to PN-1; Fig. 3) *in vitro* correlated both with IL-1 levels found in brain supernatants and with the appearance of Dil-ac-LDL(+) ameboid microglia in the cerebral cortex (Table 1). To confirm that embryonic microglia were in fact secreting IL-1, we selectively destroyed ameboid cells found in the mixed glial cell cultures using the lysosomotropic agent L-leucine methyl ester (Fig. 2D) (Thiele et al., 1983). As described earlier, L-leucine methyl ester will lyse Dil-ac-LDL(+) microglia (Fig. 2E), while sparing astroglia or oligodendroglia (Giulian and Baker, 1986). Under the conditions used, there was nearly a 95% destruction of ameboid microglia (Table 1). Embryonic brain cultures treated with L-leucine methyl ester lacked microglia and produced very low levels of IL-1 (Fig. 3). Because these microglia-free cultures contained nearly 60% GFAP(+) astroglia, we believe that astroglia are an unlikely source of IL-1 in the developing CNS (Fontana et al., 1982).

In rats, the cerebellum matures more slowly than the cerebral cortex (Valentino and Jones, 1981; Murabe and Sano, 1982; Matsumoto and Ikuta, 1985). We found that cultures obtained from the cerebellum of the E-18 rat had significant numbers of GFAP(+) astroglia but lacked Dil-ac-LDL(+) microglia. By 3

**Table 1.** Ameboid microglia in cortex and cerebellum

Age	Number of ameboid microglia/cover slip	Number of ameboid microglia after LME
<b>Ameboid microglia in developing cerebral cortex</b>		
E-14	300 $\pm$ 100	N.D.
E-16	1500 $\pm$ 400	300 $\pm$ 200
E-18	6600 $\pm$ 500	100 $\pm$ 100
PN-1	5500 $\pm$ 600	100 $\pm$ 100
PN-15	2800 $\pm$ 1100	200 $\pm$ 200
<b>Ameboid microglia in developing cerebellum</b>		
E-18	200 $\pm$ 100	N.D.
E-20	300 $\pm$ 200	100 $\pm$ 100
PN-1	1200 $\pm$ 600	100 $\pm$ 100
PN-7	7700 $\pm$ 1400	200 $\pm$ 200
PN-10	7500 $\pm$ 1000	200 $\pm$ 100
PN-15	2100 $\pm$ 150	300 $\pm$ 200

Freshly isolated dissociated cells from rats of the indicated ages were placed in 35 mm dishes containing a glass coverslip and incubated for 6 hr with Dil-ac-LDL (5  $\mu\text{g}/\text{ml}$ ) in order to identify ameboid microglia. Cell numbers were estimated by determining Dil-ac-LDL(+) cells found in 10 randomly selected fields (0.314  $\text{mm}^2$ ) and corrected for the area of the 22 mm coverslip. Identical cultures were treated for 12 hr with 5 mM L-leucine methyl ester (LME) in order to destroy ameboid microglia selectively (see Fig. 2). N.D., not detected.

d after birth, however, ameboid microglia did appear in the cerebellum, with the greatest number of Dil-ac-LDL(+) cells appearing by the end of the first postnatal week (Table 1). The differences in the timing of appearance for ameboid cells in the cerebral and cerebellar tissues allowed us to examine further the role of microglia in the production of brain IL-1. As described above, we used *S. aureus* to elicit the release of IL-1 from cultured brain cells. In the case of cerebellar cultures, the peak of stimulated IL-1 release was around PN-7 (Fig. 3, bottom), which contrasts with the earlier peak of IL-1 release in the cerebral cortex at E-18–20 (Fig. 3, top). The elimination of ameboid cells by prior treatment of cerebellar cultures with L-leucine methyl ester substantially reduced the production of IL-1.

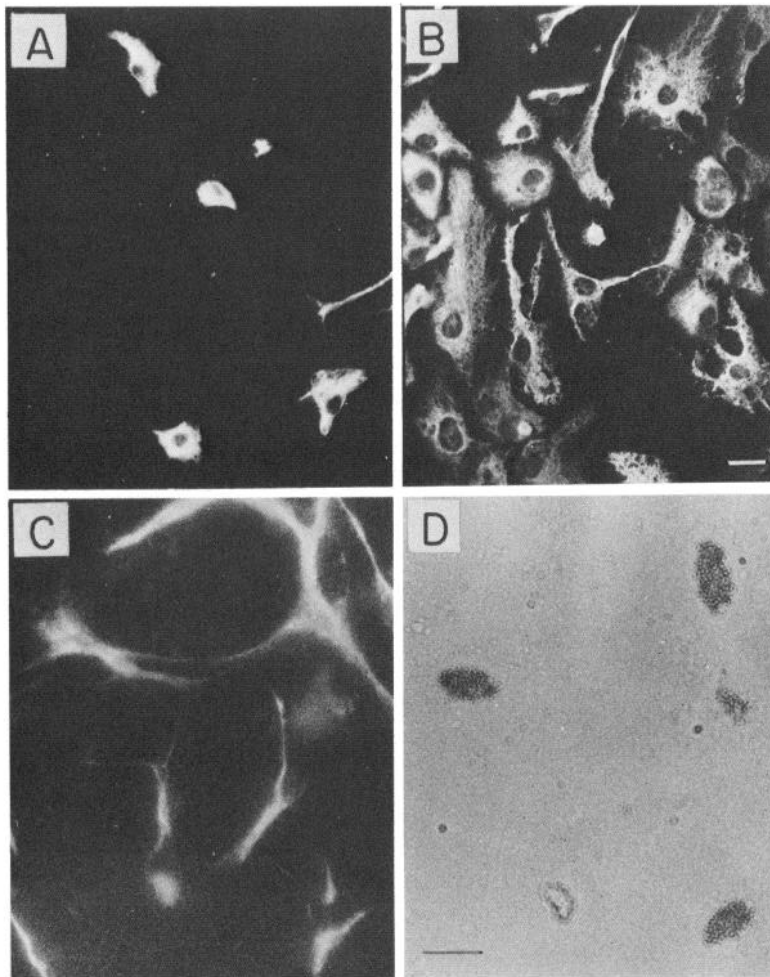
#### *Interleukin-1 as a mitogen for embryonic astroglia*

Although the developing brain contained both IL-1 and IL-1-secreting cells, it remained to be demonstrated that embryonic GFAP(+) astroglia responded to IL-1. Mixed populations of cerebral cortical cells cultured from embryonic rats of different

**Table 2.** Embryonic astroglia growth in the presence of murine recombinant IL-1

Age	Fold increase in GFAP-positive astroglia
E-14	1.5 $\pm$ 0.4
E-16	3.3 $\pm$ 0.2
E-20	7.0 $\pm$ 0.6

Mixed glia cell cultures ( $10^6$  cells/35 mm dish) from cerebral cortex of embryonic rats were grown in 1.5 ml defined medium for 72 hr in the presence of 5 units of recombinant murine IL-1 in 10  $\mu\text{l}$  PBS. The mean number of GFAP(+) astroglia/ $\text{mm}^2$  was determined by scoring 10 randomly selected fields (0.314  $\text{mm}^2$ ) per culture. A minimum of 6 cultures were used per experimental group. Data are expressed as the mean fold increase  $\pm$  SE in GFAP(+) cells in treated versus control cultures. Control cultures were incubated for the same period in 1.5 ml defined medium, with the addition of 10  $\mu\text{l}$  PBS.



**Figure 4.** Stimulation of GFAP(+) astroglia in fetal cultures of dissociated cerebral cortex. Ten units of recombinant human IL-1 $\alpha$  were incubated with E-20 cortical cell cultures for 72 hr. As shown here, there was a 5–8-fold increase in GFAP(+) cells in IL-1-treated cultures (B), as compared to unstimulated control cultures (A). About 70% of the IL-1-treated GFAP(+) astroglia, viewed by fluorescence microscopy (C), show incorporation of  $^3\text{H}$ -thymidine (D, bright-field microscopy). Bar, 20  $\mu\text{m}$ .

ages were incubated for 72 hr with recombinant murine IL-1. After incubation with recombinant murine IL-1, we observed increased numbers of GFAP(+) astroglia in all embryonic stages tested with E-20 cells, showing a 7-fold elevation in the cell number (Table 2). rhIL-1 $\alpha$  also stimulated the proliferation of GFAP(+) astroglia 5–8-fold in E-20 cultures (Fig. 4). Cultures treated with rhIL-1 $\alpha$  showed that about 70% of E-20 GFAP(+) astroglia had incorporated high concentrations of  $^3\text{H}$ -thymidine (Fig. 4), while less than 20% of cells in control cultures were labeled. In agreement with earlier reports (Giulian and Lachman, 1985; Giulian et al., 1986a), neither type of recombinant IL-1 altered the number of cultured galactocerebroside(+) oligodendroglia or Dil-ac-LDL(+) microglia (data not shown). We did not assess the effects of IL-1 upon GFAP(–) or GC(–) precursor cells.

## Discussion

By employing *in vitro* techniques and selective destruction of cells, we identify ameboid microglia as a principal source of IL-1 in the CNS during the perinatal period. Based on these observations, coupled with the fact that IL-1 serves as a mitogen for GFAP(+) astroglia, we propose that ameboid microglia, upon stimulation, help to regulate astroglial growth within specific sites of the normally developing brain. The mechanisms that activate microglia remain uncertain. Perhaps debris associated with cell death and extraneous axonal projections (In-

nocenti et al., 1983a, b; Killackey, 1984) act as phagocytic signals that induce microglia to release astroglial growth factors. IL-1-stimulated astroglia may, in turn, help to direct pathways of axonal outgrowth or to stabilize newly formed circuits. In this way, we believe microglia play an important role in the maturation of the CNS.

During development, ameboid microglia seem to disappear from areas they once heavily populated (Rio Hortega, 1932; Ling et al., 1982). Such a decline in the ameboid cell population may result from death, emigration, or differentiation of microglia. Current work favors the third possibility, for several investigators note ameboid cells undergoing morphologic changes with the formation of long, thin branches (Rio Hortega, 1932; Ling et al., 1982; Perry et al., 1985). As ameboid cells differentiate into ramified microglia, they lose nonspecific esterase activity (Ling et al., 1982) and phagocytic function (Rio Hortega, 1932). We have observed similar changes in culture as ameboid microglia transform into ramified microglia with loss of ac-LDL receptors, nonspecific esterase, and phagocytic activity (Giulian and Baker, 1986). Such transformations from “macrophage-like” microglia into “quiescent” ramified microglia may account for the apparent loss of ameboid cells in maturing brain and the associated decline in IL-1 production.

Our hypothesis that microglia regulate astroglial growth is also supported by the recent discovery that recombinant IL-1 stimulates astrogliosis *in vivo* (Giulian, 1987). Further study

with mRNA probes *in situ* may help to elucidate the sites of brain IL-1 production and action. This is the first report to suggest a role for IL-1 in a developmental process and the first to link the immune system with growth of the immature brain. Our observations pose the possibility that IL-1-secreting cells help to mediate structural development in other organs.

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