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Interleukin-1 Injected into Mammalian Brain Stimulates Astrogliosis and Neovascularization

Dana Giulian, Janet Woodward, Douglas G. Young, Joseph F. Krebs, and Lawrence B. Lachman[1]

Department of Neurology and Program of Neuroscience, Baylor College of Medicine, and [1]Department of Cell Biology, The University of Texas, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77030

Interleukin-1 (IL-1), a protein produced by mononuclear phagocytes, helps to initiate the inflammatory response through its action upon a diverse population of cells. Recently this immunomodulator has been detected at sites of traumatized brain. As reported here, recombinant forms of IL-1 injected into the cerebral cortex of adult rats elicit not only astrogliosis but also new blood vessel growth. These responses are typical of brain injury and suggest that IL-1-secreting inflammatory cells may mediate wound healing in the CNS.

Injury to the CNS of man often leads to irreversible structural changes and permanent loss of function. The cellular events at wound sites in the brain include the rapid appearance of inflammatory cells (Rio-Hortega, 1932; Ling, 1981; Giulian, 1987) and the subsequent growth of astroglia (Latov et al., 1979; Bignami and Dahl, 1976). The resulting dense collection of astroglial processes may prevent recovery of function by inhibiting the regeneration of neurons (Aguayo et al., 1981; Parés and Levine, 1982; Reier et al., 1983) and by retarding new myelin membrane formation (Blakemore and Crang, 1986). The mechanisms that control astrogliosis after brain injury are unknown.

Interleukin-1 (IL-1) is a pluripotent immunomodulator released by mononuclear phagocytes (Dinarello, 1984). It is believed that IL-1 acts as a growth factor to stimulate the proliferation of lymphocytes (Dinarello, 1984) and fibroblasts at sites of inflammation (Leibovich and Ross, 1976; Postlewaite et al., 1984). Recent work has shown that stab wounds within the CNS also contain high concentrations of IL-1 (Giulian and Lachman, 1985). We report here that recombinant IL-1, when infused into mammalian brain, will elicit astrogliosis and new blood vessel growth at the site of injection. Our observations suggest that secretion products from the immune system help to regulate wound healing in damaged brain.

Materials and Methods

Injection of immunomodulators into brain. Adult female rats (250–300 gm; Holtzman, Madison, WI) received 2 μl intracerebral injections containing either 2 units of recombinant human IL-1β (rh-IL-1β), 5 units of recombinant murine IL-1 (rm-IL-1), 10 units of recombinant human interleukin-2 (rh-IL-2), or 100 units of recombinant murine gamma interferon (rm-IF-γ). We obtained rm-IL-1 as a gift from Dr. Peter LoMedico, Hoffman-LaRoche; rh-IL-1β was purchased from Cistron, rm-IF-γ purchased from Genentech, and rh-IL-2 purchased from Cetus. All factors showed less than 0.05 ng/ml of endotoxin, as detected by the Limulus amoebocyte lysate assay (Associates of Cape Cod). Control injections of 2 μl containing only the vehicle, PBS (pH 7.4), 100 ng cytochrome C in PBS, or 100 ng human serum albumin in PBS were placed at an identical position in the contralateral hemisphere.

Rats were anesthetized with 0.8–1.2 ml/kg of a cocktail containing 8.6 mg/ml xylazine (Miles Laboratories, Shawnee, KS), 1.4 mg/ml acepromazine (Tech America Group, Shawnee, KS), and 42.9 mg/ml ketamine HCl (Bristol-Myers, Syracuse, NY). Samples were injected intracerebrally through a burr hole in the skull using a 5 microliter syringe (Hamilton, Reno, NE) mounted on a stereotaxic device (David Kopf Instruments, Tujunga, CA). Injections were placed 4.0 mm caudal to bregma, 2.0 mm lateral from the midline suture, and at a depth of 1.2 mm from the surface of the brain (Fig. 1). The rate of injection was 1 μl/min, and at completion of the injection 2 min were allowed before removing the syringe needle from the brain. All solutions contained a suspension of 5 μm polystyrene microspheres (0.25% vol/vol) (Microspheres 15714, Polysciences, Warrington, PA) that was used to locate the site of injection.

Histology. The extent of astrogliosis was quantitated by scoring the number of nuclei for astroglia containing glial fibrillary acidic protein (GFAP) at the sites of injection. Five days after injection, animals were anesthetized and perfused with 50–75 ml of heparin–PBS solution (500 USP U/liter; Elkins-Sinn, Cherry Hill, NJ), followed by 40–50 ml of 3% formaldehyde in PBS (EM Science, Cherry Hill, NJ). The tissue was washed 3 times in PBS and repeatedly rinsed with a final solution of PBS containing 20% sucrose and 10% glycerol. The tissue was trimmed, frozen in O.C.T. compound (Tissue-Tek, Naperville, IL), and sectioned serially in the coronal plane (20 μm thickness) using a freezing microtome (Minotome, Needham Heights, MA) at −20°C. The tissue sections were mounted onto agar-coated slides, treated with 0.1% Triton X-100 for 30 min at 37°C, and washed with PBS. Tissue sections were incubated with 5% normal goat serum for 30 min at 37°C, followed by washing with PBS. The primary antibody, rabbit immunoglobulins to bovine GFAP (Dako, Santa Barbara, CA), was diluted 1:100 in PBS containing 0.1% Triton X-100 and applied to the slides for 2 hr at 37°C. After rinsing in PBS, biotinylated anti-rabbit IgG (10 mg/ml; Vector Lab, Burlingame, CA) was applied for 30 min at 37°C, followed by a 30 min incubation with HRP complex (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA). The slides were incubated in 1 mg/ml diaminobenzidine tetrahydrochloride (Sigma) diluted 1:1 with 3% hydrogen peroxide in Tris-HCl (pH 8.2). After 10 min, the reaction was stopped with water. Peroxidase-stained sections were counterstained with hematoxylin to identify nuclei. The GFAP(+) astroglia were also identified by indirect immunofluorescence using rhodamine-labeled goat anti-rabbit IgG (Bignami and Dahl, 1976; Giulian et al., 1986a) on sections prepared as described above. All tissue sections that contained an injection site were identified by the presence of polystyrene microspheres viewed by dark-field microscopy.

Peroxidase-stained GFAP(+) astroglia found at control and experimental injection sites were examined by a light microscope with a drawing tube attachment (250×). Ten to twelve tissue sections, all containing polystyrene microspheres, were used from each animal. The position of the needle tract, the location of the polystyrene microspheres, and the position of nuclei for GFAP(+) astroglia were traced onto paper. The drawings of these tissue sections, extending from the most rostral

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Correspondence should be addressed to D. Giulian, Department of Neurology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.

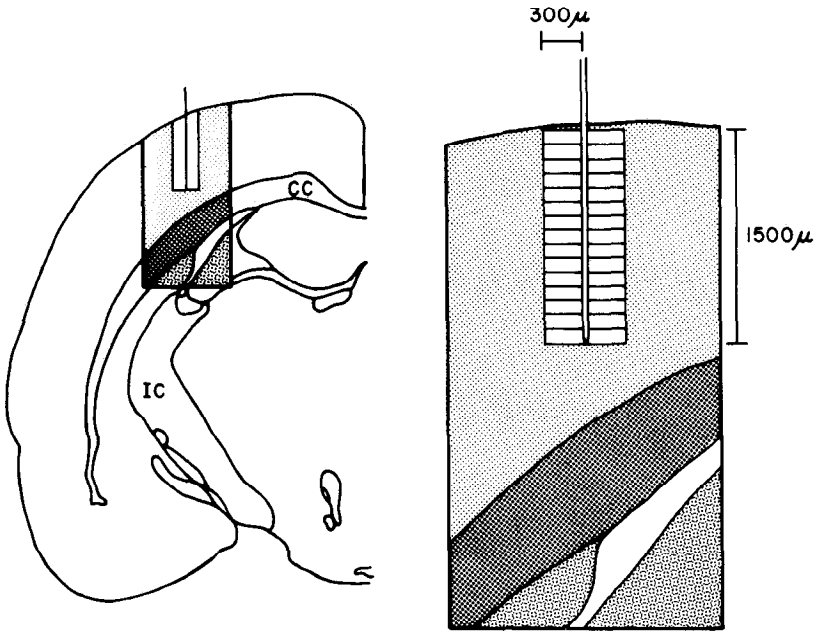

Figure 1. Diagrams to illustrate the site and depth of intracerebral injection. *A*, Two microliter injections were placed 4.0 mm caudal to bregma, 2.0 mm lateral from the midline, at a depth of 1.2 mm. Serial sections 20 μm in thickness were taken throughout the injection site and stained for GFAP. Enlarged *insert* shows that GFAP(+) cell numbers were determined from 300 μm distance from the center of the injection site at increasing depths from the brain surface. Center of the injection site was located by coinjected polystyrene microspheres.

to the most caudal positions of the injection site, were overlayed onto a plastic sheet and the total number of astroglia determined for each animal. The number of cell nuclei from 3 or more animals for each experimental group was calculated and averaged, and scores were expressed as the mean number of nuclei/(100 μm)² per tissue section.

We used the method of Gomori (1937) to stain reticulum along vessel walls. Indirect immunofluorescence techniques using anti-rabbit IgG-L-rhodamine were used to identify laminin (rabbit antiserum 1:1250 from BRL, Gaithersburg, MD) and factor VIII (rabbit antiserum 1:4, from Ortho Diagnostics, Raritan, NJ).

Determination of IL-1 levels in injured brain. In order to determine the physiologic levels of IL-1 in the cerebral cortex of injured adult rat, we inflicted single stab wounds to the depth of 1.2 mm from the surface of the brain using a flame-heated #26 needle mounted in a stereotaxic device (Giulian, 1987). Cylindrical biopsies of cerebral cortex (2 mm diameter, 5.0 ± 0.2 mg wet weight) were recovered after injury and placed in 1.0 ml of chemically defined medium (Giulian et al., 1986a) for 15 hr at 37°C. The amount of IL-1 secreted into the conditioned medium was estimated by measuring biologic activity using ^{3}H-thymidine incorporation by the D10.G cell line (Lachman et al., 1985). Units of IL-1 were calculated from a dose–response curve based upon rh-IL-1α from Genzme.

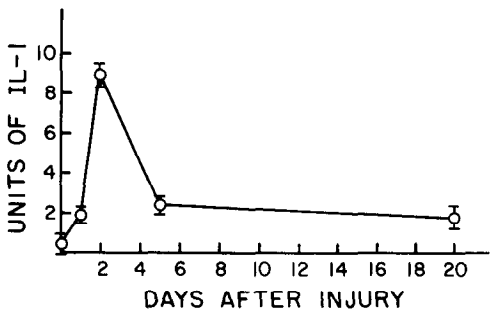

Figure 2. The appearance of IL-1 within the brain after penetrating injury. Stab wounds inflicted in the cerebral cortex of adult rats were biopsied at various times after injury and monitored for the secretion of IL-1. Normal brain released little biologic activity, as measured by the D.10.G cell line assay (<0.5 units). In contrast, significant amounts of IL-1 were produced by tissue isolated 24 hr after stab wounding. The peak of IL-1 production occurred at 2 d, with elevations persisting for at least 20 d. Data are units expressed as mean values ± SE. Each value was based on a minimum of 6 biopsies.

Results

Although IL-1 secretion in a normal adult brain biopsy is near the limits of detection, we observe nearly 2 units of secreted biologic activity within 1 d after a stab wound (Fig. 2). The peak of brain IL-1 secretion (9.0 ± 0.6 units) occurred at 2 d post-injury, corresponding to that time when large numbers of mononuclear phagocytes appear at the site of damage (Giulian, 1987). Assuming that these observations reflected the physiologic concentrations of IL-1 after penetrating injury to the brain, we tested the ability of 2–5 units of recombinant IL-1 to elicit astrogliosis *in vivo*. The location and depth of the injection into the cerebral cortex matched that of the stab wound.

Five days after injection, red cells and inflammatory cells could be identified within the needle tracts of all experimental or control injection sites. In all groups GFAP(+) astroglia were found along the needle tracts, although striking increases in both cell number and intensity of GFAP staining were noted only in those sites injected with IL-1 (Figs. 3, *A*, *B*). The number of polystyrene microspheres injected into each animal was approximately the same in all groups, and there was no correlation

Table 1. Cylindrical biopsies of the injection sites were obtained from unfixed, heparin-PBS-perfused brains

Treatment (*n*)	Glutamine synthetase activity (U/mg)
rh-IL-1β (14)	0.0868* ± 0.0100
rh-IL-2 (14)	0.0609 ± 0.0057
Cytochrome C (14)	0.0608 ± 0.0076
Control without injection (13)	0.0503 ± 0.0060

Specific enzymic activity of glutamine synthetase (Rowe et al., 1970) was measured in biopsies 5 d after injection, with activity expressed in units (U = μmol of γ-glutamyl hydroxamic acid/min) per milligram protein ± SEM. Factors injected include 2 units rh-IL-1β, 10 units rh-IL-2, or 100 ng cytochrome C. Only IL-1 stimulated a significant increase in enzymic activity when compared to controls.

* Student's $t = 3.13$, $df = 26$, $p < 0.01$. *n*, Number of biopsies examined.

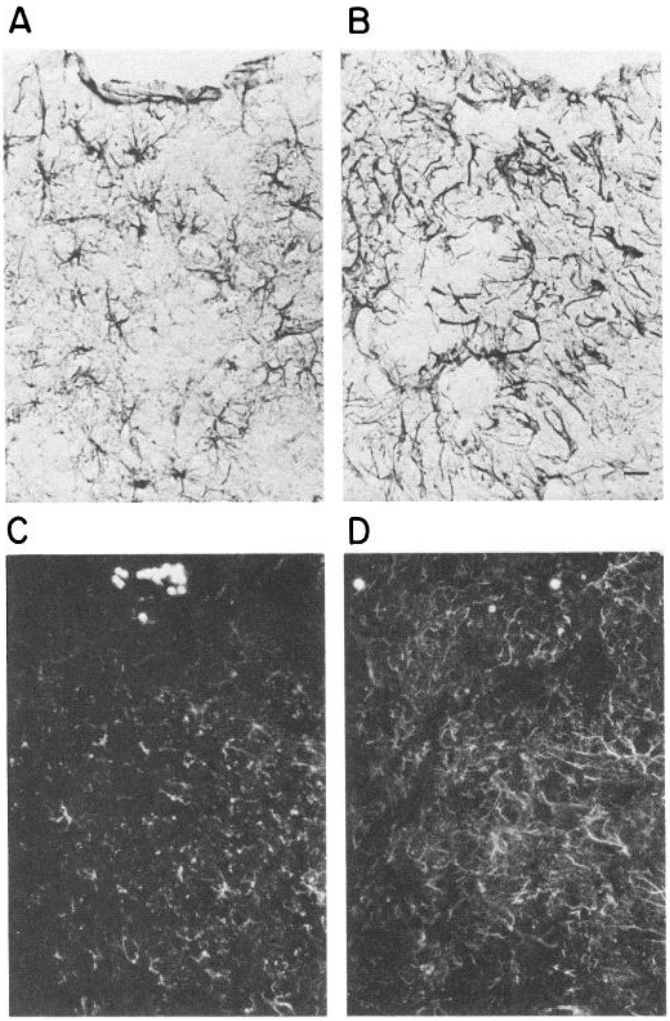

Figure 3. Photomicrographs of tissue sections stained for GFAP from brains injected 5 d earlier with PBS (*A, C*) or 5 units of rm-IL-1 (*B, D*). IL-1 induced an increase in GFAP(+) astroglia and hypertrophy of GFAP(+) astroglial processes. Astroglia were visualized by immunoperoxidase labeling of antibody to GFAP (*upper panels*) or by immunofluorescence with rhodamine-labeled antibody to GFAP (*lower panels*). The site of injection was located by the presence of polystyrene microspheres (*arrows* in lower panels). Bar, 20 μm.

between the number of beads and numbers of astroglia found at the injection sites (Fig. 3, *C, D*).

Quantitation of astroglia showed that increased cell numbers typically extended about 300–400 μm to either side of the center of the injection site (Fig. 4). For this reason, we determined the mean number of nuclei from GFAP(+) astroglia for a distance of 300 μm on either side of the injection, to a depth of 1100 μm from the brain's surface. These cell counts were averaged for all animals of a group, with each factor injection site compared with the contralateral control injection site. For this study, we examined more than 30,000 GFAP(+) astroglia found at 34 injection sites. As shown in Figure 5, rm-IL-1 and rh-IL-1β increased the number of GFAP(+) astroglia 3-fold above that found at control injection sites or above those animals receiving rh-IL-2 or rm-IF-γ. It should be noted that this scoring method could not distinguish cell migration, cell proliferation, or accumulation of GFAP as possible mechanisms to account for the

Table 2. Blood vessels at sites of intracerebral injection

Treatment (*n*)	Total blood vessel area at injection site (μm²)
Control injection (50)	20,100 ± 1700
rm-IL-1 (16)	69,800* ± 9100
rh-IL-2 (13)	20,200 ± 2600
rm-IF-γ (21)	28,700 ± 2600
Control without injection (21)	11,600 ± 1000

Area of reticulum-stained blood vessels found at the site of intracerebral injection. Five days after injection, brain sections, 20 μm in thickness, were stained for reticulum and the total vessel area measured by a Zeiss Videoplan Analyzer. The region evaluated was determined by the position of polystyrene microspheres 300 μm to either side of the center of the injection, to a depth of 1100 μm from the surface of the brain. Factors injected include 2 μl volumes containing 5 units of rm-IL-1, 10 units of rh-IL-2, or 20 units of rm-IF-γ. Control injections of 2 μl contained 100 ng equine cytochrome C in PBS. Data are expressed as mean areas in μm² ± SE. Only IL-1 stimulated a significant increase in the formation of blood vessels when compared to control injection.

* Student's $t = 5.30$, $df = 64$, $p < 0.001$. *n*, Number of sections scored.

increase in numbers of GFAP(+) astroglia. Other control injections, including PBS, PBS with human serum albumin, or PBS with cytochrome C, did not increase astroglial numbers (Fig. 5).

In addition to the histological changes associated with astrogliosis, increased levels of glutamine synthetase, an astroglial enzyme (Norenberg, 1979; Hallermayer et al., 1981), are known to occur in traumatized mammalian brain (Politis and Miller, 1985). For this reason, we also examined glutamine synthetase levels at sites of IL-1 injections. We found that rh-IL-1β, but not rh-IL-2, stimulated significant increases in specific enzymic activity as compared to control injections of cytochrome C (Table 1). All injected sites showed greater enzymic activity than that found in normal uninjected tissue controls. The IL-1-induced increase in glutamine synthetase activity is similar to elevations found in crushed optic nerve of rat (Politis and Miller, 1985), and supports the idea that IL-1 has an astroglial-stimulating effect *in vivo* that mimics the naturally occurring astroglial response to brain injury. Although IL-1 stimulates the activity of glutamine synthetase, it does not alter levels of the oligodendroglial enzyme 2′,3′ cyclic nucleotide 3′ phosphohydrolase when tested *in vitro* and *in vivo* (data not shown). Further evidence of the specificity of the action of IL-1 comes from *in vitro* studies that demonstrated that IL-1 stimulates the growth of astroglia, but not of oligodendroglia (Giulian and Lachman, 1985; Giulian et al., 1986b).

We also found an increased number of astroglial processes abutting blood vessels at sites of IL-1 injection. Since neovascularization is a common finding at sites of brain injury (Yoshida et al., 1986), we monitored blood vessel formation near injection sites by staining for reticulum (Gomori, 1937), for laminin (Foidart et al., 1980), and for factor VIII (Hoyer et al., 1973). All 3 techniques showed increased vessel numbers, as well as increased vessel tortuosity at sites injected with IL-1 (Fig. 6). The reticulum stain demonstrated a 3–4-fold increase in total vessel area at IL-1 injection sites compared to control cytochrome C injections (Table 2). The increase in numbers and in tortuosity of blood vessels induced by IL-1 is similar to those changes associated with vessels found at sites of brain infarction (Yoshida et al., 1986).

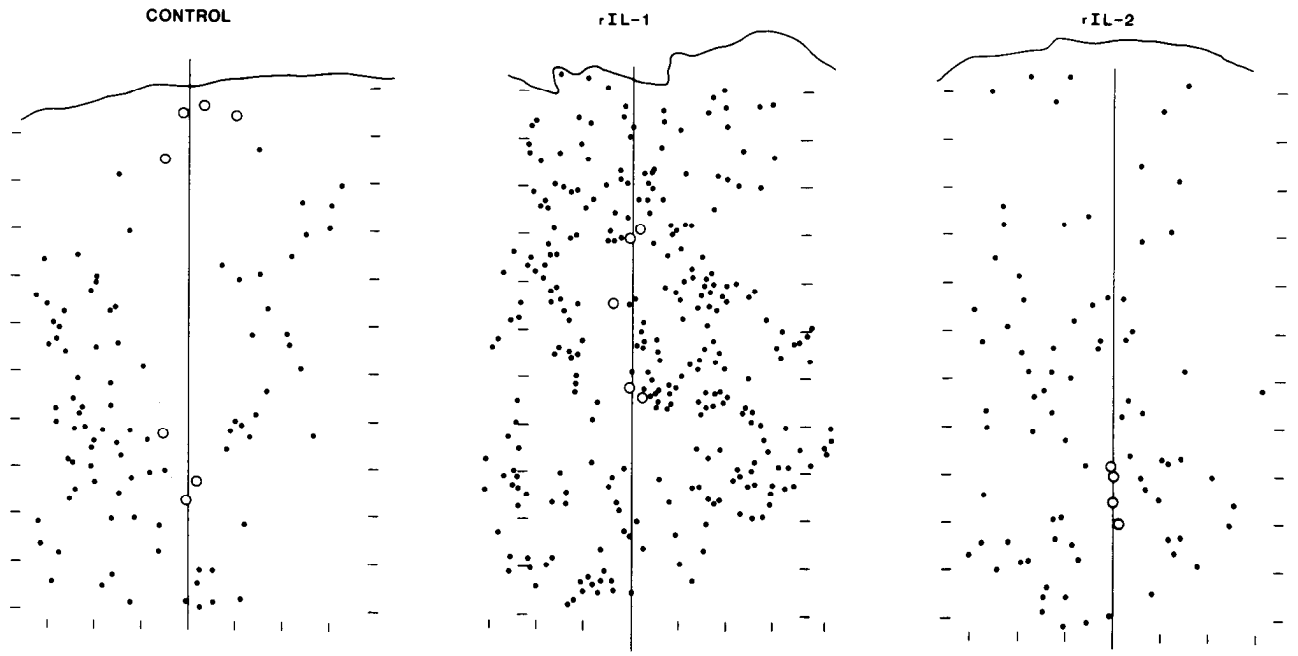

Figure 4. Camera lucida drawings of IL-1-induced astrogliosis. Astroglia found at injection sites for rm-IL-1, rh-IL-2, and PBS control injections were viewed at 250× following staining with GFAP. *Open circles* indicate 5 μm polystryene microspheres that were used to determine the center of each injection. *Closed circles* indicate the location of nuclei of GFAP(+) astroglia. *Bars* depict 100 μm increments from the surface of brain in the vertical axis and from the center of injection site in the horizontal axis. These data, taken from a single tissue section, were averaged with 10–12 additional sections from each animal to give a mean cell score, as shown in Figure 5.

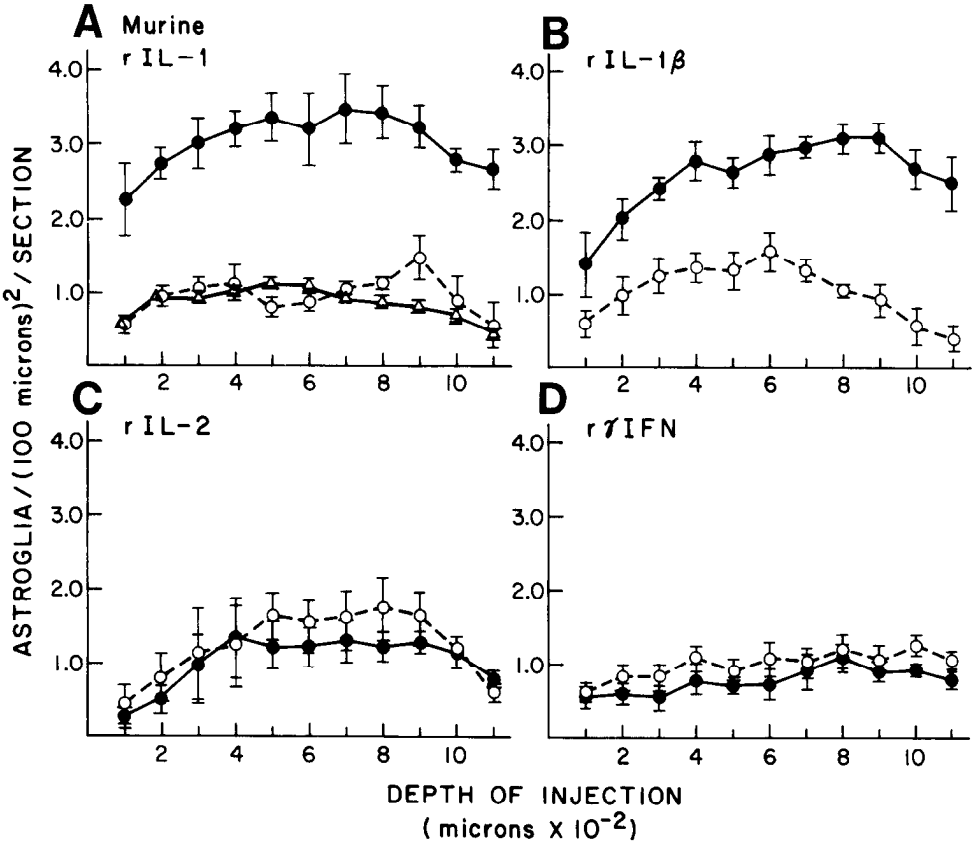

Figure 5. The number of nuclei for GFAP(+) astroglia found at the injection sites for rm-IL-1 (*A*), rh-IL-1β (*B*), rh-IL-2 (*C*), and rm-IF-γ (*D*) were expressed as the mean number of nuclei ± SEM at given depths from the surface of the brain, as found in 100 μm squares at a distance up to 300 μm from the center of the injection sites in each 20 μm-thick brain section examined. These mean values were obtained from 10–12 tissue sections cut in the coronal plane through the site of injection. Control injections in the contralateral hemisphere were scored in an identical fashion (*open circles*), and included PBS (*A, C*), 100 ng human serum albumin (*B*), or 100 ng equine cytochrome C (*D*). Data were also obtained from uninjected sites (*open triangles, A*). The injections were 5 units of rm-IL-1 (n = 6), 2 units of rh-IL-1β (n = 4), 10 units of rh-IL-2 (n = 4), or 100 units of rm-IF-γ (n = 3).

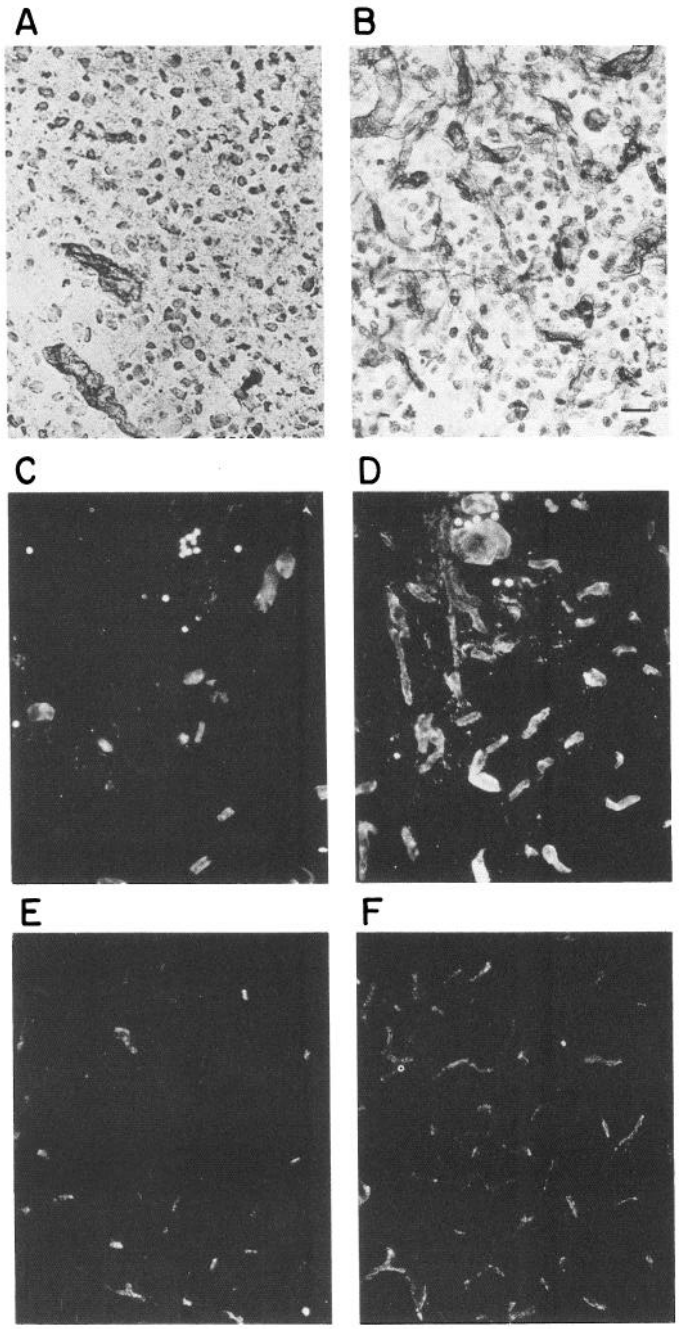

Figure 6. Photomicrographs showing blood vessels found after intracerebral injection of IL-1. The rh-IL-1β (*B*) injection showed an increase in the number of reticulum-stained vessels, as well as an increase in lumen size, when compared to PBS injection (*A*). Laminin staining of capillaries near the sites of a control injection identified basement membrane of blood vessels (*C*), while an increased number of laminin-stained capillaries were evident at the site of rh-IL-1β injection (*D*). Staining for factor VIII demonstrated that rh-IL-1β induced an increase in capillary endothelium (*F*), as compared to control injection sites (*E*). Bar, 20 μm.

Discussion

The immunomodulator IL-1 has been shown to mediate inflammatory responses outside the CNS by stimulating lymphocyte proliferation and by promoting scar formation (Dinarello, 1984). Using both native and recombinant forms, our laboratory has shown that IL-1 is also a mitogen for astroglia *in vitro* (Giulian and Lachman, 1985; Giulian et al., 1986b). Because this growth factor is found in high concentrations at sites of penetrating brain injury (Giulian and Lachman, 1985), we have proposed that it may help to mediate astrogliosis growth in the CNS. To test this hypothesis, we infused recombinant forms of various immunomodulators into the cerebral cortex of rat and monitored astroglial responses at the site of injection. As reported here, only IL-1, and not IL-2 or IF-γ, induced astrogliosis typical of brain injury. The *in vivo* action of physiologic concentrations of IL-1 upon the brain suggests that it does in fact mediate glial responses after brain injury. Second, our observations link secretion products of the immune system to tissue repair of the CNS.

What is the source of brain IL-1? Outside the CNS, mononuclear phagocytes are recognized as the most potent secretors of IL-1 (Dinarello, 1984). Study of different injury models suggests that the type of injury determines the type of inflammatory cell present. For example, penetrating wounds of the brain contain both macrophage and activated microglia (Rio-Hortega, 1932; Ling, 1981; Giulian, 1987), while axotomized cranial nerves contain only microglia (Kreutzberg and Barron, 1978). Both classes of brain mononuclear phagocyte, blood-borne macrophage and ameboid microglia, have been found to release IL-1 (Giulian and Baker, 1985, 1986; Giulian, 1987).

We also observed that IL-1, infused into the brain, stimulated blood vessel growth. Although there is no *in vitro* data to show IL-1 as an endothelial mitogen, IL-1 elicits angiogenesis in the cornea of rabbits (Prendergast et al., 1987). Futher work will be necessary to determine whether IL-1 acts directly upon brain capillaries as an angiogenic factor (Folkman and Klagsburn, 1987), or indirectly, perhaps by stimulating astroglia–endothelial cell interactions. Regardless of the cellular mechanisms, the net effect of intracerebral infusion of IL-1 is astrogliosis, with concomitant neovascularization.

Our data suggest a role for IL-1 as a regulator of cell growth and wound healing in the brain. We believe that inflammatory cells at the site of injury help to control astrogliosis and blood vessel growth in damaged CNS. Therapies to inhibit IL-1-secreting cells may therefore reduce the structural changes that occur after brain trauma.

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