The Journal of Neuroscience, December 1989, 9(12): 4416–4429

The Role of Mononuclear Phagocytes in Wound Healing After Traumatic Injury to Adult Mammalian Brain

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We monitor cellular responses to a penetrating wound in the cerebral cortex of adult rat during the first weeks after injury. Two classes of activated mononuclear phagocytes containing acetylated low-density lipoprotein (ac-LDL) receptors appear within hours at the wound site. One type of cell surrounding the lesion edge had thin, delicate processes and is identical in appearance to ramified microglia found in developing brain. Within the lesion, round cells are recognized as blood-borne macrophages when labeled by intravenous injection of carbon particles. Thus, both process-bearing reactive microglia and invading macrophages respond to brain trauma.

The greatest number of ac-LDL(+) or nonspecific esterase(+) mononuclear phagocytes appears 2 days after injury within the wound site and are associated with a peak production of the cytokine interleukin-1 (IL-1). Because intracerebral infusion of IL-1 is known to stimulate astrogliosis and neovascularization (Giulian et al., 1988), we examine the time course of injury-induced reactive astrogliosis and angiogenesis. A 5-fold increase in the number of reactive astroglia is found at 3 d and a marked neovascularization at 5 d after injury. During the first week, mononuclear phagocytes engulf particles and clear them from the wound site either by migrating to the brain surface or by entering newly formed brain vasculature.

To investigate further the role of reactive brain mononuclear phagocytes in CNS injury, we use drugs to inhibit trauma-induced inflammation. When applied *in vivo,* chloroquine or colchicine reduce the number of mononuclear phagocytes in damaged brain, help to block reactive astrogliosis and neovascularization, and slow the rate of debris clearance from sites of traumatic injury. In contrast, the glucocorticoid dexamethasone neither reduces the number of brain inflammatory cells nor hampers such responses as phagocytosis, astrogliosis, neovascularization, or debris clearance *in vivo.* Our observations show that mononuclear phagocytes play a major role in wound healing after CNS trauma with some events controlled by secretion of cytokines. Moreover, certain classes of immunosuppressive drugs may be useful in the treatment of acute brain injury.

Received Dec. 21, 1988; revised June 2, 1989; accepted June 5, 1989.

This work was funded by Grant NS25637 from NINDS.

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Many investigators have suggested that the glial environment determines in part the survival or growth of neurons after brain injury. The mechanisms by which glial cells block neuronal survival or axonal regeneration remain uncertain. One hypothesis suggests that reactive astroglia are a barrier to axonal regeneration (Reier et al., 1983), while another proposes that secreted toxic substances destroy neighboring neurons (Giulian, 1987). Because of such thinking, there has been a long-standing interest in identifying pharmacologic agents to control glial cellular responses in damaged brain (Windle and Chambers, 1950; Levine and Sowinski, 1977; Giulian, 1988). By altering the glial environment it was hoped that the survival and growth of neurons might be enhanced.

The brain of adult mammal contains 2 major classes of mononuclear phagocytes: the microglia which are resident to the nervous system and blood-borne macrophages (Rio-Hortega, 1932; Konigsmark and Sidman, 1963; Oehmichen, 1983; Giulian, 1987). Ameboid microglia first appear during embryogenesis and eventually differentiate into ramified or process-bearing cells (Rio-Hortega, 1932; Ling, 1981; Giulian and Baker, 1986). Ramified microglia are found in normal adult brain, while reactive microglia and macrophages are associated with neuropathic conditions (Oehmichen, 1983). Recent observations have shown that ameboid microglia are similar to macrophages in their ability to secrete factors (Giulian and Baker, 1985, 1986). Some of these factors may help to mediate tissue responses to CNS injury (Giulian, 1987). For example, "activated" microglia release superoxide anion (Giulian and Baker, 1986; Colton and Gilbert, 1987), a free radical implicated in neural tissue destruction. Microglia also secrete the immunomodulator interleukin-1 (IL-1; Giulian et al., 1986; Hetier et al., 1988), a cytokine which acts as a pluripotent growth factor (Dinarello, 1988). When infused into the brain, IL-1 stimulates astrogliosis and neovascularization at the site of injection (Giulian et al., 1988).

In this study, we examine cellular events after a penetrating injury to the cerebral cortex. We show that there is a close association between IL-1-secreting brain mononuclear phagocytes and wound healing in the CNS. Chloroquine and colchicine inhibit brain inflammatory responses and retard astrogliosis, neovascularization, and debris clearance at sites of CNS damage. Surprisingly, dexamethasone, a potent glucocorticoid often used in clinical neurology, did not suppress mononuclear phagocytes *in vivo,* did not reduce brain inflammation, and did not influence structural changes after trauma. Our observations support the idea that mononuclear phagocytes play an important role in organizing the tissue reaction to CNS injury. Moreover, certain immunosuppressive agents may be helpful in treatment of brain trauma.

Materials and Methods

Penetrating injury to the cerebral cortex. Adult albino rats (250–300 gm; Holtzman, Madison, WI) were deeply anesthetized by intraperitoneal injection (0.8–1.0 ml/kg of a mixture containing 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 Inc.). 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. A flame-heated 26 gauge needle, mounted in the stereotaxic device, was inserted to a depth of 1.0 mm from the surface of the brain and slowly withdrawn after a 2 min interval. In some experiments, the penetrating wound was immediately infused with a 1.0 μl suspension of fluorescently labeled polystyrene microspheres (see below) at a depth of 0.5 or 1.8 mm.

Cell cultures. Ameboid microglia were isolated from the brains of newborn rat using the method of Giulian and Baker (1986). Cells were grown on glass coverslips for 24 hr in chemically defined medium supplemented with 10% fetal calf serum and then transferred to chemically defined culture medium. Resident peritoneal macrophages were isolated from adult albino rats (Holtzman, Madison, WI) by the method of Daems (1980) and blood monocytes by ficoll/sodium diatrizoate gradients (Boyum, 1968); all cells were grown under conditions identical to those of microglia. Lipopolysaccharide W *E. coli* 055:B5 (LPS, Difco, Detroit, MI) at 10–100 μg/ml or 5 μl/ml suspension (1:1 dilution) of fixed *S. aureus* (Pansorbin, Calbiochem, La Jolla, CA) were used as monocyte activators (Nathan et al., 1980; Dinarello, 1984).

Identification of cells and histology. As reported earlier, acetylated low-density lipoprotein (ac-LDL) receptors were found in ameboid microglia (Giulian and Baker, 1985) and macrophages (Pitas, 1981) but not ramified microglia after the postnatal period (Giulian, 1987). Fluorescence microscopy was used to identify cells which accumulated ac-LDL bound to the fluorescent probe 1,1′-dioctadecyl-1,3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI). DiI-ac-LDL was obtained from Biomedical Technologies Inc., Cambridge, MA (Pitas et al., 1981). At increasing time intervals after injury, the animals were deeply anesthetized and killed by cardiac perfusion with heparin–PBS solution (500 USP units/liter). A cylindrical biopsy (2.0 × 1.0 mm) was recovered rapidly from the wound site and incubated for 12 hr at 37°C in chemically defined culture medium (Giulian et al., 1986) containing 10 μg/ml DiI-ac-LDL. The biopsies were then fixed with 3.5% formaldehyde in PBS for 1 hr, mounted under a coverslip with glycerol, and viewed using fluorescence microscopy. Mononuclear phagocytes were also identified by the presence of nonspecific esterase activity (Koski et al., 1976) or by the phagocytosis of fluorescent polystyrene microspheres (0.7 μm, Covaspheres Particles, FX Green, Duke Scientific, Palo Alto, CA) as seen in sections of 10 μm thickness cut in the coronal plane through the site of injury (Giulian et al., 1988).

Blood monocytes were labeled by intravenous injection of carbon particles prior to brain injury (Ling, 1979). One milliliter of black ink (black 17; Pelikan, Hanover, FRG) was washed 3 times with PBS and resuspended in an equal volume of PBS by sonication and injected into the femoral vein. Twenty-four hours after injection, a penetrating injury was inflicted in the cerebral cortex, and animals were killed 15–48 hr later.

Immunohistochemical techniques were used to identify astroglia containing glial fibrillary acidic protein (GFAP; Bignami and Dahl, 1976) in fixed, frozen sections of tissue cut in the coronal plane (Giulian et al., 1988). Brain capillaries were identified by the reticulum stain of Gomori (1937). The total area of capillaries (Giulian et al., 1988) seen within 300 μm of either side of the lesion were determined from serial sections (cut into 10-μm-thick sections in the coronal plane) using an image analyzer (MagicScan 2, Joyce Loble).

Bioassays. IL-1 secreted by brain biopsy samples was measured at increasing intervals after injury. Animals were deeply anesthetized, perfused with heparin–PBS, and a cylindrical biopsy (2.0 × 1.0 mm) was

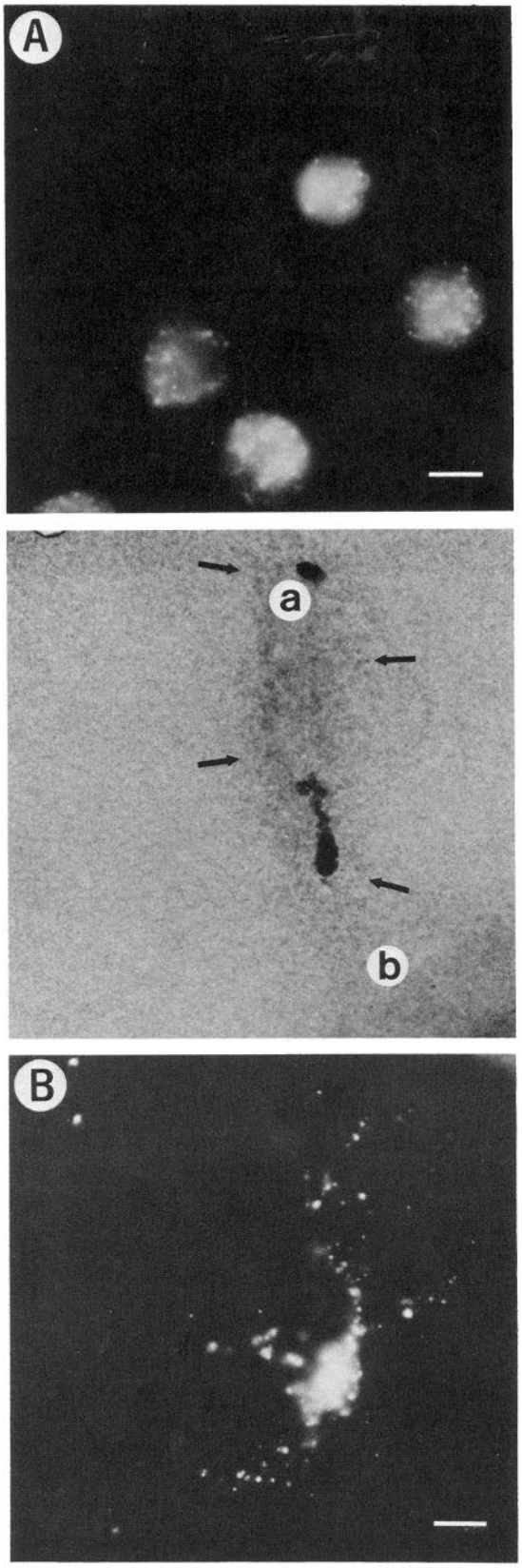


Figure 1. Mononuclear phagocytes present at the wound site 15 hr after injury. Freshly isolated brain was cut with a vibratome in the coronal plane and incubated with DiI-ac-LDL, a marker for "activated" mononuclear phagocytes. Bright-field photomicrograph at low magnification (*middle panel*) shows stab wound within the cerebral cortex. The borders of the lesion are delineated by *arrows*. *Dark areas* are sites of tissue coagulation and hemorrhage. Fluorescence photomicrographs at high magnification showed that DiI-ac-LDL(+) cells located in region *a* of the wound were round (*A*), while cells located on the lesion's edge (region *b* of the wound site shown in *middle panel*) had thin, delicate processes (*B*). As described in the text, round cells were invading blood-borne macrophages, while the process-bearing cells were reactive microglia. Scale bar, 20 μm.

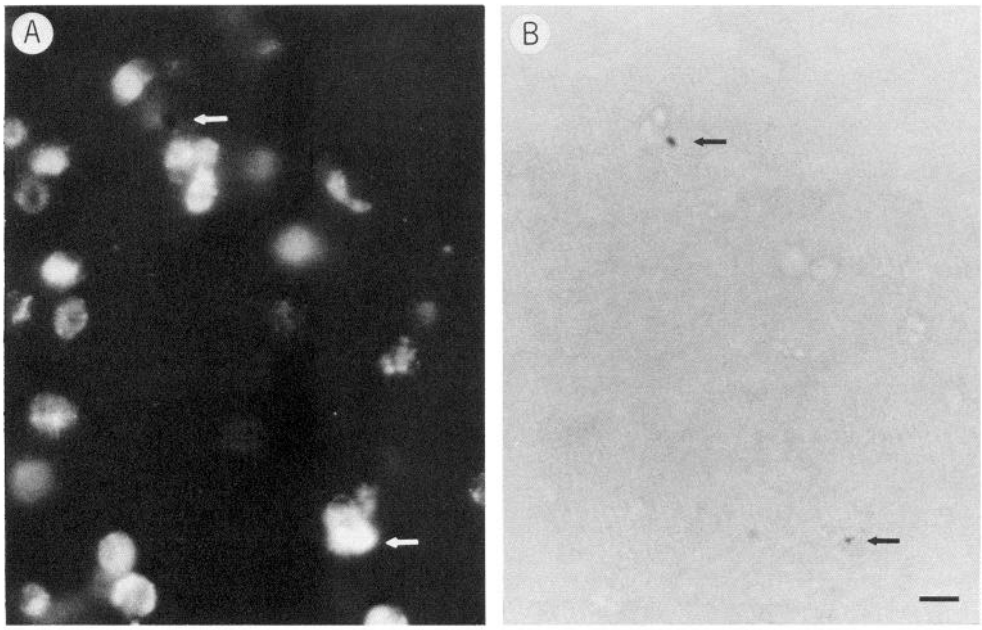


Figure 2. Photomicrographs showing the shape of invading DiI-ac-LDL(+) macrophage. Animals prior to injury received intravenous injections of carbon particles which labeled systemic mononuclear phagocytes. Fifteen hours after injury, round DiI-ac-LDL(+) cells found near the top of the lesion (*A, arrows*) contained vesicles with carbon particles (*B, arrows*). These vesicles could be seen only within round DiI-ac-LDL(+) cells (*A, arrows*) and not in process-bearing DiI-ac-LDL(+) ones (see Fig. 1*B*). Such observations suggested that round cells appearing soon after injury were invading macrophage. Scale bar, 20 μm.

removed from the surface of the brain. Two tissue samples pooled for each determination were incubated in chemically defined culture medium at 37°C for 24 hr (Giulian et al., 1988). Biologic activity for secreted brain IL-1 was determined using ^{3}H-thymidine incorporation by the D10.G4 cell line (Kaye et al., 1984; Giulian et al., 1986). Lysed cells were harvested onto glass filter paper (Skatron Harvester, Sterling, VA), and radioactivity was measured by liquid scintillation counting. Data were expressed as mean counts per min for triplicate determinations from at least 5 pooled tissue samples per group. Experiments using neutralizing anti-IL-1 antiserum in the D10.G4 assay confirmed the production of IL-1 by injured brain. We also monitored blood monocytes' release of IL-1 in the presence of either LPS (a signal requiring endocytosis) or fixed *S. aureus* (a phagocytic signal; Dinarello, 1984). Units of IL-1 biologic activity were estimated for blood monocytes using recombinant human IL-1 α (Genzyme, Boston, MA) as a standard.

Phagocytic activity was measured *in vitro* by scoring the number of fluorescently labeled polystyrene microspheres engulfed within 24 hr at 37°C by DiI-ac-LDL(+) cells. Macrophage or microglia (1×10^6/dish) grown for 24 hr on glass coverslips were identified as viable mononuclear phagocytes by prelabeling with DiI-ac-LDL 3 hr prior to incubation with drug (see Drug treatments) and microspheres in chemically defined culture medium supplemented with 5% fetal bovine serum. The number of microspheres in drug-treated cells was compared with untreated cells to determine percent inhibition of microsphere phagocytosis.

In order to test the ability of the CNS to remove debris from the injury site, we injected polystyrene microspheres (0.0005% wt/vol) suspended in 1.0 μl PBS. Phagocytic activity was measured by scoring the number of fluorescently labeled polystyrene microspheres (0.7 μm in diameter) at the site of the penetrating wound. Brains were fixed in 3.5% formaldehyde, frozen in OCT compound (Tissue-Tek, Naperville, IL), and cut with a freezing microtome (Minitome, Needham, MA) into 10-μm-thick serial sections in the coronal plane. Clearance curves were based upon the total number of microspheres counted in serial brain sections.

Endocytosis was measured by monitoring the uptake of DiI-ac-LDL into vesicles. Peritoneal macrophage or ameboid microglia (1×10^6 cells/dish) grown on glass coverslips were incubated with drugs (see Drug treatments) in chemically defined culture medium for 12 hr with DiI-ac-LDL (50 ng/ml) added during the last 6 hr of the incubation period. The percent inhibition for endocytotic activity was determined by comparing the number of cells containing DiI-ac-LDL(+) vesicles per field in drug-treated and untreated control cultures.

Drug treatments. Cytosine arabinoside, vincristine, dexamethasone, chloroquine, colchicine, and promethazine have been reported to be inhibitors of mononuclear phagocytes (Norris et al., 1977; Ahn and Harrington, 1980; MacKenzie, 1983). We screened these drugs in concentrations ranging from 10^{-6} to 10^{-11} M for *in vitro* effects upon phagocytosis and endocytosis using isolated resident peritoneal macrophages or ameboid microglia as targets. The 3 most promising drugs, chloroquine (5.0 mg/kg/d), colchicine (0.2 mg/kg/d), and dexamethasone (0.8 mg/kg/d), were then examined for effects upon astrogliosis, neovascularization, debris clearance and IL-1 secretion in adult rats (250–300 gm) after daily intraperitoneal injections.

Results

Appearance of mononuclear phagocytes at the site of injury

Under normal conditions the brain parenchyma of adult rat has few DiI-ac-LDL(+) or nonspecific esterase(+) mononuclear phagocytes (Giulian, 1987). The cerebral cortex, when injured by a penetrating wound and stained with DiI-ac-LDL, contains cells with bright fluorescently labeled vesicles at the wound site. Two distinct classes of DiI-ac-LDL(+) cells appear 5–8 hr after injury with round cells found within the lesion near the surface of the brain and process-bearing cells seen neighboring the lesion (Fig. 1).

To examine the relationship between intrinsic (cells within the brain prior to injury) and extrinsic mononuclear phagocytes (blood-borne cells), we label blood systemic mononuclear phagocytes by intravenous injection of carbon particles 24 hr prior to injury of the cerebral cortex. Within 15 hr after injury we observe DiI-ac-LDL(+) cells in the CNS that contain carbon particles. Although only a few cells are labeled with carbon particles in each animal, they are all round, not process-bearing, DiI-ac-LDL(+) cells (Fig. 2). We believe that DiI-ac-LDL(+) round cells found soon after injury are invading blood-borne macrophages as described by a number of other investigators (Konigsmark and Sidman, 1963; Oehmichen, 1983). The second type of DiI-ac-LDL(+) cells is usually located at the edge of the lesion and showed 2–4 delicately stained branches (Fig. 1). These process-bearing wound cells are identical in appearance to ramified microglia found in brain in the late postnatal period (Giulian, 1987). Reactive microglia turn up within hours after injury but decline in number such that the majority of fluorescently labeled cells are round or display short, stubby processes by the second day (Fig. 3). The loss of process-bearing

Table 1. Drug effects *in vivo* upon phagocytic activity in rat blood monocytes

Group	Mean number of microspheres/cell	% Inhibition
Control	2.2 ± 0.3 (n = 197)	—
Colchicine	1.0 ± 0.2[a] (205)	55
Dexamethasone	2.0 ± 0.5 (105)	9
Chloroquine	0.9 ± 0.1[a] (223)	59
Chloroquine + colchicine	0.3 ± 0.1[a] (172)	86

Dexamethasone (0.8 mg/kg), chloroquine (5 mg/kg), colchicine (0.2 mg/kg), or chloroquine plus colchicine (5 and 0.2 mg/kg) were given daily by intraperitoneal injections for 5 d. Isolated blood monocytes (5×10^4 cells/35 mm dish) were incubated in chemically defined medium supplemented with 5% fetal bovine serum for 24 hr at 37°C in the presence of fluorescently labeled microspheres. The data, expressed as mean number of microspheres engulfed per cell ± SEM. Dexamethasone given *in vivo* did not inhibit phagocytosis. n = number of DiI-ac-LDL(+) monocytes scored per group. Analyses by Student's t-test using the Bonferroni correction comparing drug-treated groups with normal control animals.
[a] $p < 0.001$.

cells may represent the conversion of ramified microglia to reactive ameboid forms as suggested by Rio-Hortega (1932).

Cells heavily stained for nonspecific esterase are not prominent until 48 hr after injury (Fig. 4), a pattern similar to that described by Kreutzberg and Barron (1976) and others (Konigsmark and Sidman, 1963; Ling, 1981). Thus, detection of ac-LDL receptors allows identification of activated brain mononuclear phagocytes much earlier than seen by the conventional histochemical method for esterase. Both techniques, however, show the largest numbers of mononuclear phagocytes at about 2 d after injury (Fig. 5). We conclude that reactive microglia and invading macrophage respond acutely to penetrating injury in the cerebral cortex (Fig. 6). The peak of the inflammatory response is at 2 d after injury, with many of the phagocytic cells migrating from the brain by the end of the first week.

Identification of drugs that inhibit brain mononuclear phagocytes

To explore the functional roles of CNS mononuclear phagocytes, it was first necessary to identify agents that suppress phagocytosis, secretion, or endocytosis in these cells (Giulian, 1988). We find that chloroquine and colchicine effectively hamper the ability of both cultured ameboid microglia and peritoneal macrophages to engulf fluorescently labeled microspheres (Fig. 7*A*). In general, microglia are more sensitive than peritoneal macrophages to drug suppression. Although inhibitory, vincristine at concentrations greater than 10^{-7} M is toxic *in vitro,* causing significant cell death. Dexamethasone does not inhibit phagocytosis of microspheres in mononuclear phagocytes at concentrations less than 10^{-6} M. As shown in Figure 7*B,* chloroquine and colchicine, but not dexamethasone, block endocytosis of DiI-ac-LDL. Different mechanisms of action may account for the inhibitory effects of dexamethasone, chloroquine, and colchicine upon microglia. For example, chloroquine impairs membrane-associated events, dexamethasone inhibits certain secretory functions, and colchicine blocks migration and phagocytosis by binding to microtubule proteins (Norris et al., 1977;

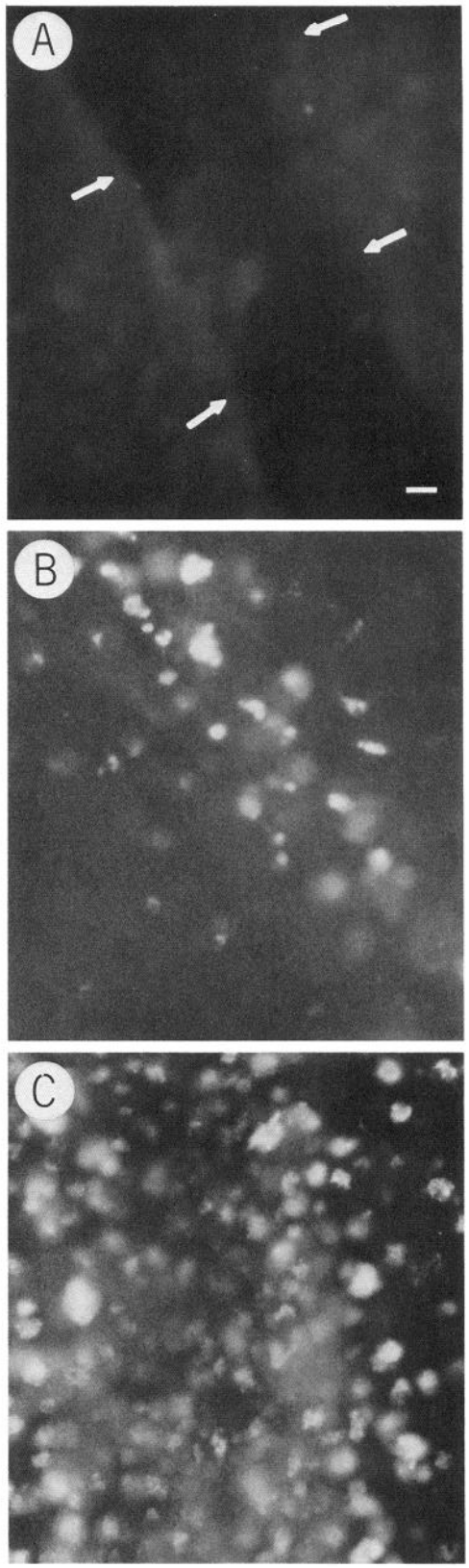


Figure 3. Time-dependent increases in DiI-ac-LDL(+) cells at the lesion site. Fluorescent photomicrographs from sections of brain cut through the wound show no labeled cells at 3 hr after injury (*A*), some by 24 hr (*B*), and large numbers by 48 hr (*C*). Wound site delineated by arrows in *A* extends from left to right in the field. Cells within the lesion tend to have round or ameboid shapes. Scale bar, 20 μm.

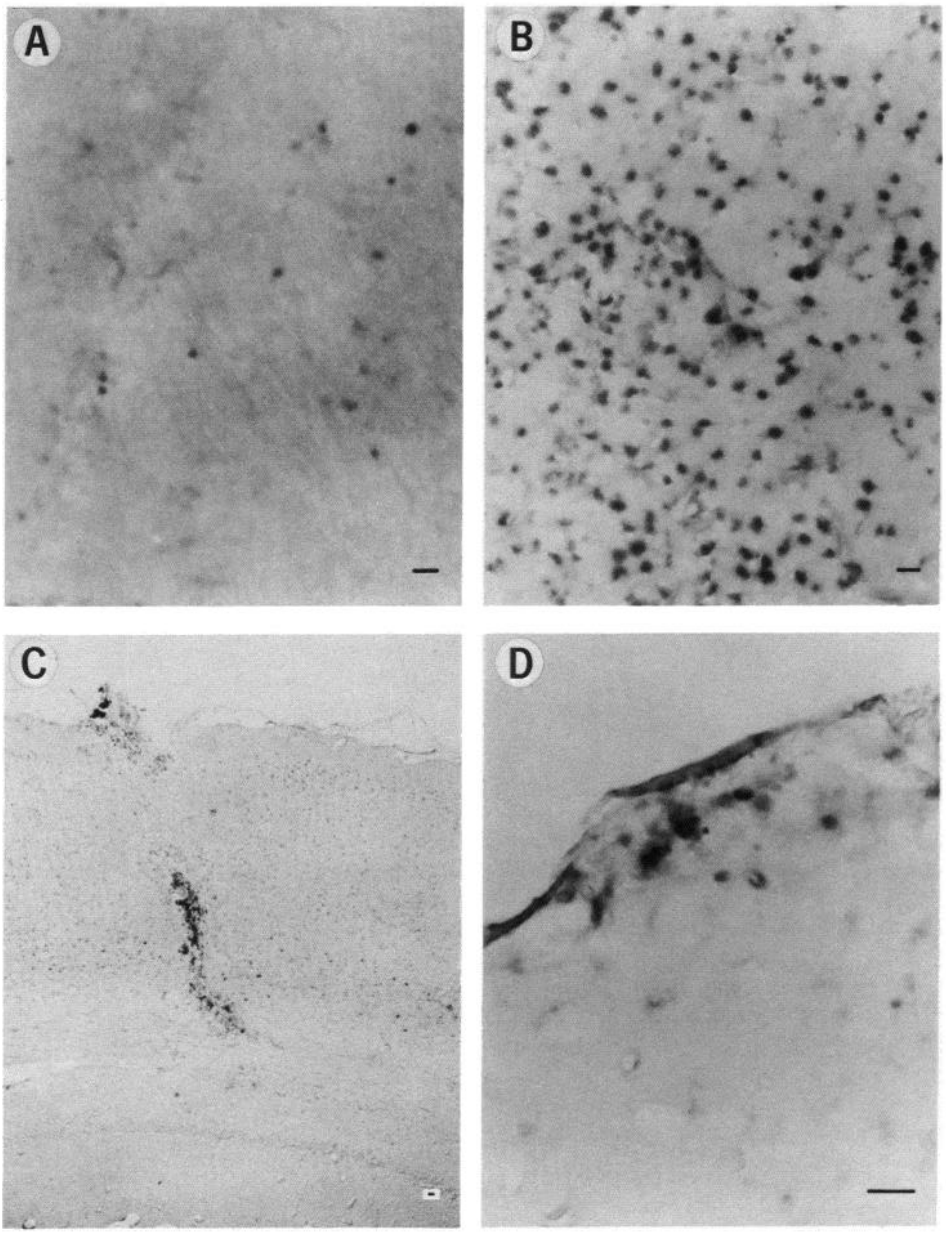


Figure 4. Photomicrographs showing the distribution of nonspecific esterase(+) mononuclear phagocytes after penetrating injury to the cerebral cortex. Although few cells were seen at the wound site by 24 hr after injury (*A*), large numbers infiltrated the lesion by 48 hr (*B*). Within 5 d the cell number declined as debris-laden cells migrated towards the meninges. A low-magnification photomicrograph illustrates groups of cells within the cortex and newly formed collection of cells in the subarachnoid spaces (*C*). By 10 d the few remaining active mononuclear phagocytes collected at the surface of the brain (*D*). This pattern of emigration was confirmed by movement of fluorescently labeled microspheres carried by the mononuclear phagocytes (see Fig. 14).

Ahn and Harrington, 1980; MacKenzie, 1983). Consistent with the putative pharmacologic actions of these drugs, we find the combination of choloroquine plus colchicine to be more effective in inhibiting phagocytosis in cultured cells than either agent alone (Fig. 8).

To determine the effectiveness of dexamethasone, chloroquine, and colchicine on mononuclear phagocytes *in vivo* we used doses well tolerated by the rat for at least 2 weeks [daily intraperitoneal injections of chloroquine (5.0 mg/kg); colchicine (0.2 mg/kg), or dexamethasone (0.8 mg/kg)]. When given *in vivo* chloroquine, colchicine, or chloroquine plus colchicine blocks phagocytosis in blood monocytes (Table 1), while dexamethasone has little effect. We find that colchicine also reduces IL-1 secretion in blood monocytes stimulated by phagocytosis but not after activation with LPS (Table 2). Chloroquine blocks IL-1 secretion that is stimulated by either the engulfment of fixed bacteria or LPS. Dexamethasone administered *in vivo* does not impair blood monocyte secretion of IL-1.

Drug effects upon inflammatory cells at the site of brain injury

As shown in Figure 4, penetrating injury to the cerebral cortex elicits a large population of inflammatory cells within 2 d after injury. There are significantly fewer DiI-ac-LDL(+) mononuclear phagocytes at the lesion site after treatment with chloroquine or colchicine (Figs. 9, 10). These drugs also decrease numbers of nonspecific esterase(+) mononuclear phagocytes; dexamethasone, however, produces no reduction in DiI-ac-LDL(+) (Figs. 9, 10) or nonspecific esterase(+) cells. Thus, chloroquine, colchicine, or a combination of chloroquine plus colchicine effectively suppresses brain inflammation after trauma, whereas dexamethasone has little effect.

Astrogliosis at the wound site

The appearance of GFAP(+)-reactive astroglia has been recognized as the prototypic glial reaction to brain injury (Bignami and Dahl, 1976). Recently, IL-1 has been implicated as a brain-

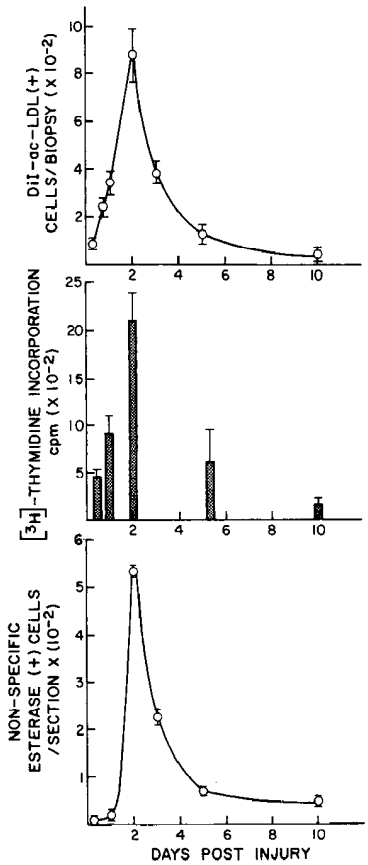


Figure 5. Mononuclear phagocytes and brain IL-1 secretion. Mononuclear phagocytes labeled by either DiI-ac-LDL (*upper panel*) or nonspecific esterase (*lower panel*) were monitored at lesion sites during the first 10 d after injury. Significant numbers of cells containing ac-LDL receptors were noted by 15 hr (*upper panel*), at which time secreted IL-1 was also detected. The peak number of inflammatory cells appeared at 2 d (as shown by either cell marker) correlated with the highest levels of IL-1 produced by wound tissue (*middle panel*). Relative cytokine values are expressed as mean counts per min ± SEM for 10 μl of secretion-containing culture medium using the ^{3}H-thymidine incorporation assay with the D10.G4 cell line.

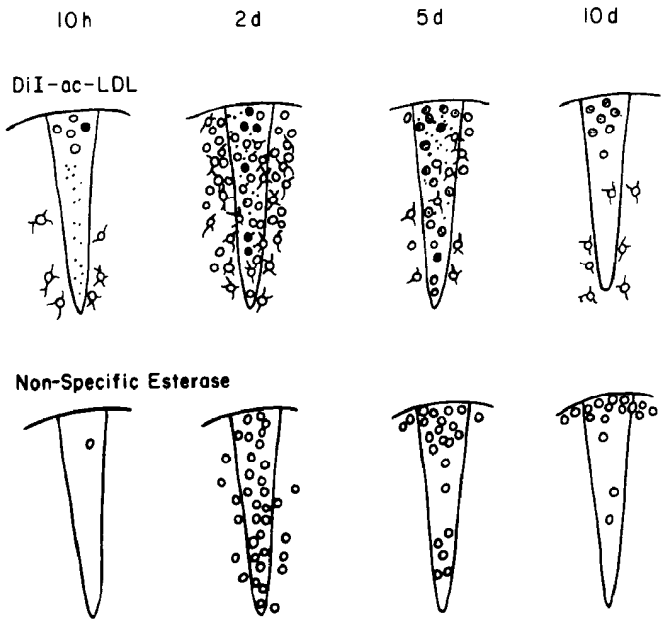
Table 2. Drug effects *in vivo* upon IL-1 secretion by rat blood monocytes

	Total units of IL-1 secreted	
Drug treatment	LPS stimulated	*S. aureus* stimulated
Control	121 ± 16	144 ± 26
	(n = 8)	(12)
Colchicine	136 ± 8	39 ± 1[b]
	(7)	(7)
Chloroquine	52 ± 13[b]	41 ± 19[a]
	(8)	(4)
Dexamethasone	134 ± 24	129 ± 14
	(5)	(4)
Chloroquine + colchicine	60 ± 2[a]	13 ± 7[b]
	(3)	(5)

Rats received dexamethasone (0.8 mg/kg), chloroquine (5.0 mg/kg), colchicine (0.2 mg/kg), or a combination of chloroquine plus colchicine in the same dosages daily by intraperitoneal injection for 5 d. Blood monocytes were isolated and plated at a density of 1.5 × 10^5 cells/ml in 24-well plates containing 1.0 ml of chemically defined culture medium. The cells were stimulated with either a suspension of fixed *S. aureus* or lipopolysaccharide (LPS). The data, total units of IL-1 produced by 1.5 × 10^5 monocytes in 24 hr, are expressed as mean values ± SEM based upon D10.G4 cell line assay using recombinant human IL-1 α as a standard. Values were obtained from triplicate determinations. n = number of cultures tested per group. As shown, chloroquine or chloroquine + colchicine effectively inhibited LPS-stimulated release of IL-1, while chloroquine, colchicine, or chloroquine + colchicine blocked IL-1 release stimulated by phagocytosis of fixed *S. aureus*. Dexamethasone given *in vivo* did not inhibit IL-1 secretion. Statistical analyses comparing controls with the drug-treated animals by Student's t-test.

[a] $p < 0.05$.

[b] $p < 0.01$.



derived growth factor released at wound sites (Giulian et al., 1988). As shown in Figure 5*B*, IL-1 is secreted within 24 hr after traumatic injury, with the peak production around 2 d or that time corresponding to the greatest number of DiI-ac-LDL(+) or nonspecific esterase(+) cells. These observations coupled with earlier *in vitro* studies suggest that "activated" brain mononuclear phagocytes are the major source of brain IL-1 (Giulian et al., 1986; Hetier et al., 1988). As illustrated in Figure 11, prominent GFAP(+) astroglia were observed by day 3 in this injury model. The 5-fold increase in GFAP(+)-reactive astroglia occurs in areas up to 300 μm from the center of the lesion (Fig. 12*A*) near collections of phagocytic cells.

The fact that IL-1 stimulates astrogliosis (Giulian et al., 1988) suggests that inhibition of cytokine secretion may alter glial

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Figure 6. Diagram to summarize the inflammatory response to penetrating injury of the cerebral cortex. Within 10 hr, invading macrophages (round cells) and reactive ramified microglia (process-bearing cells) could be seen at the wound site. Microspheres (*fine dots*) were distributed evenly over the injury site, suggesting no debris clearance. An occasional macrophage containing carbon particles (*black cells* labeled prior to brain injury by systemic injection) could be detected. Very few nonspecific esterase(+) cells were noted at this time. By 2 d, large numbers of DiI-ac-LDL(+) or nonspecific esterase(+) cells collected within the wound site. The predominant morphology was ameboid or round, although cells with short, stubby processes could be seen near and within the lesion. By 5 d, the number of mononuclear phagocytes began to decline with movement of cells to surface of the brain. Microspheres were carried out of the wound as cells emigrated from the CNS. Within 10 d, the number of inflammatory cells had markedly declined with the largest collection of cells noted at the surface of the brain.

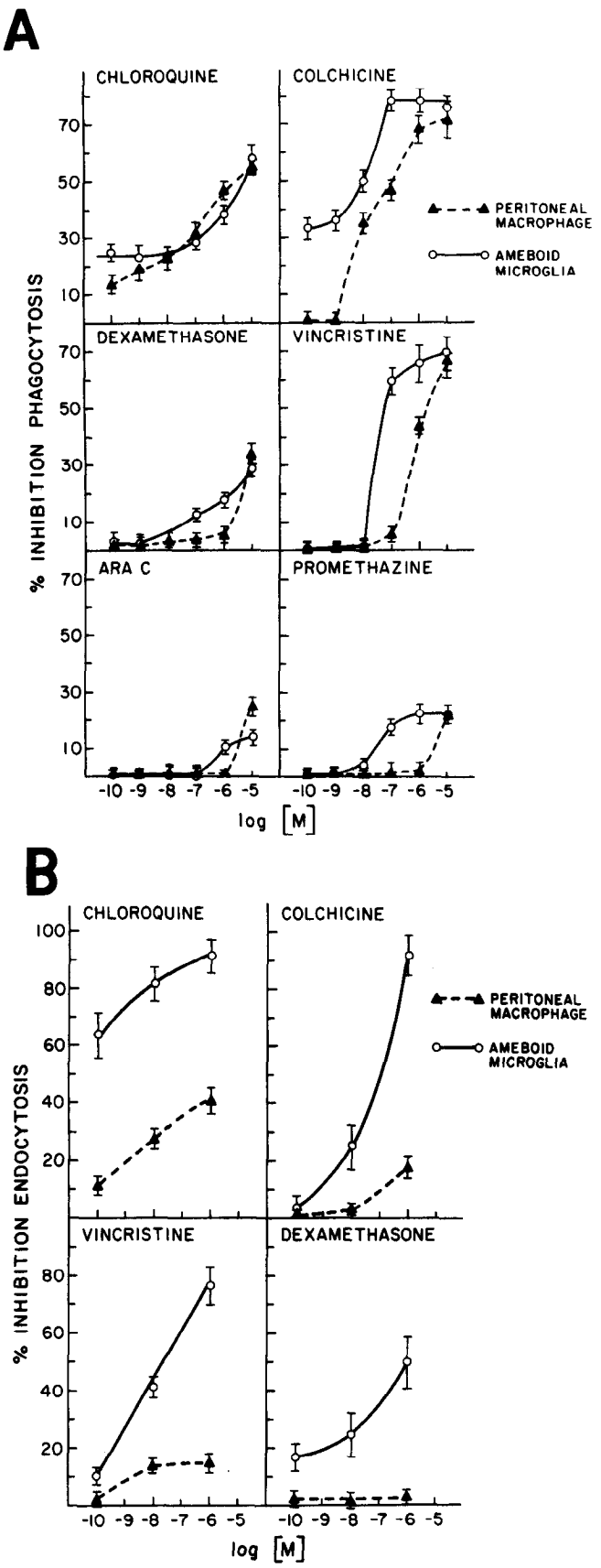


Figure 7. *A*, Quantitation of phagocytic activity in the presence of increasing concentrations of drugs. Drugs reported to inhibit monocytes or macrophages *in vitro* were tested for their ability to block phagocytosis in cultured resident peritoneal macrophages or ameboid microglia. Data, expressed as percent inhibition of phagocytosis, were based upon microsphere engulfment assay as described in Materials and Methods. Dose–response curves show that colchicine and chloroquine are the most effective inhibitors in concentrations less than 10^{-8} M. Vincristine at 10^{-7} M produced significant morphologic changes and cell death. *B*, Drug inhibition of endocytosis in cultured ameboid microglia or resident peritoneal macrophage. Endocytosis was measured by scoring the number of cells which contained vesicles of DiI-ac-LDL. Chloroquine, the most potent inhibitor, was effective at concentrations of less than 10^{-8} M. The specific inhibitory effect of vincristine was uncertain at 10^{-8} M because of apparent cytotoxic effects.

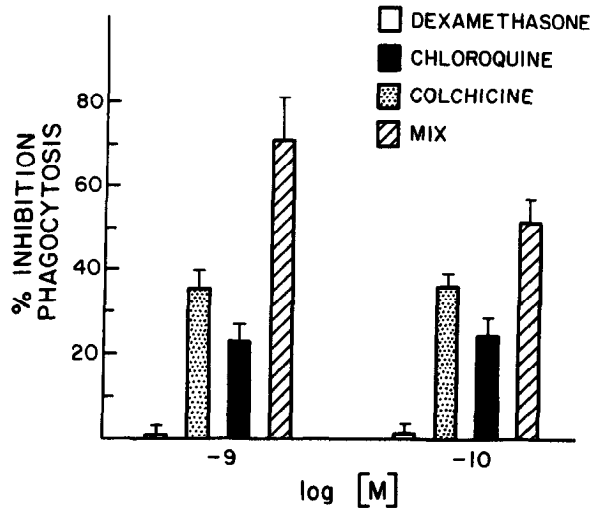


Figure 8. Combined inhibitory effect of chloroquine plus colchicine upon phagocytosis. Isolated ameboid microglia were incubated for 24 hr in chemically defined medium containing low concentrations of dexamethasone, chloroquine, colchicine, or a combination of chloroquine plus colchicine (*Mix*). Data, expressed as percent inhibition of phagocytosis, were based upon microsphere engulfment assay. As shown, a combination of chloroquine and colchicine most effectively reduced phagocytic activity.

reaction to brain injury. We find that treatment with chloroquine, an effective inhibitor of IL-1 secretion, significantly reduces the number of GFAP(+)-reactive glia found near the wound site (Fig. 12*B*); dexamethasone, a poor inhibitor of IL-1 secretion *in vivo*, does not influence reactive astrogliosis. Perhaps the number and location of reactive astroglia are linked to the levels of stimulatory factors secreted by neighboring mononuclear phagocytes.

Neovascularization at the wound site

Angiogenesis, a well-recognized tissue response to damage (Folkman and Klagsburn, 1987), occurs at around day 5 after injury (Fig. 13*A*). Most of the new blood vessel formation is noted near the lesion center, an area most densely populated with mononuclear phagocytes. Previously, our laboratory has shown that recombinant IL-1 stimulates brain neovascularization (Giulian et al., 1988). The timing and location of neovascular responses to brain injury are consistent with the notion that secreted factors, including IL-1, help to regulate growth of blood vessels abutting reactive astroglia (Fig. 11). To test this hypothesis, we suppress brain IL-1 production. Chloroquine and colchicine, but not dexamethasone, impair angiogenesis for at least 15 d after traumatic injury (data not shown). Quantitation

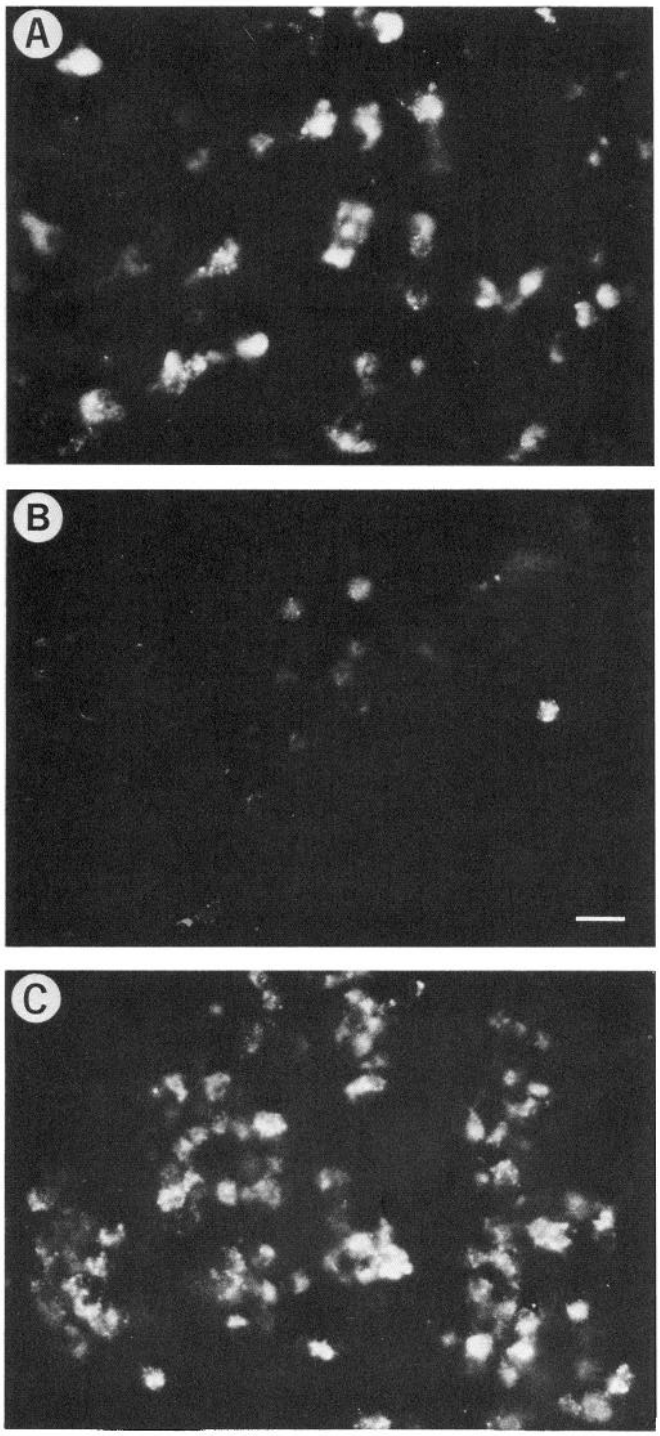


Figure 9. Fluorescence photomicrographs showing the effects of chloroquine and dexamethasone upon mononuclear phagocytes at the site of penetrating brain injury. Forty-eight hours after stab wound injury, serial sections were cut in the coronal plane through the wound site. Large numbers of DiI-ac-LDL(+) cells were found at center of wound in a control animal (*A*). As shown, chloroquine (*B*; 5 mg/kg/d) markedly reduced number of inflammatory cells, whereas dexamethasone (*C*; 0.8 mg/kg/d) did not. Scale bar, 20 μm.

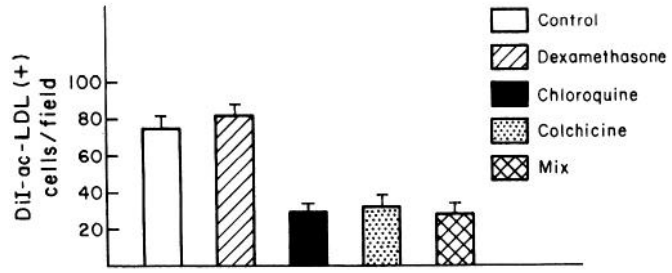


Figure 10. Quantitation of DiL-ac-LDL(+) cells at the site of injury. Biopsies obtained 48 hr after penetrating injury were incubated in chemically defined medium containing DiI-ac-LDL. These tissues were then viewed at 200× magnification with an epifluorescence microscope and the number of DiI-ac-LDL(+) cells were scored per field. The data, expressed as mean number of cells per field ± SEM, were obtained from at least 8 biopsies per group. Control animals received only a penetrating injury in the cerebral cortex. Dexamethasone, chloroquine, colchicine, or chloroquine plus colchicine (*Mix*) were given by intraperitoneal injections. A comparison of mean values from controls to the drug-treated animals by Student's *t*-test showed that chloroquine, colchicine, or the drug combination of chloroquine plus colchicine significantly reduced the number of inflammatory cells at the site of injury ($p < 0.01$ for all comparisons using the Bonferroni correction). Dexamethasone did not, however, alter the DiI-ac-LDL(+) cell population.

of the cross-sectional capillary areas (Fig. 13*B*) confirms that chloroquine or colchicine inhibit neovascularization at the wound site. In order to rule out nonspecific toxic effects of drug treatments upon vessel growth, we inject recombinant IL-1 α intracerebrally 5 d after penetrating injury to the cerebral cortex of animals receiving daily doses of chloroquine (5.0 mg/kg/d by intraperitoneal injection). Each animal received 2 μl PBS containing 5 units of recombinant IL-1 into the wound site (1.0 mm depth from the surface of the brain) of one cerebral hemisphere or 2 μl PBS alone into the wound site of the contralateral hemisphere. The chloroquine-treated PBS-infused wound sites had a mean capillary score of 31,180 ± 2900 μm^2 ($n = 21$), while the chloroquine-treated IL-1-infused wound sites had 91,200 ± 6400 μm^2 ($n = 18$; $p < 0.0001$ by Student's *t*-test). A similar result was noted for colchicine-treated animals (0.2 mg/kg/d) given intracerebral IL-1 (data not shown). Thus, chloroquine or colchicine treatments do not block new vessel growth if an intracerebral growth factor is available. Drug suppression of factor secretion by brain inflammatory cells is therefore a likely mechanism for the observed inhibition of trauma-induced neovascularization.

Debris clearance from the wound site

To examine the scavenger activity of brain inflammatory cells *in vivo,* we inject fluorescently labeled microspheres into penetrating wound sites and monitor their pattern of clearance from damaged tissue. There are striking changes in the distribution and number of microspheres within injured brain neuropil during the first 10 d. At 2 hr after injury, microspheres are found as individual particles densely collected within the injection site (Fig. 14*A*). Several days later the microspheres begin to disperse from the injection site in groups or clusters, most of which are found within DiI-ac-LDL(+) mononuclear phagocytes. Some phagocytic cells containing microspheres become autofluorescent after engulfing tissue debris (Fig. 14*D*). Within 5 d, the rate of microsphere removal from the brain increases as demonstrated by a clearance curve (Fig. 15). There is evidence for 2 routes of particle removal. In the first, DiI-ac-LDL(+) phagocytic cells migrate to the surface of the brain carrying clusters of microspheres (Fig. 16). In the second, cells engulfing particles enter blood vessels, which in some cases comprise newly formed vascular networks (Fig. 17). The microsphere clearance curve

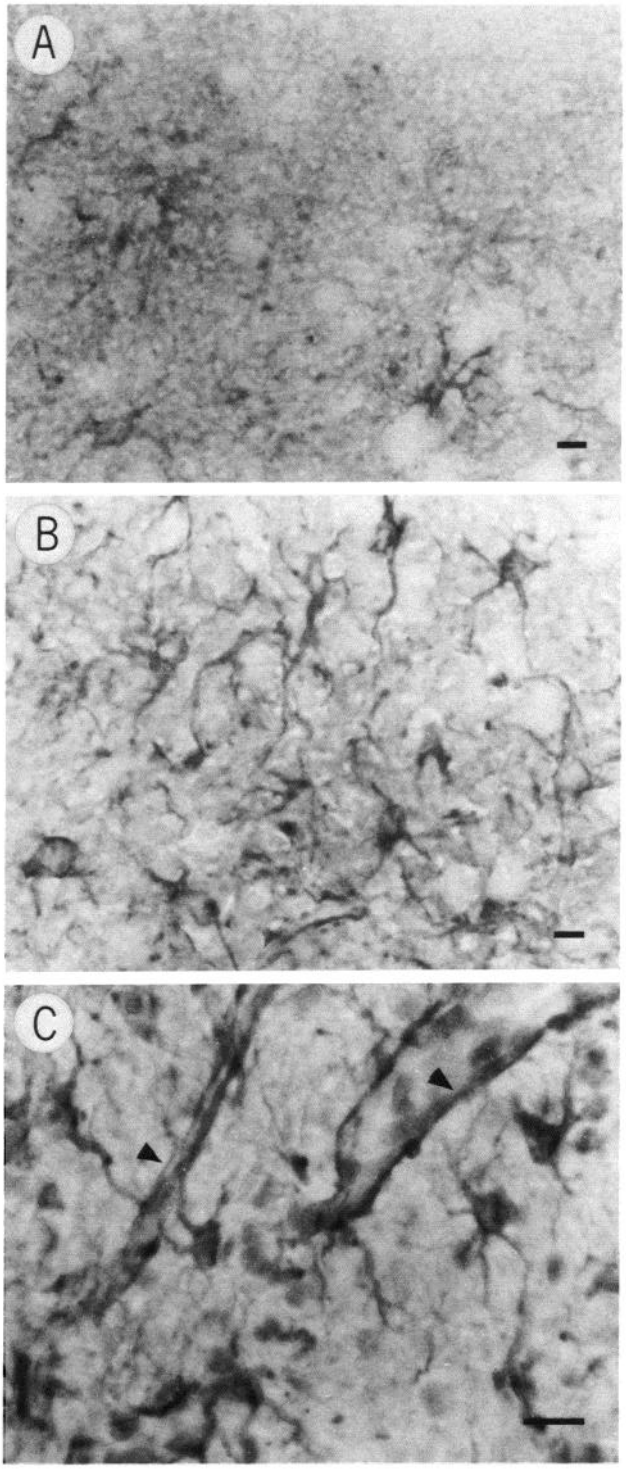


Figure 11. Reactive astrogliosis after penetrating wound. Sections of cerebral cortex cut in the coronal plane through the wound site at increasing intervals after injury. At 24 hr (*A*), a few GFAP(+) astroglia were present among an amorphous background (gray region in the photomicrograph) of damaged tissue. The astroglia seen at this time had normal shape and nuclear size with modest processes. By 72 hr, reactive GFAP(+) cells were noted (*B*) with large nuclei and prominent, darkly stained processes. The greatest density of these cells were along the edge of the wound neighboring clusters of mononuclear phagocytes. By 5 d after injury, reactive astroglia were often seen abutting capillaries (*C, arrows*). These vessels were newly formed and located primarily within the lesion. Scale bar, 20 μm.

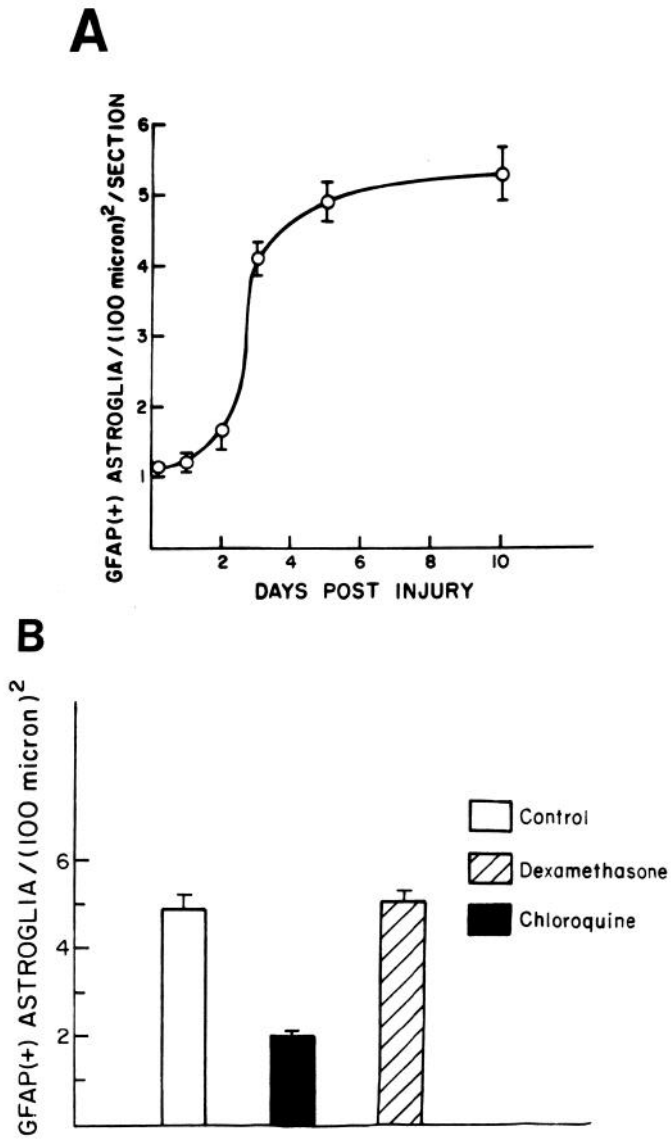


Figure 12. *A*, Number of GFAP(+) cells were determined in serial sections cut in the coronal plane through the wound site at increasing intervals after injury. Data expressed as mean cell number ± SEM per (100 μm)² were obtained at 300 μm or less from center of the lesion. *B*, Drug effects upon astrogliosis. Five days after injury, serial sections cut in the coronal plane were stained for GFAP(+) astroglia. Data, expressed as mean number of GFAP(+) cells ± SEM, were obtained from at least 10 sections from 3 animals per group. As shown, chloroquine-treated animals had a significant reduction in the number of reactive astroglia at the wound site ($p < 0.001$ by Student's *t*-test with the Bonferroni correction; Godfrey, 1985). Dexamethasone did not block reactive astrogliosis.

(Fig. 15) thus reflects a complicated series of events whereby mononuclear phagocytes recognize and engulf foreign material and carry this debris to the surface of the brain or to its vasculature (Fig. 18).

Chloroquine, an inhibitor of phagocytosis *in vitro,* effectively cripples the phagocytic system of the brain *in vivo* (Fig. 19). Chloroquine-treated animals 10 d after trauma have a large number of particles broadly distributed throughout the wound site in a pattern similar to that of untreated animals 1–2 d after injury. Significantly more particles remain in the cerebral cortex

of animals treated with chloroquine or colchicine (data not shown) than in dexamethasone-treated or control animals (Fig. 2C).

Discussion

Wound healing in the CNS represents a complicated series of cellular responses to acute tissue injury. "Activated" mononuclear phagocytes (North, 1978; Nathan et al., 1980) found within damaged neural tissue include reactive microglia and invading macrophages. As first recognized by Rio-Hortega (1932), microglia are scavengers which engulf and clear debris from wound sites. Our observations described here agree with Rio-Hortega's hypothesis that quiescent microglia of the adult CNS transform into reactive cells resembling microglia found in perinatal brain. Ultimately, most active mononuclear phagocytes disappear from the wound site. The 3 likely possibilities to account for this disappearance include cell death, cell differentiation, or cell migration. As shown here, many cells migrate to the surface of the brain and eventually emigrate by entering the subarachnoid space. Perhaps invading macrophages clear damaged tissue, hemorrhage, or abscess from the CNS in this fashion. Further work will be necessary to determine the fate of reactive microglia. One possibility (Rio-Hortega, 1932) is that the reactive microglia return to quiescent ramified forms which are DiI-ac-LDL(−). The mechanisms that activate ramified microglia and the events that lead to inactivation of reactive microglia are unknown but fundamentally important for an understanding of CNS inflammatory responses.

Mononuclear phagocytes may influence wound healing in the brain by the secretion of growth factors. We have shown previously that IL-1 released both by ameboid microglia and macrophage (Giulian et al., 1986) stimulate astroglial growth *in vitro*, astrogliosis *in vivo*, and neovascularization *in vivo* (Giulian et al., 1988). The astrogliotic reaction to trauma we observe here is similar to that noted by a number of other investigators (Bignami and Dahl, 1976; Berry et al., 1983). The appearance of astrogliosis following peak production of IL-1 at the wound site suggests an association between reactive astroglia and the secretory activity of neighboring mononuclear phagocytes. Although the long-term consequences of astrogliosis remains unclear, reactive astrocytes are thought to block axonal regeneration and remyelination (Reier et al., 1983; Blakemore et al., 1986). Stab-injured animals treated with chloroquine have suppressed IL-1 production and significant decreases in brain angiogenesis at the wound site. To confirm that reduction of new vessel growth is due to suppression of IL-1, we infused recombinant IL-1 α into the wound sites of rats treated with chloroquine. It was clear that IL-1, when present in these drug-treated animals, still stimulated vascular growth. Thus, drug suppression of secretion products, including brain IL-1, reduces new vessel formation at sites of trauma. However, since microglia secrete at least 2 astroglia-stimulating growth factors other than IL-1 (Giulian and Baker, 1985), it is unlikely that IL-1 is the only inflammatory cell-released factor which influences astroglial growth or neovascularization at trauma sites (Leibovich and Wiseman, 1988).

Reactive microglia and invading macrophages may impair function of the nervous system by several different mechanisms including the secretion of the cytokine tumor necrosis factor (TNF) and the production of cytotoxins (Frei et al., 1987; Giulian, 1987). Recently, TNF has been associated with the destruction of myelin and oligodendroglia (Selmaj and Raine, 1988). Free radicals, arachidonic acid metabolites, and proteases (Giulian, 1987) secreted by microglia have also been implicated as mediators of neural tissue damage (Raichle, 1983; Pope and Nixon, 1984; Chen et al., 1986). Alternatively, brain mononuclear phagocytes may hamper survival of injured neurons by such contact-mediated events as phagocytosis and direct cell killing (Giulian, 1987; Henson and Johnston, 1987).

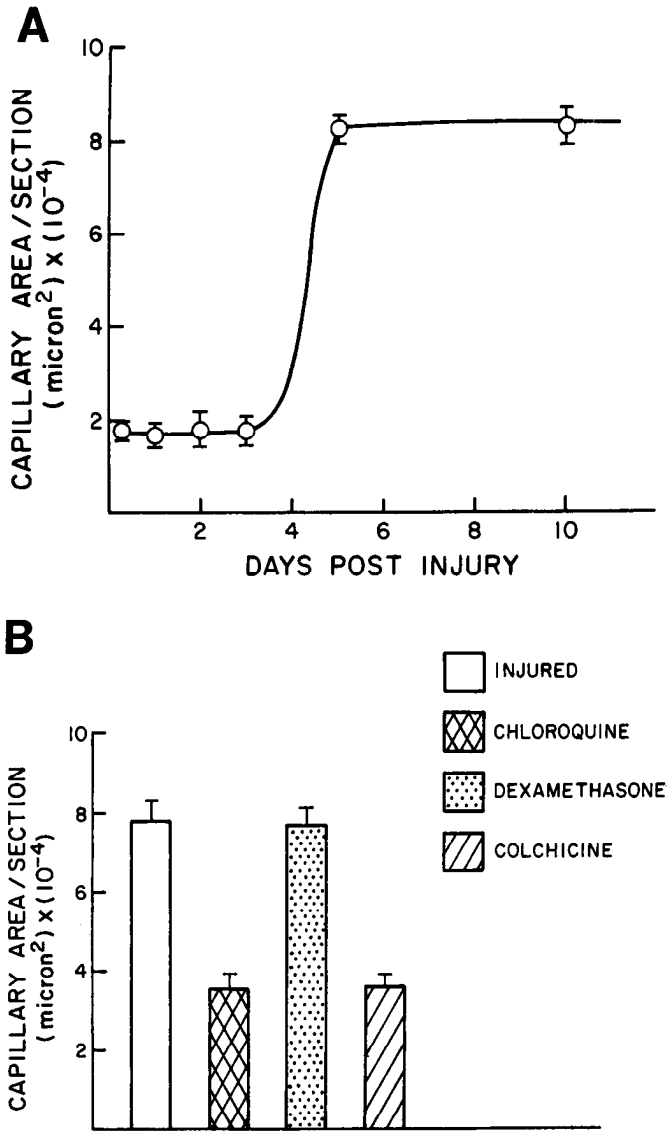


Figure 13. *A*, Quantitation of blood vessels at the penetrating wound site. Cross-sectional areas of reticulum-stained capillaries within 300 μm of the center of the lesion were determined by image analyzer. Data, expressed as mean area in $\mu m^2 \pm$ SEM per brain section, were obtained from at least 25 sections from each of 3 or more animals at a given time point. Significant increases in vascular areas were noted by 5 d after injury. *B*, Quantitation of drug effects upon neovascularization 5 d after penetrating injury to the cerebral cortex. Data, expressed as mean values in $\mu m^2 \pm$ SEM, were obtained from 10 sections from 3 animals in each group. Control animals (*injured*) had significantly greater capillary areas when compared by Student's *t*-test to animals treated with colchicine ($p < 0.001$) or chloroquine ($p < 0.01$). Dexamethasone did not block neovascularization.

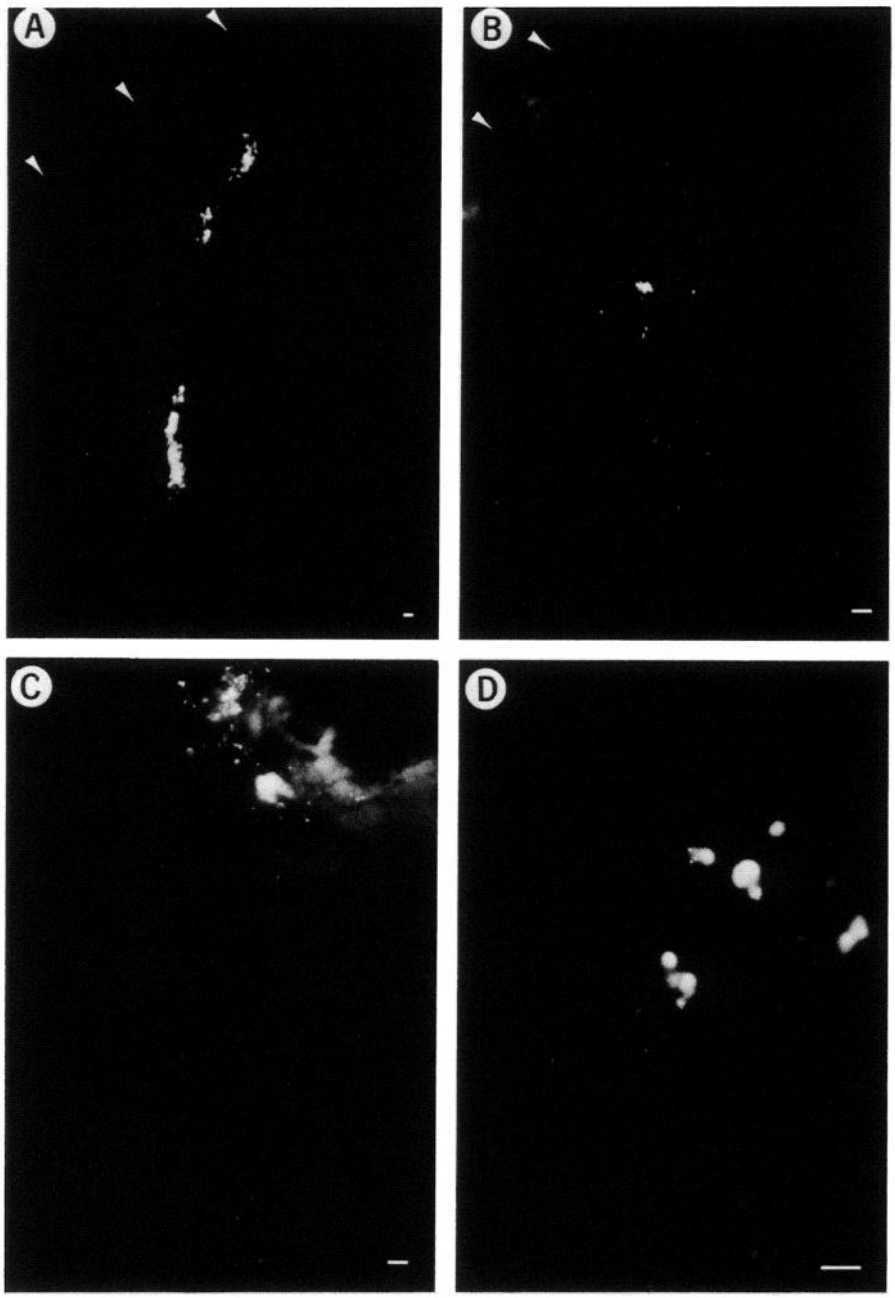
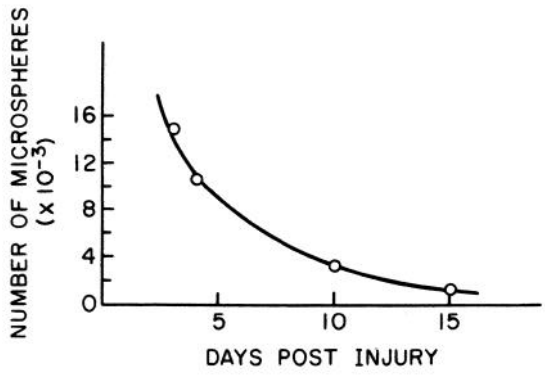


Figure 14. Fluorescent photomicrographs showing the clearance of microspheres from penetrating wound site. Initially, injected microspheres were found in massive collection within the center of the lesion (*A*, low magnification, $t = 2$ hr). By 5 d (*B*), microspheres had dispersed with small clusters of microspheres moving towards the surface of the brain (*arrows*). Most of the microspheres were cleared from brain neuropil within 10 d after injury (*C*). During this later phase, clusters of particles were found in autofluorescent debris-laden mononuclear phagocytes (*D*). Scale bar, 20 μm.



In view of the complex behaviors exhibited by activated brain mononuclear phagocytes, it was unlikely that blockade of a single cellular event would effectively suppress brain inflammation. For this reason we sought to inhibit endocytosis, secretion, and phagocytosis in brain phagocytes as a way to impede activating or chemotaxic signals, cell movement and engulfment, and the production of cytotoxins. As described here, chloroquine and colchicine reduced the number of inflammatory cells at sites of

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Figure 15. Clearance curve of microspheres from penetrating wound in the cerebral cortex. Fluorescently labeled microspheres were infused at a depth of 0.5 mm from the surface of the brain into the wound site immediately after injury. Data, expressed as total number of particles found in serial sections of brain, were obtained from at least 15 serial sections with 2 animals used per time point.

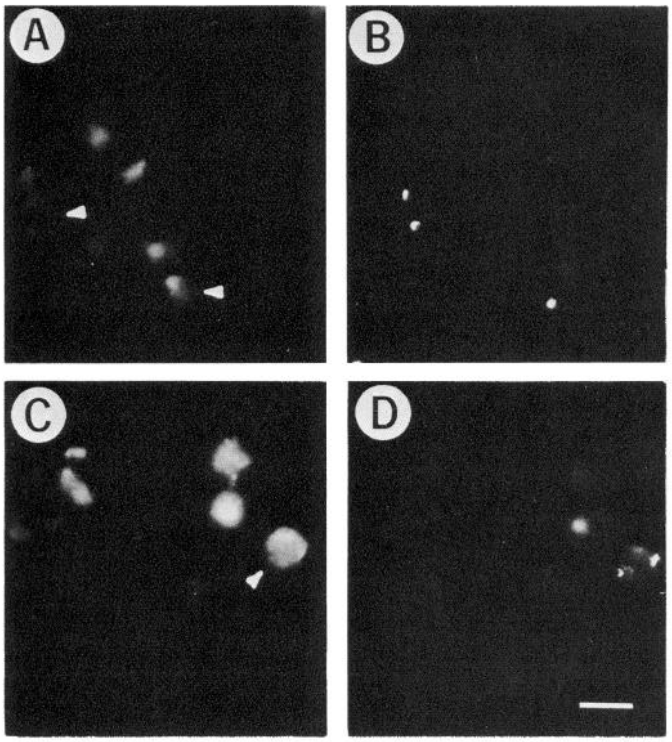


Figure 16. Clearance of microspheres by migrating inflammatory cells. Photomicrographs show DiI-ac-LDL(+) mononuclear phagocytes (*arrows, A* and *C*) which contain fluorescently labeled microspheres (*B* and *D*). Groups of these cells collected at the surface of the brain beyond 5 d after injury. Scale bar, 20 μm.

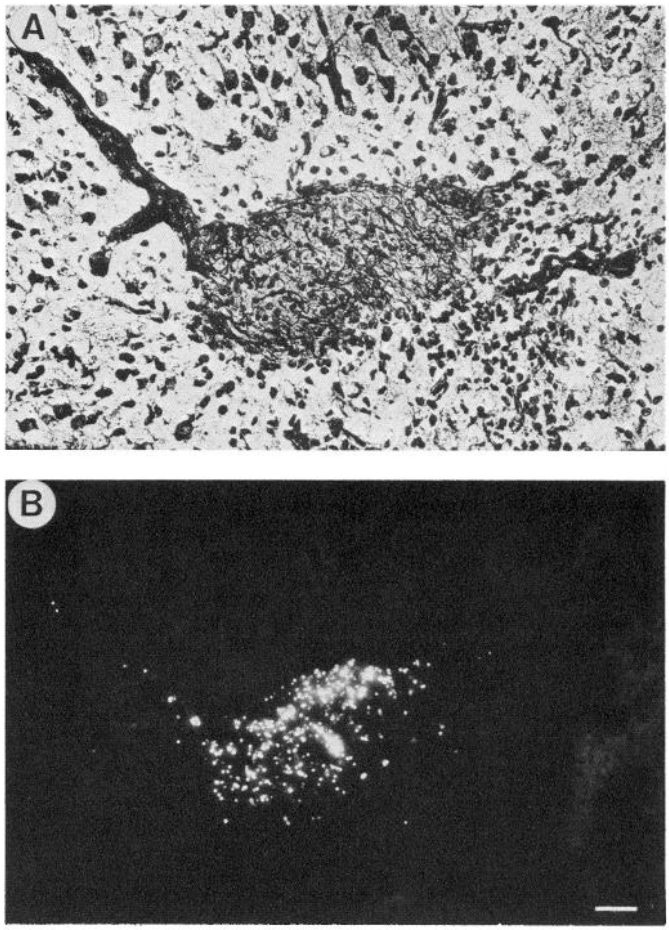
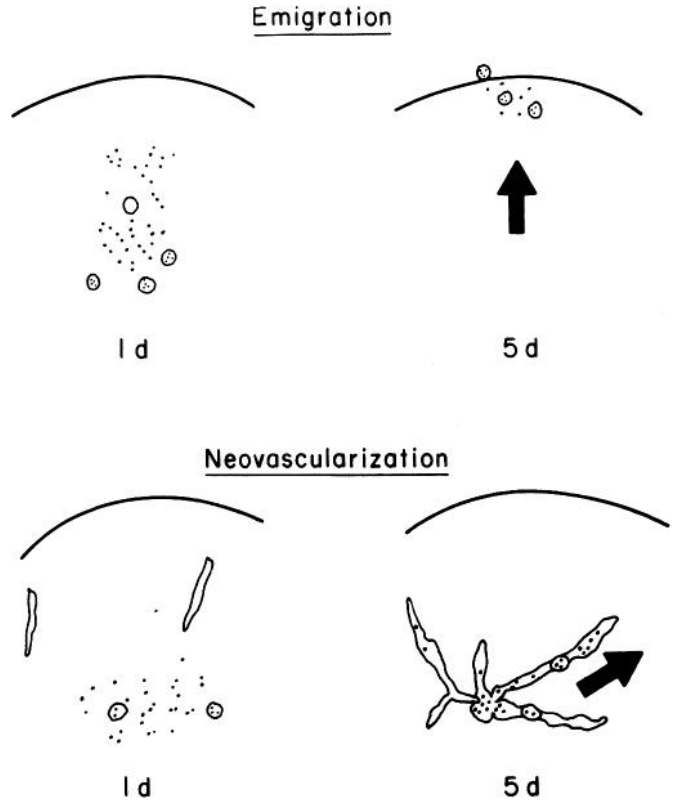


Figure 17. Wound neovascularization and clearance of microspheres. Microspheres were injected 1.8 mm into a wound site located in the cerebral cortex. This photomicrograph obtained from an animal at 10 d after injury shows a reticulum-stained vascular network deep in the brain (*A*) which contains a dense collection of microspheres (*B*). These particles were originally distributed throughout the neighboring neuropil.



injury, reduced clearance of debris, reduced trauma-induced astrogliosis, and reduced trauma-induced neovascularization. It is important to note that chloroquine or dexamethasone each has a wide range of pharmacologic effects upon cells of the brain (Norris et al., 1977; MacKenzie, 1983; Wildfeuer, 1983), while colchicine is thought to retard phagocytosis and chemotaxis by binding to cytoskeleton proteins (Ahn and Harrington, 1980). We were surprised to find that dexamethasone, long used in clinical neurology for treatment of brain inflammation (Anderson and Cranford, 1979; Norris and Hachinski, 1986), had little apparent effect upon blood monocytes or brain mononuclear phagocytes *in vivo*. Furthermore, dexamethasone did not influence astrogliosis or neovascularization at sites of brain injury. Our observations cast doubt upon the usefulness of glucocor-

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Figure 18. Diagram illustrating 2 mechanisms for debris clearance. Most phagocytic cells collect debris and migrate to surface of the brain. This clearance process was noted particularly after superficial infusion of particles. When placed more deeply into the cerebral cortex, particles were also found in newly formed vascular networks.

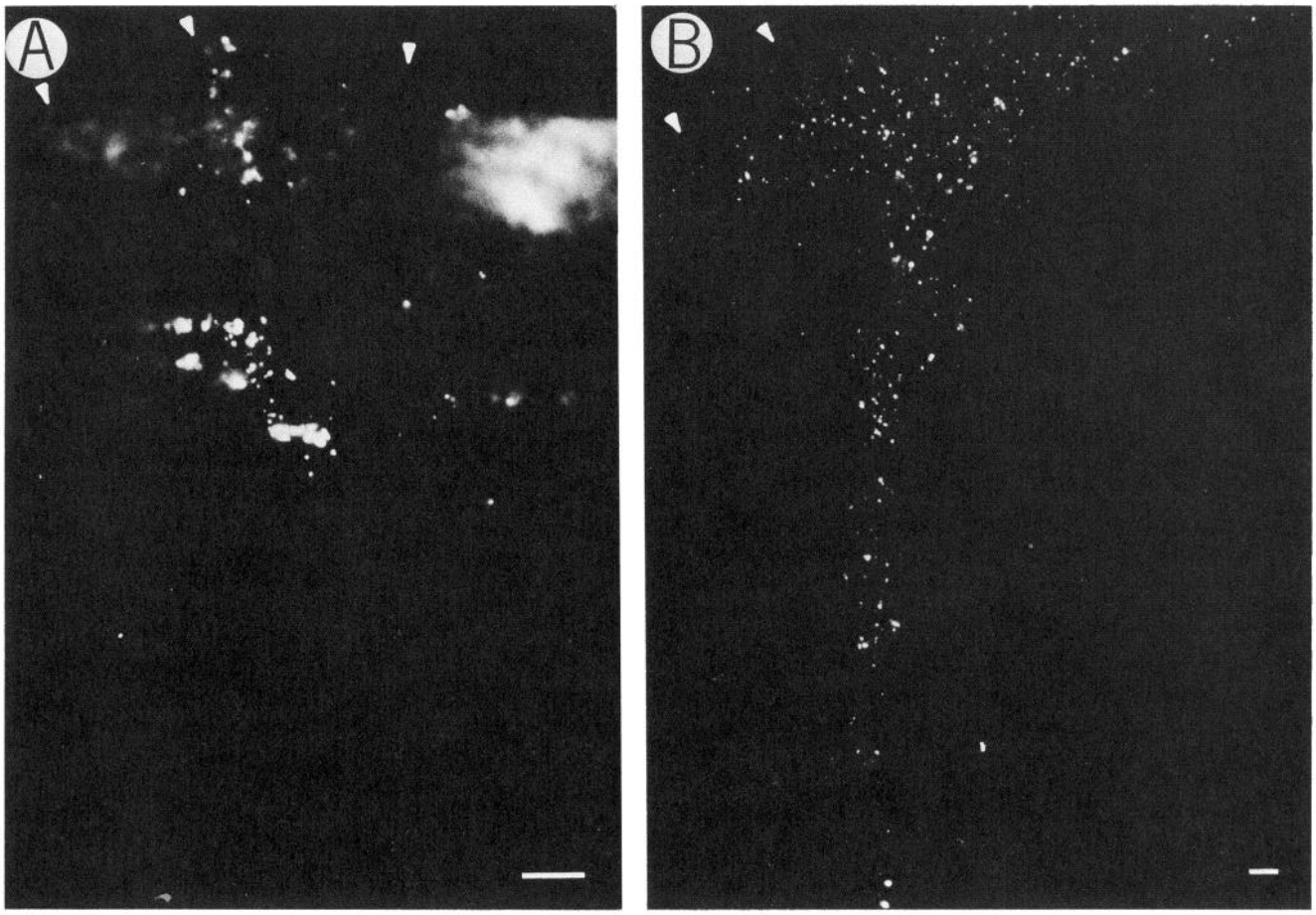

Figure 19. Chloroquine effects upon the pattern of microsphere clearance. Fluorescence photomicrographs of coronal brain sections showing microsphere clearance from wound site 10 d after injury. *A,* Control animal has few microspheres most of which are in clusters near the surface of the brain. These particle collections are within mononuclear phagocytes moving towards the meninges. *B,* Chloroquine treatment retards microsphere clearance. As shown here, more particles compared with control animals are present evenly distributed throughout the wound site. This pattern of microsphere distribution resembles that seen during the first few days after injury in control animals. *Arrows* delineate the surface of the brain. Scale bar, 20 μm.

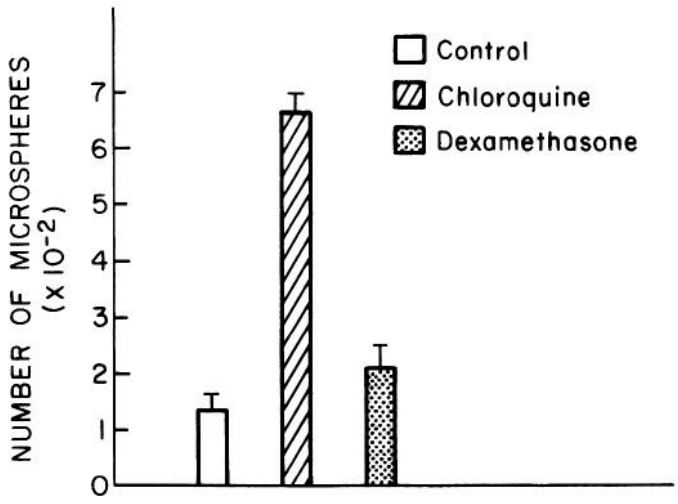

Figure 20. Microsphere clearance after treatment with chloroquine or dexamethasone. Fifteen days after injury, brains were cut serially, and the total number of fluorescently labeled microspheres was determined for each animal. Data, expressed as mean number of microspheres per animal, were obtained from at least 5 animals per group. Statistical analyses by Student's *t*-test shows that chloroquine significantly retarded particle clearance ($p < 0.0001$). Dexamethasone did not influence microsphere removal.

ticoids in the treatment of inflammatory responses associated with acute brain trauma.

We conclude that mononuclear phagocytes help to mediate tissue reactions to brain injury. Manipulation of CNS inflammatory responses retards histological changes that normally occur after trauma. Although the pattern of wound healing in the traumatized brain is altered by drug suppression, it is not known if such suppression will improve recovery of neurological function. Recently, we have found that chloroquine and colchicine promote neuronal survival and recovery of motor function after ischemic stroke (Giulian and Robertson, 1989). Perhaps inhibition of brain mononuclear phagocytes limits the extent of neural tissue damage and may therefore offer a new approach in the treatment of neurological diseases.

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