Neutrophil Depletion Reduces Blood-Brain Barrier Breakdown, Axon Injury, and Inflammation After Intracerebral Hemorrhage

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Abstract
Neutrophils are thought to contribute to damage after intracerebral hemorrhage (ICH), but there is little direct evidence for this. We depleted circulating blood neutrophils with an anti-polymorphonuclear leukocyte antibody (anti-PMN) before inducing ICH in the rat striatum. Neutrophil infiltration, which was mainly at the edge of the hematoma, was decreased by more than 60% by anti-PMN mediated depletion. We then analyzed neutrophil contributions to BBB breakdown, white matter damage (axons and myelin), and glial and inflammatory responses, both spatially and temporally. Neutrophil depletion reduced BBB leakiness in the peri-hematoma region. Matrix metalloproteinase 9, which is thought to contribute to BBB breakdown, was restricted to neutrophils after ICH and was thus reduced by neutrophil depletion. Early perihematomal axonal injury seen at 1 and 3 days after ICH was decreased by depleting neutrophils, and at later times (7 and 14 days), the astrocytic and microglia/macrophage responses were reduced in the perihematomata region and the surrounding striatum. Detailed spatial analysis showed that neutrophil depletion reduced infiltration of activated microglia/macrophages in the peri-hematoma white matter tracts and decreased myelin fragmentation and axon damage. These results show that, in experimental ICH, neutrophils produce matrix metalloproteinase 9 and contribute to blood vessel disruption, BBB breakdown, axon damage, and astrocytic and microglial/macrophage responses that evolve after ICH.

Key Words: Axonal injury, Blood-brain barrier breakdown, Experimental intracerebral hemorrhage, Glial scar, Matrix metalloproteinase 9, Microglia/macrophage activation, Myelin damage, Neutrophils.

INTRODUCTION
Intracerebral hemorrhage (ICH) is a devastating form of stroke that affects more than 2 million people per year (1). Although ICH is less prevalent than ischemic stroke, there is up to 40% mortality in the first month (2) and the prognosis for survivors is worse. There is a crucial need for more research on factors that initiate and propagate damage after ICH. One such process is the CNS inflammatory response, which correlates with deterioration and poor functional recovery in humans (3).

Inflammation after ICH involves infiltration of neutrophils and macrophages, activation of microglia and astrocytes, and consequent production of inflammatory mediators, including cytokines, reactive oxygen species, and matrix metalloproteases (MMPs) (4). After ICH, the hematoma becomes surrounded by a glial scar that is composed of activated microglia, macrophages, and reactive astrocytes. The role of the glial scar is still widely debated largely because these cells have the potential to secrete many mediators; some of these can exacerbate damage and others promote resolution of the injury (5–9). Part of the difficulty in determining roles of inflammation after ICH is the complexity and choice of outcomes for analysis. Most experimental ICH studies have focused on neuron death; however, it is increasingly evident that blood-brain barrier (BBB) breakdown and edema are key determinants of the functional outcome. In addition, more research is needed into the pathophysiology of white matter (WM) damage after ICH and after ischemic stroke (10–12). We and others have begun to analyze the relationship between inflammation, BBB breakdown, and progressive WM damage over the course of several days after experimental ICH (13–15).

In animal models of ICH, extracellular matrix degradation and BBB breakdown by MMPs can exacerbate injury (16, 17), and in humans, MMP-9 protein levels in brain and blood correlate with the severity of injury (18–21). Consequently, numerous experimental ICH studies have examined MMP-9 production (4, 22–24). Neutrophils are one of the richest cell sources of MMP-9 (25), and although they are expected to exacerbate damage, specific contributions of neutrophils to ICH pathology are largely unknown. We previously found that the antibiotic, minocycline, reduced neutrophil infiltration, BBB breakdown, and edema after ICH (26). Because minocycline has complex actions, including direct effects on microglia (27) and neurons (28), these outcomes cannot simply be ascribed to decreased neutrophil entry. Here, we have assessed the specific contribution of neutrophils by depleting them from the blood before inducing ICH.

MATERIALS AND METHODS
Intracerebral Hemorrhage With and Without Neutrophil Depletion
All procedures were approved by the University Health Network animal care committee in accordance with guidelines established by the Canadian Council on Animal Care. Adult (350–450 g; 3–4 months old, n = 36) male Sprague-Dawley
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Rats were anesthetized using isoflurane (3% induction and 1.5% maintenance) and placed in a small-animal stereotoxic instrument (David Kopf Instruments, Tujunga, CA). Under aseptic conditions, skin coverings were cleared and a 1-mm-diameter burr hole was drilled in the skull (0.1 mm anterior and 3 mm lateral to bregma), and a 30-gauge needle was then lowered into the right caudate putamen (6 mm ventral to the skull surface).

Intracerebral hemorrhage was induced in the striatum using a well-established method (29) that we have extensively used (30, 31). A micropump (Micro4; World Precision Instruments, Sarasota, FL) delivered 0.2 U of bacterial type IV collagenase (Sigma-Aldrich, Oakville, Canada) in 0.5 μL of saline at a rate of 250 nL/min, after which the needle was left in place for 5 minutes to prevent solution reflux. The core body temperature was maintained at 36.5°C using an electric heating pad throughout surgery and recovery. Animals regained consciousness within 10 minutes. As expected, all animals displayed an ipsilateral turning bias within hours of ICH onset. Their ability to eat, drink, and clean themselves was not impaired, and no rats died as a result of surgery or ICH induction. We strove to use the minimum number of animals while allowing statistical analysis of a large number of parameters.

Neutrophil depletion was achieved by intravenous tail vein injection of 0.8 mL of polyclonal rabbit anti-rat polymorphonuclear neutrophil (anti-PMN) serum (Cedarlane Labs, Burlington, Canada) 24 hours before ICH induction. This technique has been widely used to delineate the role of neutrophils in both peripheral damage and CNS injuries.

Animals were divided into untreated (n = 12) and anti-PMN–treated groups (n = 24) and killed at 1, 3, 7, or 14 days after ICH using an overdose of the anesthetic isoflurane (untreated controls, 3 per time point; neutrophil depleted, 6 per time point). The extent of neutrophil depletion was determined for each animal using an automated blood cell counter (Hemavet model 950FS; Drew Scientific, Oxford, CT). To determine baseline circulating blood cell numbers, 100 μL of blood was drawn from the saphenous vein of each animal immediately before anti-PMN serum injection. At 24 hours after anti-PMN serum injection, another 100 μL of blood was drawn to assess changes in circulating blood values. For untreated control animals, baseline cell counts were made, but no anti-PMN serum was injected before the induction of ICH.

Tissue Preparation and Immunohistochemistry

Animals were perfused with 4% paraformaldehyde (EMD Biosciences, Gibbstown, NJ) and 2% sucrose in PBS (pH 7.5). The isolated brains were stored in this fixative at 4°C for 24 hours, then in 10% sucrose for 24 hours, and finally in 30% sucrose until they were sectioned (~48 hours). Coronal slices were made at 3, 6, and 9 mm from the anterior end of the brain. Frozen brain sections (16 μm thick) were made using a cryostat (Model CM350S; Leica, Richmond Hill, Canada) and stored at ~40°C until used.

Immunohistochemistry was used to assess the spatial and temporal evolution of inflammation, neuron loss, WM damage, and BBB breakdown. Individual brain sections were circled with a PAP pen and then incubated (overnight at 4°C) in PBS containing 3% normal donkey serum, 0.3% Triton X-100, and the appropriate primary antibody, as follows. Neutrophils were labeled with rabbit polyclonal anti-myeloperoxidase (MPO, 1:200; Dako-Canada, Mississauga, Canada). Myelin basic protein (MBP) was labeled with a mouse monoclonal anti-MBP antibody (1:100; Sigma-Aldrich), and regions of myelin damage or demyelination were identified with a rabbit polyclonal antibody that recognizes degraded MBP (dMBP, 1:250; Chemicon, Temecula, CA). Axons were labeled with a mouse monoclonal antibody against neurofilament 200 (NF200, 1:1000; Sigma-Aldrich). Accumulation of amyloid precursor protein (APP; a marker of axonal damage) was detected with rabbit polyclonal anti-APP (1:250; Zymed, San Francisco, CA), which does not recognize other amyloid forms (e.g. Aβ). Microglia and macrophages were labeled with rabbit polyclonal anti-ionized calcium-binding adapter 1 (Iba1, 1:1000; Wako, Chuo-ku, Osaka, Japan), and a mouse monoclonal anti-ED1 (1:100; Serotec, Raleigh, NC) was used to label CD68 in the lysosomes of activated phagocytic microglia/macrophages. (We use this collective term when rounded-up activated microglia cannot be distinguished from blood-derived macrophages.) Astrocytes were selectively labeled with a mouse monoclonal antibody against glial fibrillary acidic protein (GFAP, 1:500; Sigma-Aldrich). Neurons were labeled with a rabbit polyclonal antibody against collagen type IV (CgIV; 1:250; Abcam, Cambridge, MA). Microvessels were labeled with a rabbit polyclonal antibody against collagen type IV (CgIV: 1:250; Abcam, Cambridge, MA), a major constituent of the basal lamina in cerebral blood vessels (32). Extravasation of immunoglobulin G (IgG) was detected with a fluorescein isothiocyanate–conjugated monoclonal anti-IgG antibody (1:100; Abbiotec, San Diego, CA). To determine the location and cellular source of MMP-9, double labeling was done with one of several cell markers and an antibody against MMP-9 (1:10; R&D, Minneapolis, MN). For secondary antibody labeling, the sections were washed in PBS (3×, 10 minutes each) and then incubated (2 hours, room temperature) in PBS containing 3% normal donkey serum, 0.3% Triton X-100, and both a DyLight A594-conjugated donkey anti-rabbit antibody (red) and a DyLight A488-conjugated donkey anti-mouse antibody (green) (Jackson Immunoresearch, West Grove, PA). The sections were washed in PBS (3×, 10 minutes each), then overlapped using Vectashield with 4’-6-diamidino-2-phenylindole (Vector Labs, Burlington, Canada) and examined with a confocal fluorescence microscope (LSM 510 META; Zeiss, Oberkochen, Germany).

Quantifying Damage Parameters After ICH

To ensure consistency of staining intensities for quantification, for a given antibody all immunohistochemistry was carried out on the same day, with the same reagents, and all images were captured using the same pinhole size, intensity, and contrast settings and with short exposure times to avoid fading. Cell counts, staining areas, and staining intensities were determined in 1 coronal section per animal, with 4 sampling boxes each, averaged in each of 3 regions (hematoma core, perihematoma, surrounding striatum), unless otherwise stated. We delineated the hematoma boundary from an adjacent serial section stained for either MBP or GFAP, and the presence of blood readily identified the hematoma. These sample sites are illustrated by the colored boxes in Figure 9A, as follows: For sampling inside the core, one side of each box was
aligned with the hematoma boundary (white boxes). Perihematoma sample sites were entirely outside the hematoma, with one side of each box aligned with the hematoma boundary (black boxes). When sampling the surrounding ipsilateral striatum further from the lesion, each box neither aligned with nor touched the hematoma (empty boxes). When appropriate for a given stain, data were then compared with the average of 4 sample boxes from the undamaged contralateral striatum of the same animal. All sampling boxes were 369 × 369 μm² unless otherwise stated. For all measurements of staining area, the background fluorescence was subtracted, and the fraction of stained area was calculated by ImageJ, version 1.33k (National Institutes of Health, Bethesda, MD). The sampling regions and sizes differed depending on the damage parameter being assessed.

Infiltrating neutrophils (MPO-labeled) were counted at 1 and 3 days after ICH by an observer who was blinded to the treatment conditions. Densities were determined within sampling boxes that straddled the hematoma edge. Blood vessel integrity, axon damage, and astrocytic reactions were monitored in the perihematoma at 1, 3, 7, and 14 days after ICH. Axon damage was quantified as the area of APP staining. Microvessel breakdown was seen as a loss of CgIV staining; both vessel numbers and CgIV stained areas were compared with the contralateral striatum. Vessel leakiness was monitored from the area of extravasated IgG and as the number of IgG-positive vessels in sections double labeled with CgIV. Because IgG and APP staining were not detected in the contralateral striatum, these comparisons were precluded. To account for both astrocyte accumulation and increases in GFAP expression in reactive astrocytes, both the areas and intensities of staining in the perihematoma were compared with the contralateral striatum. Staining was quantified by subtracting the background fluorescence, converting the images to a grayscale, and then calculating the mean gray value using ImageJ software. Astrocyte cell counting was not feasible owing to their complex morphology. Accumulation of microglia and macrophages was quantified as the area of Iba1 staining in each region (hematoma core, perihematoma, surrounding striatum) at 1, 3, 7, and 14 days after ICH and compared with microglia in the contralateral striatum. Individual cell counting within the glial scar was not feasible because such large numbers of microglia/macrophages accumulate. Activated phagocytic microglia/macrophages were labeled with anti-ED1 and quantified at 3, 7, and 14 days in the perihematoma and surrounding striatum (ED-1 was not detected at Day 1). In the core, all microglia/macrophages became phagocytic and ED1-labeled. ED1 staining was not detected in the contralateral striatum at any time.

**Statistical Analysis**

For analyses of cell densities or staining areas, mean values within a region (core, perihematoma, surrounding region in the ipsilateral striatum; contralateral striatum) were first determined for the 4 sampling boxes indicated for each stain. The summarized data for the number of animals indicated are presented as mean ± SEM. Statistical differences were determined by the unpaired 2-tailed Student t test (Fig. 1A), by 1-way analysis of variance (ANOVA; Fig. 1B), or by 2-way ANOVA (Figs. 1D, 4, 5C, 7C, 9B–F), when assessing 2 independent variables (time and location). All ANOVAs were followed by the Bonferroni correction, and differences were considered to be statistically significant if p < 0.05.

The damaged ipsilateral striatum was compared with the undamaged contralateral striatum for microvessel integrity, astrocyte reactivity, and activated microglia/macrophages. Before doing this, we compared the contralateral striata of control (n = 12) and anti-PMN–treated (n = 24) animals, and because they did not differ, the mean values for all 36 animals are shown as dashed lines on the graphs.

**RESULTS**

**Anti-PMN Treatment Reduces Circulating and Infiltrating Neutrophils**

First, blood was drawn from all 36 animals to determine baseline circulating cell counts. Then, anti-PMN antiserum was injected into the treatment group animals and blood was subsequently drawn from all treated animals to quantify the depletion of circulating blood neutrophils in the treatment group. Anti-PMN treatment reduced blood neutrophils by ~87% at 24 hours (from 4.0 ± 0.3 to 0.5 ± 0.1 × 10³ cells/μL; Fig. 1A). As expected, neutrophil depletion was transient, with evidence of repopulation by 48 hours (Fig. 1B). Intracerebral hemorrhage was then induced in all animals, and they were then killed after 1, 3, 7, or 14 days. We and others have shown an early and prominent infiltration of neutrophils in this ICH model (30, 33, 34). After anti-PMN treatment, the neutrophil density at the edge of the hematoma was decreased by 66% on Day 1 (from 367 ± 31 to 126 ± 13 cells/mm²) and by 59% on Day 3 (from 501 ± 18 to 204 ± 26 cells/mm²) (Figs. 1C, D).

**Neutrophil Depletion Reduces MMP-9 and BBB Breakdown**

On Days 1 and 3 after ICH, control rats had substantial MMP-9 immunostaining, mainly at the edge of the hematoma (Fig. 2), but also in the core and occasionally in the surrounding striatum. Essentially all of the MMP-9-labeled cells also expressed MPO, indicating that infiltrating neutrophils were the source of MMP-9. Not all MPO-positive cells were MMP-9-positive, but we do not think this represents MPO expression in cells other than neutrophils. MPO can be expressed in other phagocytes, but its level in activated microglia/macrophages was apparently below our immunostaining detection limits. That is, we did not see MPO staining in highly activated Iba1 or ED1-stained microglia/macrophages, and MPO staining was no longer detected at 7 and 14 days, times when large numbers of highly activated microglia/macrophages were present at the hematoma edge. As expected for MMP-9 restriction to neutrophils, at later times (Days 7 and 14, not shown), it was no longer detected in the damaged striatum, and no MMP-9 staining was detected in the contralateral striatum at any time examined. Because anti-PMN treatment reduced the number of neutrophils (Fig. 1D), it is not surprising that MMP-9 staining was reduced.

Matrix metalloprotease 9 upregulation has been correlated with BBB disruption after ICH (24, 35, 36). As in our earlier study (26), we assessed the physical integrity of the BBB by examining disruption of the basal lamina component,
CgIV, and extravasation of the plasma protein IgG (Fig. 3). Quantification of all time points is shown in Figure 4. Immunoglobulin G staining was not detected in the contralateral striatum (Fig. 3A) at any time examined. On Day 1 (Fig. 3B) and Day 3 (Fig. 3C), IgG was present both inside and outside damaged microvessels in the hematoma and perihematoma. Very little IgG remained at Day 7 and it was undetectable by Day 14 (not illustrated). Neutrophil-depleted animals had less IgG staining in the perihematoma than control animals (Figs. 3 and 4A). The density of IgG-positive microvessels was reduced by 64% on Day 1 (from 66 ± 4 to 24 ± 3/mm²) and by 64% on Day 3 (from 103 ± 17 to 37 ± 13/mm²). The area of staining was reduced by 91% on Day 1 (from 2.3% ± 0.2% to 0.2% ± 0.05% of area) and 78% on Day 3 (from 3.6% ± 0.5% to 0.8% ± 0.3% of area; Fig. 4A). Compared with the undamaged contralateral striatum (Fig. 3A), the core and perihematoma had fewer intact CgIV-stained microvessels (Figs. 3B, C) at all times examined. Microvessel density in the peri-hematoma (184 ± 25/mm²) was 56% of the contralateral value (331 ± 5/mm²) on Day 1 and 86% on Day 14 (285 ± 33/mm²; Fig. 4B). This apparent recovery of vessel density corresponds with the reduced hematoma size, edema, and ventricular expansion seen at these later times (37). Despite reduced vessel density, the proportional area of CgIV staining increased (Fig. 4B) owing to an increase in girth of the surviving vessels. This change in microvessel architecture was most evident on Days 1 and 3, whereas by Day 14, the morphology was similar to that in the contralateral striatum. Neutrophil depletion had no effect on the density of surviving microvessels or area of CgIV staining (Fig. 4B).

Neutrophil Depletion Reduces WM Damage

White matter damage was assessed by examining the integrity of axons and their myelin sheaths. In healthy CNS neurons, APP is present throughout axons, but fast anterograde transport maintains a low concentration that is difficult to detect by immunohistochemistry. Amyloid precursor protein accumulates when axon transport is compromised (38, 39) and is particularly useful because it detects more subtle damage than routine histopathologic stains (40). We did not detect APP staining in the striatum of healthy saline-injected animals or in the contralateral striatum at any time examined after ICH induction. In contrast, in the perihematoma, APP staining was detected as early as Day 1 (Fig. 5A), and by Day 3, it extended into the surrounding striatum.

**FIGURE 1.** Anti-polymorphonuclear leukocyte antibody (anti-PMN) treatment reduces circulating and infiltrating neutrophils. Values reported as mean ± SEM. (A) Blood neutrophils were counted before (“pre”) and 24 hours after (“post”) intravenous injection of anti-PMN serum. *** p < 0.001; n = 24, by unpaired 2-tailed Student t test. (B) Repopulation of circulating neutrophils after anti-PMN treatment (n = 3); statistical differences determined by 1-way ANOVA and Bonferroni post hoc test (*, p < 0.05; **, p < 0.01). (C) Confocal images showing myeloperoxidase (MPO)-positive neutrophils at the edge of the hematoma in control and anti-PMN-treated animals on Days 1 and 3 after intracerebral hemorrhage (ICH) onset. Scale bars = 100 μm. (D) The density of infiltrating neutrophils (anti-MPO) was determined at the edge of the hematoma on Days 1 and 3 after ICH onset. Four 369 × 369-μm² sampling boxes from each animal were averaged for each group (controls, n = 12; anti-PMN, n = 24). Statistical differences (***, p < 0.001) were determined by 1-way ANOVA and Bonferroni post hoc test.
FIGURE 2. Neutrophil depletion reduces matrix metalloproteinase-9 (MMP-9) after intracerebral hemorrhage (ICH). Confocal images taken at the edge of the hematoma in control and anti-PMN–treated animals on Days 1 and 3 after ICH. Sections were labeled for neutrophils with myeloperoxidase (MPO; red), and MMP-9 (green). Scale bars = 100 μm.
FIGURE 3. Blood-brain barrier breakdown after intracerebral hemorrhage (ICH), with and without neutrophil depletion. (A–C) Extravasation of the plasma protein, immunoglobulin G is indicated with an anti-IgG antibody (IgG; green). Microvessels are labeled with an antibody against collagen type IV (CgIV; red). Scale bars = 100 μm. Images were taken from the contralateral striatum of a control animal on Day 1 (A) and from the edge of the hematoma (H) in control and anti–polymorphonuclear leukocyte antibody (anti-PMN)–treated animals on Days 1 (B) and 3 (C) after ICH. Arrows indicate examples of vessels that were counted when quantifying the number of IgG-positive microvessels.
In neutrophil-depleted animals, APP staining was reduced by 62% on Day 1 (from 2.9% to 1.1% of area) and by 53% on Day 3 (from 3.8% to 1.8% of area; Fig. 5C). Little or no APP was detected in the hematoma core in either treatment group at any times examined (Days 1 and 3 shown in Fig. 5A).

The progression of axon damage (APP accumulation) was compared with myelin damage using antibodies against normal MBP and dMBP at 1, 3, 7, and 14 days after ICH. This analysis was facilitated by the structure of the rat striatum, with myelinated tracts that extend in the rostral-caudal axis. With cryostat sections taken every 16 μm, the same tracts could be identified in adjacent serial sections and we could cross-correlate staining with multiple antibodies. To compare the locations of axon and myelin damage, a brain section from each animal was double labeled for APP and MBP, and the next serial section was double-labeled for MBP and dMBP. On Day 1 (not shown) and Day 3 (Fig. 6A), many WM tracts stained strongly for APP but were devoid of dMBP. Specifically, axon damage extended into the surrounding striatum on Day 3, whereas damaged myelin was restricted to the core and edge of the hematoma.

**FIGURE 4.** Neutrophil depletion reduces BBB breakdown after intracerebral hemorrhage (ICH). Immunoglobulin G (IgG) and collagen type IV (CgIV) staining in the perihematoma on Days 1, 3, 7, and 14 after ICH is compared between control (n=3 at each time point) and anti-polymorphonuclear leukocyte antibody (anti-PMN)–treated animals (n = 6 at each time point). Values are mean ± SEM of measurements from four 369 × 369 μm² sampling areas from each animal, averaged for each treatment group. (A) IgG staining is indicated as the density of IgG-labeled microvessels (left panel), and as a percentage of the total area examined (right panel). (B) CgIV staining was used to calculate the density of microvessels (left panel) and area fraction of vessel staining (right panel). The dashed lines indicate mean values for the contralateral striatum of all animals (n = 36). Statistical differences are based on 2-way ANOVA with Bonferroni post hoc tests, where “c” indicates difference from the contralateral striatum, and the asterisks indicate differences between control and neutrophil-depleted animals: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

FIGURE 5. Neutrophil depletion reduces early axon damage. Axon damage was monitored from accumulation of amyloid precursor protein (anti-APP antibody; red) and compared with the location of normal myelin basic protein (anti-MBP antibody; green). (A, B) Color-separated and merged confocal images of double-labeled sections at the edge of the hematoma (H) in control and anti-polymorphonuclear leukocyte antibody (anti-PMN)–treated animals on Days 1 and 3 after intracerebral hemorrhage (ICH). Scale bars = 100 μm. (C) Summary of changes in the area fraction of APP staining (expressed as percent of total area examined) in the perihematoma. Values are mean ± SEM from four 369 × 369 μm² sampling areas from the perihematoma of each animal and averaged for each group (control, n = 12; anti-PMN, n = 24). Statistical comparisons were made with 2-way ANOVA and Bonferroni post hoc tests: *, p < 0.05; **, p < 0.01.

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FIGURE 6. Relationship between axon and myelin damage. (A) Representative confocal image showing the location of axon damage (anti-APP antibody; red) and normal myelin basic protein (anti-MBP antibody; green) in a control animal on Day 3 after intracerebral hemorrhage (ICH). The adjacent serial section was double-labeled for normal MBP (green) and damaged MBP (anti-dMBP antibody; red). The same axon bundle is circled in the upper and lower panels. Arrows indicate axon bundles with strong APP labeling but very little dMBP. (B) Confocal images of sections double-labeled for normal MBP (green) and damaged MBP (red) in control and neutrophil-depleted (anti-PMN) animals on Days 1 and 3. The hematoma core (H) is to the left in each panel. Scale bars = 100 μm.
Neutrophil Depletion Reduces ICH Damage

A.

Day 3

control

anti-PMN

Day 7

control

anti-PMN

Day 14

control

anti-PMN

B.

contralateral

control

anti-PMN

C.

GFAP: peri-hematoma

Area fraction (% of total area)

3d 7d 14d

Mean gray value

3d 7d 14d

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Within the hematoma core, staining for normal MBP was fragmented and diffuse at Day 1 and no longer detectable on Day 3 (Fig. 6B). Over the same period, WM tracts in the core and edge of the hematoma contained damaged myelin (dMBP-positive), and these bundles had disintegrated by Day 7. No dMBP was detected in the undamaged contralateral striatum or in saline-injected animals. In both control and neutrophil-depleted rats, MBP staining became increasingly fragmented with time in the perihematoma and in the surrounding striatum; however, dMBP was restricted to the core and edge of the hematoma at all times examined. The architecture of WM tracts precluded accurate analysis of staining area or intensity in the perihematoma and in the surrounding striatum. That is, the tracts vary considerably in diameter throughout the striatum (increasing toward the posterior) and perihematomatric tracts shift position as the hematoma resolves during the 14-day time course. Therefore, the diameter of MBP-positive tracts in the damaged striatum no longer matches the contralateral side (our internal control), and this confounds area and intensity analysis.

**Neutrophil Depletion Reduces Inflammation and Alters Glial Scar Formation**

Intracerebral hemorrhage is followed by formation of a prominent glial scar consisting of reactive astrocytes, activated microglia, and macrophages (4, 30, 33, 34, 37). In this rat ICH model, an astrocytic boundary had developed at the edge of the hematoma by Day 3 (Fig. 7A). Within this glial scar, many astrocytes displayed a reactive phenotype with retracted processes and increased staining for GFAP (Fig. 7, inset marked a); GFAP staining was absent from the hematoma core. On Day 1 (not shown), astrocytes at the edge of the hematoma displayed a normal morphology like those in the contralateral striatum (Fig. 7B, inset marked b'), whereas those inside the hematoma appeared fragmented. Staining intensity of GFAP is a measure of astrocyte reactivity, and the proportional area of staining reflects the number of cells. As expected, both the area and intensity of GFAP staining increased in the perihematoma during the first week after ICH (Fig. 7C). In neutrophil-depleted animals, the glial scar was reduced as follows: the GFAP stained area was reduced by 27% on Day 7 (from 22% ± 2% to 16% ± 2% of area) and by 33% on Day 14 (from 21% ± 3% to 14% ± 2% of area), and staining intensity was reduced by 23% on Day 7 (from 2.6 ± 0.1 to 2.0 ± 0.1 mean pixel intensity) and by 24% on Day 14 (from 2.5 ± 0.2 to 1.9 ± 0.1 mean pixel intensity).

Activated microglia were broadly distributed in the perihematoma and in the surrounding striatum by Day 1 after ICH (not shown). When further activated, the initially ramified microglia retract their processes and become morphologically indistinguishable from macrophages (41). We use the term “activated microglia/macrophages” for these cells. An antibody against Iba-1 was used to label both microglia and macrophages. By Day 3, the time at which reactive astrocytes clearly delineated the hematoma (Fig. 7), a prominent band of Iba1-positive activated microglia/macrophages surrounded the hematoma (Fig. 8). At later times (Days 7 and 14), the core and edge of the hematoma became filled with Iba1-positive activated microglia/macrophages. We then monitored activated phagocytic microglia/macrophages with an antibody against the lysosomal protein ED1. As expected, ED1 was not detected in the contralateral striatum at any time examined, nor was it detected in any brain region on Day 1 after ICH; however, ED1 staining clearly increased in the core and perihematoma from Day 3 to Day 14 and in the surrounding striatum between Days 7 and 14 (Fig. 9).

The area occupied by Iba1-positive cells increased in the core and perihematoma and in the surrounding striatum. It was significantly higher than in the contralateral striatum at all times examined (Figs. 9, B–D). Neutrophil-depleted animals had less Iba1 staining in the perihematoma on Day 7 (reduced by 39%; from 4.1% ± 1% to 2.5% ± 0.4% of area), and although the mean was reduced 50% on Day 14 (from 2.2% ± 0.5% to 1.1% ± 0.2% of area), it did not reach statistical significance. Microglia/macrophage staining in the surrounding striatum was reduced by 52% on Day 7 (from 2.7% ± 0.4% to 1.3% ± 0.2% of area) and 53% on Day 14 (from 1.6% ± 0.2% to 0.75% ± 0.1% of area). Neutrophil depletion did not affect microglia/macrophage Iba1 staining in the core, and on Day 3, staining did not differ in any region. ED1 staining for activated phagocytic microglia/macrophages was more dramatically affected by neutrophil depletion (Figs. 9E, F). In the perihematoma, ED1 was reduced by 54% on Day 3 (from 2.6% ± 0.2% to 1.2% ± 0.1% of area), by 45% on Day 7 (from 3.1% ± 0.4% to 1.7% ± 0.2% of area), and by 52% on Day 14 (from 2.1% ± 0.4% to 1.0% ± 0.01% of area). In the surrounding striatum, ED1 was reduced by 58% on Day 7 (from 1.2% ± 0.1% to 0.5% ± 0.1% of area) and by 73% on Day 14 (from 1.1% ± 0.4% to 0.3% ± 0.03% of area).

**Neutrophil Depletion Alters the Relationship Between Inflammatory Cells and WM Damage**

We next investigated the temporal and spatial relationship between activated microglia/macrophages and the extensive perihematomatric axon damage. Brain sections were double labeled for Iba1 and ED1, and the adjacent serial section was double-labeled for ED1 and APP (Fig. 10). On Day 1, we did not detect ED1 staining; however, there was widespread axon...
FIGURE 8. Accumulation of activated microglia/macrophages after intracerebral hemorrhage (ICH). Confocal images of double-labeled sections from control and anti-PMN-treated animals on Days 3, 7, and 14 after ICH onset. Microglia and macrophages were labeled with an antibody against ionized calcium-binding adapter 1 (Iba1; red). Activated phagocytic microglia and macrophages were labeled with an antibody against the lysosomal marker, ED1 (green). The white dotted line shows the approximate position of the edge of the hematoma (H). Scale bars = 100 μm. Areas marked by the yellow boxes are shown at higher magnification (right panels) to illustrate the morphology of highly activated microglia/macrophages (panel 1) and ameboid microglia (panel 2) further from the hematoma. Note: The gain was adjusted to show the less brightly stained microglia further from the lesion; thus, staining of activated microglia/macrophages at the edge of the hematoma appears saturated.
FIGURE 9. Neutrophil depletion alters the microglia/macrophage response. (A) A section stained with Cresyl violet from Day 1 after intracerebral hemorrhage (ICH) shows a schematic of the 16 sampling boxes (to scale) used for analyzing the area of staining: 4 each in the core (C; white boxes), perihematoma (black boxes); surrounding striatum and undamaged contralateral striatum. Scale bar = 1 mm. (B-D) Temporal and spatial analysis of Iba1-labeled microglia/macrophages with and without neutrophil deletion (anti-PMN treatment). The area fraction is expressed as a percent of the total area examined. Iba1 staining was analyzed in the hematoma core (B), perihematoma (C), and surrounding striatum (D) and compared with the average value from the contralateral striatum (n = 36; dashed line). (E, F) Temporal and spatial analysis of ED1 staining; expressed as a percent of total area examined in the perihematoma (E) and in the surrounding striatum (F). Values are shown as mean ± SEM for four 369 × 369 µm² sampling boxes from each region for each animal and then averaged for each treatment group (control, n = 12; anti-PMN, n = 24). Statistical comparisons were made with 2-way ANOVA with Bonferroni post hoc tests. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
damage, as judged by APP accumulation (Fig. 5A). On Day 3, when axon damage was maximal, ED1-positive cells had infiltrated the APP-positive WM tracts at the edge of the hematoma. In contrast, in the surrounding striatum, many APP-positive WM tracts were devoid of ED1-positive cells. Thus, phagocytic microglia/macrophages selectively infiltrated APP-positive WM tracts at the edge of the hematoma but were excluded from bundles further from the hematoma. By Day 7, APP immunostaining was barely detectable and ED1-positive cells filled the space previously occupied by APP-positive WM tracts in both control and anti-PMN-treated rats (Fig. 10). Anti-PMN-treated rats had less extensive axonal damage and fewer ED1-positive cells within axon bundles in the perihematoma and in the surrounding striatum (not shown).

To further relate the presence of activated microglia/macrophages to the structural disintegration of WM tracts, brain sections were labeled for Iba1 and MBP, and the adjacent serial section was labeled for Iba1 and NF200 (axons; Fig. 11). Myelin basic protein and NF200 label different components of WM tracts; however, at low magnification, their staining pattern is very similar; thus, we could readily identify the same WM tracts in serial sections and compare the degree of myelin fragmentation and axon loss with microglia/macrophage infiltration. Figure 11 shows Day 7 after ICH, the time at which control animals had the greatest numbers of Iba1-positive cells in the perihematoma. In the contralateral striatum (Fig. 11A), note the structural integrity of the WM tracts (MBP-positive, NF200-positive),

FIGURE 10. Spatial and temporal correlation of microglia/macrophages with axon damage. Representative confocal images from control rats on Days 3 and 7 after intracerebral hemorrhage (ICH). Left, Activated microglia/macrophages were labeled for Iba1 (red) and the lysosomal protein, ED1 (green). Middle, The adjacent serial sections were labeled for ED1 (green) and amyloid precursor protein (APP; red). Right, Higher magnification images of the boxed regions. Scale bars = 100 μm (left, middle); 10 μm (right).
FIGURE 11. Detailed relationship between microglia/macrophages and white matter damage. (A–C) Representative confocal images on Day 7 after intracerebral hemorrhage (ICH) taken from the contralateral striatum of a control rat (A), from the perihematoma in the same animal (B), and from a neutrophil-depleted (anti-PMN-treated) rat (C). For clarity, the left panels show color-separated images, and panels to the right show merged images with Iba1 staining of microglia/macrophages (red). Within each panel, 1 brain section was labeled for normal myelin basic protein (MBP; green); the adjacent serial section was labeled for neurofilament 200 (NF200; green). Images are shown at lower magnification (scale bars = 100 μm), and the boxed regions are shown at higher magnification (scale bars = 10 μm).
the ramified morphology of the microglia (Iba1-positive) and their exclusion from the tracts. In the perihematoma of the control animal after ICH (Fig. 11B), MBP staining was recognizable as bundles; however, at higher magnification, the myelin was clearly fragmented. NF200 staining for axons was diffuse and not recognizable as bundles. In the merged images with Iba1 staining, the fragmented bundles were infiltrated with many activated microglia/macrophages. In neutrophil-depleted animals, there was a dramatic sparing of WM and a reduced microglia/macrophage response in the perihematoma (Fig. 11C). Note the striking difference in WM tracts compared with control animals. Bundles were clearly recognizable with NF200 staining of axons and MBP staining showed more structurally intact WM tracts, with less myelin fragmentation. Not only do the merged images with Iba1 show less infiltration of bundles by microglia/macrophages, but the infiltrating cells displayed a less activated morphology, with more amoeboid processes.

**DISCUSSION**

We show that anti-PMN treatment greatly reduced the number of circulating neutrophils and the number that infiltrated the striatum after ICH, with several salient outcomes: (i) MMP-9 expression, which was mainly in neutrophils at the edge of the hematoma after ICH, was greatly reduced. (ii) Neutrophil depletion reduced BBB breakdown at early times (Days 1 and 3) in the peri-hematoma; with less leakiness to proteins. (iii) Inflammation and glial scarring were reduced; there were lower densities of activated microglia/macrophages in the perihematoma (Days 3, 7, and 14) and in the surrounding striatum (Days 7 and 14) and fewer reactive astrocytes with high GFAP expression (Days 7 and 14). (iv) Axonal injury was reduced at early times (Days 1 and 3). (v) Detailed spatial analyses showed that activated phagocytic microglia/macrophages infiltrated the perihematomal WM tracts on Days 7 and 14, and their numbers correlated with myelin fragmentation and axon loss. Neutrophil depletion reduced the number of microglia/macrophages within the tracts in the perihematoma, which corresponded with more structurally intact myelin and less pronounced axon loss. Together, these results show that neutrophils are involved in MMP-9 production and BBB breakdown, in axon injury, and in the evolving glial and inflammatory responses after ICH.

After ICH, neutrophils rapidly enter the brain and then die within the first few days (4). Because these immune cells can produce MMPs, proinflammatory cytokines, and reactive oxygen species (42), they are widely regarded as capable of damaging neurons and compromising the BBB (24, 42–44). Direct evidence of their roles after ischemic stroke has been obtained by depleting circulating neutrophils, but this approach has not previously been applied to models of ICH. To deplete blood neutrophils, we intravenously injected an antibody specific for PMNs. The rats became clinically neutropenic, and the 97% reduction in blood neutrophils was comparable to previous studies using the same depletion method in other damage models (45–48). After ICH, neutrophils in the striatum were reduced by 59% to 66%, detected on Days 1 and 3 only, and were mainly restricted to the edge of the hematoma.

Breakdown of the BBB, which normally limits movement of fluid, macromolecules, and cells into the brain, contributes to vasogenic edema and the evolving damage after ICH (49). Neutrophils and MMPs have been widely implicated in BBB breakdown. After experimental ICH broad-spectrum MMP inhibitors reduced BBB disruption and edema (23, 50), and MMP-9 expression correlated with both outcomes (49). Using the collagenase model of ICH, we previously provided indirect evidence, based on rescue by minocycline treatment, for neutrophil involvement in BBB breakdown, microvessel degradation, protein leakiness, and edema (26). However, minocycline is a broad-spectrum anti-inflammatory drug that acts on several other cell types, including reducing the number of activated microglia/macrophages after ICH (30). Because these cells are spatially and temporally associated with CgIV breakdown and microvessel loss in the perihematoma and are a major source of MMP-12 after ICH (26, 51), effects of minocycline cannot be ascribed solely to neutrophils. The present study provides crucial evidence for the role of MMP-9–loaded neutrophils, which were spatially and temporally associated with BBB breakdown. Neutrophil depletion reduced microvessel leakiness in the perihematoma. Experimental differences preclude detailed comparisons with other studies. For example, decreased edema reported after ICH was induced by inserting a microballoon (without bleeding) and leukocytes and platelets were nonelectronically depleted by whole-body irradiation (52). In experimental studies of postischemic hemorrhagic transformation, neutrophil depletion had no effect in 1 report but reduced t-PA–induced ICH in another (47, 53).

On the basis of temporal and spatial correlations, our recent study of ischemic stroke implicated neutrophils as an early initiator of WM damage (54), but direct effects of neutrophil depletion on WM damage have not been assessed in models of ischemic or hemorrhagic stroke. Here, we assessed APP accumulation, which can occur with both reversible and irreversible axon damage (55), and NF200 staining to monitor loss of axons. Amyloid precursor protein–positive damaged axons surrounded the hematoma at Day 1 and extended into the surrounding striatum by Day 3. Axon loss (i.e. reduced NF200 staining) was observed by Day 7. Neutrophil depletion reduced axon damage, implicating neutrophils in this earliest observed aspect of WM injury after ICH.

Plasma proteins diffusing through the BBB have the potential to damage WM. We found that neutrophil depletion decreased IgG extravasation and WM injury. Fibrinogen deposition correlated with axon damage after spinal cord injury (56), and injections of plasma into cerebral WM evoked edema, proinflammatory cytokine gene expression, and WM damage (57). Proteins involved in blood clotting and lysis are apparently harmful to the brain; plasmin or thrombin injection evoked leukocyte infiltration, microglial activation, and cell apoptosis (58). In addition, the high metabolic demands of axons might make them especially vulnerable to transport defects, oxidative damage, and mechanical injury (59).

Axon damage can occur in the absence of demyelination (13, 54, 60, 61). Thus, the observed temporal and spatial distribution of myelin damage is interesting. Within the hematoma, decreased staining for normal MBP and increased
staining for dMBP began by Day 1 and were complete by Day 3. During the same time course, the perihematoma contained MBP-labeled WM tracts that were fragmented and also labeled for dMBP, but this WM damage never extended into the surrounding striatum. Neutrophil depletion did not affect the distribution or progression of myelin damage. The most likely explanation is that the regions with myelin damage were exposed to blood components, such as thrombin and hemoglobin degradation products. Although neutrophil depletion decreased reactive astrocytes around the hematoma at later times (Days 7 and 14), there remained a well-organized glial scar, with no apparent differences in its spatial/temporal organization capable of limiting diffusion of toxic substances into the surrounding striatum.

With the burgeoning interest in inflammation after experimental ICH (49), it has become clear that although neutrophil infiltration is one of the earliest inflammatory responses (4, 26), a prominent microglial/macrophage response develops during the first few days (30, 33, 34, 37). Neutrophils can produce inflammatory mediators that help recruit monocytes/macrophages (62), thus amplifying the inflammation. Although several studies have addressed neutrophil depletion in other CNS damage models, we found none addressing WM injury, astrocyte, or microglia/macrophage responses after ICH, and we found none concerning activated phagocytic ED1-positive microglia/macrophages in any model of CNS injury.

We found that neutrophil depletion reduced microglia/macrophage responses in the perihematoma and in the surrounding striatum, but our results suggest differing contributions of neutrophils and microglia/macrophages. In agreement with our previous study, axon damage occurred before microglia/macrophages infiltrated the WM tracts; hence, these cells apparently did not initiate the damage (13). Moreover, although neutrophil depletion reduced axon damage (Days 1 and 3), it did not alter the density of activated microglia/macrophages in the perihematoma or in the surrounding striatum at these early times. At later times (Days 7 and 14), myelin appeared fragmented, and axons were lost. These events correlated with infiltration of activated microglia/macrophages into the WM tracts and were reduced in neutrophil-depleted animals. The simplest explanation for reduced infiltration of ED1-positive microglia/macrophages into WM tracts in neutrophil-depleted animals is reduced axon damage decreasing the need for phagocytosis. Overall, early axon damage in the perihematoma and in the surrounding striatum might promote infiltration of activated microglia/macrophages at later times, leading to phagocytosis and axon loss. In contrast, all neurons and astrocytes died within the hematoma core; thus, it is not surprising that the density of phagocytic microglia/macrophages did not differ between control and neutrophil-depleted animals.

Intracerebral hemorrhage is a devastating injury that leads to BBB breakdown, edema, and damage to both gray matter and WM but lacks effective therapies beyond reducing the bleeding and edema. The evolving inflammatory response is correlated with deterioration and poor functional recovery, but too little is known about the roles of specific immune cells and their products in injury to gray matter or WM. This is apparently the first neutrophil depletion study in an animal model of ICH, and as such, it begins to directly address their roles.

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