Systemic Complement Depletion Diminishes Perihematomal Brain Edema in Rats

Guohua Xi, MD; Ya Hua, MD; Richard F. Keep, PhD; John G. Younger, MD; Julian T. Hoff, MD

Background and Purpose—The complement cascade is activated after experimental intracerebral hemorrhage (ICH). It remains unclear, however, whether depleting the complement system will improve injury resulting from ICH. This study investigated the effects of systemic complement depletion on brain edema formation after ICH.

Methods—Fifty-six pentobarbital-anesthetized Sprague-Dawley rats were used. Treatment animals were complement-depleted with cobra venom factor (CVF) (intraperitoneally). Control rats received an equal volume of saline injection (intraperitoneally). In both treatment and control rats, autologous blood (100 μL) was infused stereotaxically into the right basal ganglia. Rats were killed 2, 24, or 72 hours later for brain water, ion, and tumor necrosis factor-α (TNF-α) measurements, for Western blot analysis, and for immunohistochemical studies. Brain edema was quantitated by wet/dry weight. TNF-α levels were measured by enzyme-linked immunosorbent assay. Western blot analysis was applied for C9 semiquantification. Immunohistochemistry was used to detect complement C3d, C5a, C9, and myeloperoxidase.

Results—Perihematomal brain edema was reduced by systemic complement depletion at 24 hours (78.8±0.6% versus 81.5±0.8% in control, \( P<0.01 \)) and 72 hours (81.5±1.5% versus 83.6±0.9% in control, \( P<0.05 \)), while cerebellar water content was unaffected (78.2±0.3% versus 78.0±0.1%). Complement depletion reduced TNF-α production 2 hours after ICH. Immunocytochemistry showed that complement depletion significantly reduced perihematomal C9 deposition, C3d production, and the number of C5a- and myeloperoxidase-positive cells.

Conclusions—Complement depletion by CVF attenuates brain edema in ICH, indicating that complement activation plays an important role in ICH-induced brain edema. Preventing complement activation may be effective in the treatment of ICH. (Stroke. 2001;32:162-167.)

Key Words: brain edema ■ cerebral hemorrhage ■ complement ■ tumor necrosis factor ■ rats

Intracerebral hemorrhage (ICH) is a common and often fatal stroke subtype that produces severe neurological deficits in survivors with no effective treatment. Edema formation after ICH contributes to these outcomes.1 Erythrocyte lysis is a potential cause of delayed edema formation,2 and complement activation and membrane attack complex (MAC) may play a role in clot lysis. Recently, our studies have found that the complement cascade is activated after ICH and that N-acetylheparin, an inhibitor of complement activation, reduces ICH-related brain edema.3,4

The complement system is an important self-defense immune system. Functions of complement activation include attraction and activation of phagocytes, opsonization, phagocytosis, and lysis of cells and gram-negative bacteria. However, it may cause tissue injury if it is activated inappropriately. Complement activation and complement-mediated brain injury have been found in a variety of central nervous system diseases, including brain trauma, cerebral ischemia, and subarachnoid hemorrhage.5 Inhibition of complement activation by soluble recombinant complement receptor 1 suppresses inflammation and demyelination in experimental allergic encephalomyelitis,6 reduces neutrophil accumulation after traumatic brain injury,7 attenuates neutrophil recruitment, and reduces cerebral infarction volume in a middle cerebral artery occlusion/reperfusion model.8

Cobra venom factor (CVF) is a nontoxic glycoprotein that is purified from the cobra venom and has been cloned by Fritzinger et al.9 CVF binding factor B forms a stable C3/C5 convertase, which hydrolyzes complement components in an uncontrolled manner and causes complement depletion. Systemic depletion of complement by CVF attenuates experimental cerebral vasospasm after subarachnoid hemorrhage,10 reduces ischemic brain damage, and improves cerebral blood flow and somatosensory evoked potentials in a model of cerebral ischemia with reperfusion.11 In addition, systemic depletion of complement with CVF reduces the recruitment of inflammatory cells in experimental allergic encephalomyelitis,12 diminishes macrophage infiltration and activation during wallerian degeneration and axonal regeneration,13 and attenuates inflammation and demyelination in adoptive transfer experimental allergic neuritis.14

Received July 19, 2000; final revision received September 22, 2000; accepted September 28, 2000.
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162
The purpose of the present study was to investigate whether systemic complement depletion by CVF attenuates brain edema formation after ICH and whether it reduces the inflammatory response that follows ICH. We chose to measure brain water content, proinflammatory cytokine tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), the complement cascade activation indicator C3d, anaphylatoxin C5a, a key complement component for MAC formation C9, and the neutrophil recruitment marker myeloperoxidase (MPO) in the ICH model with and without CVF treatment.

Materials and Methods

Animal Preparation and Intracerebral Infusion

The protocols for these animal studies were approved by the University of Michigan Committee on the Use and Care of Animals. A total of 56 male Sprague-Dawley rats (weight, 300 to 400 g; Charles River Laboratories) anesthetized with pentobarbital (40 mg/kg IP) were used in the present study. Aseptic precautions were used in all surgical procedures. The right femoral artery was catheterized for continuous blood pressure monitoring and blood sampling. Blood was obtained from the catheter for analysis of pH, PaO\(_2\), PaCO\(_2\), hematocrit, and glucose and as the source for the intracerebral blood infusion. Body temperature was maintained at 37.5°C with the use of a feedback-controlled heating pad. The animals were positioned in a stereotaxic frame (Kopf Instrument), and a cranial burr hole (1 mm) was drilled on the right, at the coronal suture 4.0 mm lateral to the midline. Autologous blood was withdrawn from the right femoral artery and infused (100 \(\mu\)L) immediately into the right caudate nucleus through a 26-gauge needle at a rate of 10 \(\mu\)L/min with a microinfusion pump (Harvard Apparatus Inc). The coordinates were 0.2 mm anterior, 5.5 mm ventral, and 4.0 mm lateral to the bregma. After intracerebral infusion, the needle was removed, and the skin incision was closed with suture.

Experimental Groups

The experiments were divided into 6 parts. Each part consisted of a control group and a CVF-treated group. All rats received intracerebral 100-\(\mu\L\) autologous blood infusion and were killed at different time points for different purposes. In the first and second parts, rats (\(n=4\) to 5 per group) were killed 2, 24, or 72 hours later for brain water and ion contents. In the third and fourth parts, 4 groups of 2 to 3 animals each were investigated. They were killed 24 or 72 hours later for complement C3d, C5a, C9, and MPO immunohistochemistry. In the fifth part, rats (\(n=6\) per group) were killed 2 hours after ICH, and the brains were used for TNF-\(\alpha\) measurement. In the sixth part, brains were removed for C9 Western blot analysis.

Brain Water and Ion Contents Measurement

Animals were anesthetized and decapitated 2, 24, or 72 hours after ICH. The brains were removed, and a coronal brain slice 4 mm from the frontal pole was cut approximately 3 mm thick with a blade. The brain slice was divided into ipsilateral and contralateral cortex and ipsilateral and contralateral basal ganglia. The cerebellum was obtained to serve as control. The 5 brain samples were immediately weighed on an electronic analytical balance (model AE 100, Mettler Instrument Co) to obtain the wet weight. Brain samples were then dried in a gravity oven weight (Blue M. Electric Co) at 100°C for 24 hours to obtain the dry weight. The water content was determined as (Wet Weight−Dry Weight)/Wet Weight. The dehydrated samples were digested in 1 mL of 1 mol/L nitric acid for 1 week. The sodium and potassium contents of this solution were measured with the automatic flame photometer (model IL943, Instrumentation Laboratory Inc). Ion content was expressed in milliequivalents per kilogram of dehydrated brain tissue (mEq/kg dry wt).

Western Blotting Analysis

One or 3 days after ICH, rats were reanesthetized with pentobarbital (60 mg/kg IP) and then underwent transcardiac perfusion with saline before decapitation. A coronal brain slice was cut as described for water content measurements. Western blot analysis was performed as previously described. Briefly, 2 \(\mu\)g protein was run on 7.5% polyacrylamide gels with a 4% stacking gel (SDS-PAGE). The primary antibody (rabbit anti-C9 polyclonal antibody) was received as a gift from Dr P. Morgan, University of Wales. The second antibody, peroxidase-conjugated goat anti-rabbit antibody, was purchased from Amersham. The antigen-antibody complexes were visualized with the ECL chemiluminescence system (Amersham) and exposed to a Kodak X-OMAT film. The relative densities of C9 protein bands were analyzed with the NIH image program (NIH Image Version 1.61).

Immunohistochemistry

Our method for immunohistochemistry has been described previously. Briefly, rats were reanesthetized with pentobarbital (60 mg/kg IP) and were perfused with 4% paraformaldehyde in 0.1 mol/L PBS, pH 7.4. The brains were removed and kept in 4% paraformaldehyde for 6 hours, then immersed in 25% sucrose for 3 to 4 days at 4°C. The brains were embedded in OCT compound (Sakura Finetek U.S.A. Inc) and sectioned on a cryostat (18 \(\mu\)m thick). Sections were stained with the avidin-biotin-peroxidase complex method. The primary antibodies were rabbit anti-human C3d polyclonal antibody (DAKO), goat anti-rat C5a polyclonal antibody (gift from Dr P.A. Ward, University of Michigan), rabbit anti-rat C9 polyclonal antibody (gift from Dr P. Morgan, University of Wales), and rabbit anti-human MPO polyclonal antibody (DAKO). Normal rabbit IgG or goat IgG was used as a negative control.

Brain Tissue TNF-\(\alpha\) Concentration Measurement

Two hours after ICH, rats were reanesthetized with pentobarbital (60 mg/kg IP) and decapitated. Basal ganglia samples were taken as described for water content measurements. The tissues were diluted (40\% wt/vol) with 0.01 mol/L PBS, pH 7.4, containing the protease inhibitor cocktail (Roche), and homogenized. The homogenates were then centrifuged at 7500 \(g\) for 20 minutes at 4°C. TNF-\(\alpha\) in the supernatant was determined with the use of an enzyme-linked immunosorbent assay kit for rat TNF-\(\alpha\) (Endogen). Results were expressed as picograms per gram of brain tissue.

Complement Depletion and CH\(_{50}\) Measurement

Circulating complement was depleted with the use of CVF, as previously described. Briefly, whole venom (Naja naja atra, Sigma Chemical Co) was dialyzed for 24 hours against 40 mmol/L PBS, then fractionated by anion exchange chromatography on a diethylaminoethyl cellulose column (DE52, Whatman International). Animals were given intraperitoneal injections of 25 \(\mu\)L of CVF in 1.0 mL of saline 36, 24, and 12 hours before induction of ICH.

To confirm complement depletion in CVF-treated animals, total serum hemolytic activity was measured before injury by the CH\(_{50}\) technique. Serial dilutions of serum were incubated for 1 hour at 37°C with sheep erythrocytes (Colorado Serum Co) that had been sensitized with rabbit anti-sheep hemolysin (Colorado Serum Co). The reciprocal of the serum dilution that resulted in 50% hemolysis was recorded as the CH\(_{50}\) value. To assess the degree of depletion further, C3 titers were measured by double immunodiffusion with the use of rabbit anti-rat C3 IgG (United States Biochemical Corp).

Statistical Analysis

All data in this study are presented as mean±SD. Data were analyzed with Student’s \(t\) test. Significance levels were measured at \(P<0.05\).

Results

Physiological data were measured before intracerebral blood infusion. The mean values of blood pH, blood gases, mean
arterial blood pressure, hematocrit, and blood glucose were controlled within normal ranges (Table 1). In all animals treated with the CVF protocol, total serum hemolytic activity was undetectable (CH50, <20). Complement C3 titers were in each case \#1:160 (with \#1:640 being normal in our laboratory).

Two hours after ICH, clot weights were 31.2\pm3.8 mg in the control group and 32.0\pm4.6 mg in the CVF-treated group. Brain water contents in the ipsilateral basal ganglia increased slightly in both the control group (79.6\pm0.2%) and the CVF-treated group (79.9\pm0.6%). There were no significant differences between the 2 groups in clot weight or brain edema at this time point.

Complement depletion by CVF significantly reduced brain edema in the ipsilateral basal ganglia (78.8\pm0.6% versus 81.5\pm0.8% in control; \(P<0.01\)) 24 hours after ICH (Figure 1A). The reduction of water content was associated with reduction of sodium ion accumulation (243\pm68 versus 369\pm59 mEq/kg dry wt in control; \(P<0.001\); Figure 1B) and reduction of potassium ion loss (373\pm19 versus 317\pm24 mEq/kg dry wt in control; \(P<0.05\)). Complement-depleted rats also had less edema (81.5\pm1.5% versus 83.6\pm0.9% in control; \(P<0.05\); Figure 2) in the ipsilateral basal ganglia 72 hours after ICH, while cerebellar water content was unaffected (78.2\pm0.3% versus 78.0\pm0.1% in control; \(P>0.05\)).

C3d immunoreactivity, an indicator of complement cascade activation, was detected around the hematoma and in the clot 3 days after ICH in non–CVF-treated rats (Figure 3B). However, there were only a few C3d-immunoreactive particles found in the clot in complement-depleted animals (Figure 3A). Perihematomal complement C5a-positive cells were also detected after ICH in non–CVF-treated rats, with most of the C5a-positive cells appearing to be neutrophils (Figure 3F). In contrast, it was difficult to find C5a-positive cells around the hematoma in CVF-treated rats (Figure 3E).

Although Western blot analysis for complement C9 showed no differences between the CVF-treated and control groups in either the ipsilateral basal ganglia (3020\pm561 pixels versus 3427\pm880 pixels in control; \(P>0.05\)) or the ipsilateral cortex (2884\pm525 pixels versus 3700\pm945 pixels; \(P>0.05\)) 24 hours after ICH, significant complement C9 deposition was found on the neurons around the hematoma (Figure 3D) in non–CVF-treated animals but not in those treated with CVF (Figure 3C).

In control rats, perihematomal TNF-\(\alpha\) concentration peaked 2 hours after ICH and returned to baseline at 24 hours, where it remained for at least 7 days (G. Xi, MD, et al, unpublished data, 2000). In contrast, systemic complement depletion reduced perihematomal TNF-\(\alpha\) production at 2 hours after ICH (243\pm68 versus 471\pm237 pg/g in control; \(P<0.05\); Table 2).

### Table 1. Physiological Parameters

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control Group</th>
<th>CVF-Treated Group</th>
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<tbody>
<tr>
<td>MABP, mm Hg</td>
<td>111\pm8</td>
<td>113\pm11</td>
</tr>
<tr>
<td>pH</td>
<td>7.42\pm0.04</td>
<td>7.43\pm0.05</td>
</tr>
<tr>
<td>PaO2, mm Hg</td>
<td>80.0\pm3.7</td>
<td>80.2\pm6.8</td>
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<tr>
<td>PaCO2, mm Hg</td>
<td>45.3\pm4.8</td>
<td>44.6\pm4.9</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>41\pm2</td>
<td>41\pm3</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>6.9\pm1.1</td>
<td>6.7\pm1.0</td>
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Values are mean\(\pm\)SD; \(n=26\) rats in the control group and \(n=28\) rats in the CVF-treated group. MABP indicates mean arterial blood pressure.
In non–CVF-treated rats, MPO immunoreactivity was detected around the hematoma 24 hours after ICH. There was a marked increase of MPO immunoreactivity on the third day after ICH (Figure 4A). This increase of MPO immunoreactivity was blocked completely by treatment with CVF (Figure 4B).

**Discussion**

Our recent studies have demonstrated that the complement cascade is activated after ICH and that N-acetylheparin, an inhibitor of complement activation, reduces perihematomal brain edema.3,4 The present study demonstrates that systemic complement depletion by CVF also reduces perihematomal edema formation 1 or 3 days after ICH. The reduction of brain edema in CVF-treated rats is associated with significantly lower TNF-α levels, less C3d production, fewer C5a- and MPO-positive cells, and a marked decrease of C9 deposition on cell membranes around the hematoma. All these data suggest that complement activation after ICH is involved in brain edema formation through an inflammatory response or MAC formation. Inhibiting complement activation may provide a novel treatment for brain edema after ICH.

![Figure 3](https://example.com/fig3.png)

**Figure 3.** Complement C3d, C5a, and C9 immunoreactivity in the clot or around the hematoma 72 hours after intracerebral infusion of 100 μL blood. Shown are complement C3d-immunoreactive minute particles in the clot of CVF-treated (A) and control (B) rats; perihematomal complement C9 immunoreactivity in CVF-treated (C) and control (D) rats; and complement C5a immunoreactivity around hematoma in CVF-treated (E) and control (F) rats. Examples of positive cells are indicated by arrows. Bar=40 μm.

<table>
<thead>
<tr>
<th>Group</th>
<th>Contralateral</th>
<th>Ipsilateral</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>98 ± 39</td>
<td>471 ± 237</td>
</tr>
<tr>
<td>CVF-treated</td>
<td>135 ± 21</td>
<td>243 ± 68</td>
</tr>
<tr>
<td><em>P</em></td>
<td>&gt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
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Values are mean ± SD, expressed in picograms per gram; *n* = 6.

After ICH, the complement cascade is activated locally in or around the hematoma in our rat model.4 C3d is a segment of complement C3 and an indicator of complement activation.19 The absence of C3d around the hematoma in CVF-treated rats confirms that the complement system is depleted not only in the blood (C3 titers) but also in the brain.

Complement depletion significantly reduced edema formation at both 24 and 72 hours after ICH. It also attenuated the increase in brain sodium content and the loss of brain potassium normally associated with ICH-induced edema.2,20 These 2 time points were chosen because there are multiple causes for edema formation after ICH.2 For example, the clotting cascade has a major role in early edema formation,20–22 while erythrocyte lysis plays a role later.2 That complement depletion affects edema formation at both time points suggests that the complement cascade triggers multiple mechanisms.

Complement-mediated brain injury is probably related to the inflammatory response. Indeed, inflammation has been found after experimental ICH.23 After activation of the complement cascade, anaphylatoxins C3a and C5a are generated. C3a and C5a cause blood-brain barrier leakage by degranulating mast cells and leukocytes. In addition, both C3a and C5a stimulate the synthesis of TNF-α in inflammatory cells.24,25 C5a is also a potent chemoattractant for polymorphonuclear leukocytes and contributes to inflammatory cell injury.26 Inflammatory cells respond to very low concentrations of C5a (nanomolar) with chemotaxis and upregulation of adhesion molecules. Mulligan et al16,27 reported that an anti-rat C5a antibody blocked C5a-mediated...
upregulation of intercellular adhesion molecule-1 (ICAM-1) and P-selectin in 2 rat lung vascular injury models. Our group has shown that ICAM-1 is upregulated after ICH. The present results show that the numbers of MPO-positive cells and C5a-positive cells are dramatically reduced in CVF-treated animals. These results are supported by the report of Piddlesden et al., who found that complement depletion by CVF significantly reduces the inflammatory response and eliminates perivascular inflammatory cells in experimental allergic encephalomyelitis.

C5a may also contribute to changes in blood-brain barrier integrity by binding to receptors expressed on endothelial cells. Expression of the receptor for complement C5a is upregulated on reactive astrocytes, microglia, and endothelial cells in the inflamed human central nervous system. Moreover, direct injection of C5a into the rabbit subarachnoid space contributes to neuronal dysfunction, and C5a induces apoptosis in neuroblastoma cells in vitro.

TNF-α and interleukin-1 are 2 major proinflammatory cytokines that are elevated after many central nervous system diseases, such as cerebral ischemia and brain trauma. Activation of the complement system can stimulate microglia and macrophages to release TNF-α and interleukin-1. TNF-α and interleukin-1 thereby recruit neutrophils by stimulating endothelial cells to produce ICAM-1 and E-selectin. After tethering, rolling, and adhesion, neutrophils can migrate into the brain parenchyma, release proteases and oxidases, and cause secondary brain injury. Barone et al. reported that exogenous TNF-α exacerbates brain injury and that blocking TNF-α activity with anti–TNF-α monoclonal antibody or soluble TNF receptor I reduces infarct volume after middle cerebral artery occlusion in the rat. TNF-α itself also increases matrix metalloproteinases production and blood-brain barrier permeability.

MAC formation after complement activation may exacerbate ICH-induced brain injury. The MAC is a macromolecular complex that consists of C5b to C9, forms a transmembrane pore, and eventually causes cell lysis. Of interest, Czermak et al. found that MAC and C5a act synergistically to enhance chemokine generation. The reduction of brain edema by complement depletion after ICH may be accomplished, at least in part, through a reduction in MAC formation. In ICH, MAC may contribute to brain edema formation by inducing erythrocyte lysis and hemoglobin release. Hemoglobin is neurotoxic and can induce brain edema. Peterson et al. demonstrated that hemolysis of aged erythrocytes is inhibited by complement depletion. MAC may also directly insert into neurons, astrocytes, and endothelial cells, causing neuronal injury and blood-brain barrier disruption. Although our data have shown that complement depletion does not reduce C9 content (by Western blot analysis) around the hematoma significantly, it does result in less perihematomal C9 deposition on parenchymal cells around the hematoma. Cell membrane C9 deposition is an indicator of MAC formation. The reduction of C9 deposition in CVF-treated animals indicates that complement depletion by CVF does not deplete C9 but abolishes MAC formation in our ICH model. Interestingly, abolition of C9 deposition in the central nervous system after CVF treatment has also been reported in acute antibody-mediated demyelinating experimental allergic encephalomyelitis. In addition, Kilgore and coworkers found that the infarct size is significantly reduced in hearts from C6-deficient rabbits in their ischemia/reperfusion model and that hereditary C6-deficient rabbits are not able to form MAC. They also reported that N-acetylheparin, an inhibitor of complement activation, reduces MAC content and myocardial infarct volume in the myocardial ischemia/reperfusion model. We have shown the N-acetylheparin also reduces brain edema formation induced by ICH.

Our previous studies have shown that the coagulation cascade (and thrombin in particular) plays a major role in early brain edema formation after ICH. Intraparenchymal infusion of thrombin causes blood-brain barrier disruption and inflammation. There are a number of interactions between the complement cascade and the coagulation system. These interactions around the hematoma may regulate the final production of thrombin. For example, MAC assembly damages cell membranes, which increases tissue factor (factor III) activity. An increase of tissue factor activity can then enhance the extrinsic coagulation pathway to increase thrombin production. In addition, TNF-α can activate the coagulation cascade by activating tissue factor. However, the clot sizes were the same in the control and CVF-treated groups. This suggests that complement depletion by CVF does not affect clot formation after ICH directly; it may limit the amount of thrombin produced from prothrombin entry into the brain after blood-brain barrier breakdown by reducing tissue factor activity.

In summary, we have shown that complement depletion by CVF reduces brain edema after ICH. The reduction of brain edema is associated with inhibition of inflammatory response and inhibition of MAC-mediated erythrocyte lysis and brain injury. Further studies using either anti-C5a neutralizing antibody or C6-deficiency rats may clarify the precise mechanisms.
Acknowledgments

This study was supported by grants NS-17760 and NS-39866 from the National Institutes of Health.

References

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Stroke. 2001;32:162-167
doi: 10.1161/01.STR.32.1.162
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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