A. SPECIFIC AIMS

Brain edema plays an important role in the secondary brain injury following intracerebral hemorrhage (ICH). It is the long-term goal of our laboratory to identify brain edema formation mechanisms after ICH. Previous studies indicate that edema formation following ICH may involve several phases. These include a very early phase (first several hours) involving hydrostatic pressure, clot retraction, and transient ischemia around the clot, a second phase (first day) involving the clotting cascade and thrombin production and a third phase (about day 3 in the rat) involving erythrocyte lysis and hemoglobin induced toxicity. Because of the delay in onset, this third phase may be more amenable to therapeutic intervention by either altering erythrocyte lysis or limiting hemoglobin-induced toxicity. In this proposal, we will test the following hypotheses focusing on this third phase:

HYPOTHESIS 1. Erythrocyte lysis and hemoglobin release causes delayed brain edema formation following intracerebral hemorrhage by either reducing blood flow, increasing blood-brain barrier (BBB) permeability or by direct cytoxicity.
Specific Aim 1. The effect of an intraparenchymal infusion of autologous packed erythrocytes, lysed erythrocytes, and hemoglobin (oxy- and methemoglobin) will be tested. Regional cerebral blood flow (CBF), blood-brain barrier integrity, and brain edema will be measured at different time points.
HYPOTHESIS 2. Hemoglobin breakdown products play an important role in hemoglobin-induced brain edema; blocking or inducing heme oxygenase (HO) will modify brain edema formation.
Specific Aim 2a. Brain edema, cerebral blood flow and blood-brain barrier permeability will be measured after infusing hemoglobin breakdown products (heme, biliverdin, bilirubin and iron) into the parenchyma of brain.
Specific Aim 2b. To determine whether inhibition of heme oxygenase (HO) with tin protoporphyrin (SnPP), tin meso-porphyrin (SnMP) or zinc protoporphyrin (ZnPP) or induction of HO-1 activity with $SnCl_2$ modifies edema formation following intraparenchymal infusion of blood or blood components.
HYPOTHESIS 3. Erythrocyte lysis in the hematoma following ICH is due to complement activation and membrane attack complex (MAC) formation. Activation of the complement system will also exacerbate brain damage.
Specific Aim 3a. To determine whether inhibition of MAC complex formation prevents red blood cell lysis and neuronal injury after ICH.
Specific Aim 3b. To examine whether complement system activation exacerbates neuronal injury following an intraparenchymal injection of blood components (i.e. in the absence of the red blood cell lysis effect).
Specific Aim 3c. To determine whether complement activation exacerbates hemorrhagic brain injury by stimulating neutrophil influx into brain.

B. BACKGROUND AND SIGNIFICANCE

B1. Clinical Problem of Intracerebral Hemorrhage

Each year approximately 500,000 people suffer a stroke in our country. The causes of stroke are, in general, either hemorrhagic or nonhemorrhagic. Intracerebral hemorrhage is a common and often fatal stroke subtype (1). About 15% of patients die from spontaneous ICH. Edema following ICH contributes to these outcomes causing both acute herniation-related deaths and long-term neurologic deficits (2, 3).

Spontaneous intracerebral hemorrhage, from a variety of sources, causes instantaneous mass effect, disruption of surrounding brain, and often an early neurological death. The clinical diagnosis is usually not difficult and CT or MRI confirmation of the lesion, its location and some of its characteristics are readily apparent. If the patient survives the initial ictus the clinical condition often stabilizes for a number of hours. However, about 15% of the 150,000 cases per year in the United States then develop progressive neurological deterioration and early death (1). Serial MRI or CT scans usually document edema in adjacent brain around the clot, clot retraction, and changing radiographic signs as the clot begins to lyse (4). At times during the course of the illness, particularly in those patients with a deteriorating condition, the issue of clot evacuation is considered by clinicians, patients,

and families. The expected quality of life for the patient, with and without removal of the hematoma, is nearly always the ultimate question. Will neurological function improve with clot removal? Will deterioration cease? Will long term outcome be better with surgical evacuation of the hematoma or with resolution of the hematoma by natural surgical techniques? Are there alternatives to surgery and if so what are they? Surprisingly, answers to the above questions are often vague and poorly founded on factual information.

Sir Wiley McKissock focused attention on intracerebral hemorrhage years ago when he designed a clinical study of the merits of surgical versus non-surgical treatment of intracerebral hematomas (5, 6). Many clinical studies followed but generally they did not produce conclusive guidelines for the clinician managing individual patient problems. Conservative management has generally been recommended in situations where mass effect has not been significant and neurological function has remained stable from the beginning. Operative intervention has usually been reserved for situations where neurological function deteriorates as a result of expanding mass effect. Treatment decisions are generally pragmatic, based upon the quality of life that can be expected with the various treatments available.

Clinical investigations have attempted to solve the management riddle many times, but specific guidelines for treatment remain elusive (7, 8, 9, 10, 11, 12). Clinical protocols with feasible and reproducible endpoints continue to be problematic despite sophisticated diagnostic tools, and safer, more focused treatments that are currently available (2, 13, 14, 15).

The purpose of our project is to investigate the causes of edema formation and brain injury following ICH. The long term goal of these studies is to inhibit brain edema formation and limit brain injury after intracerebral hemorrhage.

B2. Perihematomal Brain Edema

Perihematomal edema is commonly observed during the acute and subacute stage following ICH. It appears as hypodensity around a hematoma on CT scan and as hyperintensity on T2-weighted magnetic resonance images (4, 16). Several mechanisms have been suggested to be responsible for perihematomal edema development, including hydrostatic pressure during hematoma formation and clot retraction (17, 18), coagulation cascade and thrombin production (19, 20, 21, 22, 23), erythrocytes lysis and hemoglobin toxicity (24), ischemia (25, 26, 27), and reperfusion injury (28). Additional leakage of blood into the hematoma may compound the problem (29).

Brott et al.(17) suggested that perihematomal edema in ICH patients as early as three hours following symptom onset may be due to clot retraction. That idea was confirmed with an experimental ICH model (18). In the porcine ICH model, they found marked perihematomal edema at one hour after blood infusion with an intact blood-brain barrier. Their findings were supported by the study of Hayman et al.(30), which indicted the hematocrit in the hematoma is approximately 70-90%. In addition, Enzmann et al.(31) found there was an acellular, proteinaceous liquid zone between the hematoma and brain tissue in a dog ICH model. Finally, removal of a clot an hour after it was placed, aborts subsequent edema formation 24 hours later in our own rat model (32).

We have demonstrated that thrombin, a serine protease and an essential component in the coagulation cascade, is responsible for early brain edema formation following ICH (20). Thrombin-induced brain edema results partly from a direct opening of the blood-brain barrier (22). In addition to its effect on brain edema, direct intracerebral infusion of thrombin causes inflammation, scar formation and reactive gliosis in the brain (33).

The role of ischemia in brain edema formation is controversial. Experiments have shown that cerebral blood flow adjacent to a hematoma decreases. Perihematomal CBF falls below 25 ml/100g/minute (34), but the reduction lasts less than 10 minutes and returns to baseline within three hours (25). Our previous work also showed that 50% reduction of CBF develops around the hematoma only at the first hour and returns to control levels in four hours (35). Although Ropper and Zervas (36) injected a large volume (more than 200 μL) of blood into the rat caudate nucleus within one second, the lowest CBF values were 25-30 mL/100 g/minute. Generally speaking, the acceptable CBF threshold for ischemic injury is 15-20 mL/100 g/minute (37). These results indicate that critical levels and durations of hypoperfusion do not occur following ICH. Recently, reports showed that an energy deficit is not present around the hematoma (38) and no ischemic penumbra is present in experimental ICH (39).

Edema after ICH is not simply from mass effect. Sinar et al. (26) demonstrated that microballoon insertion, a model for testing mass effect alone, reduced CBF to less than 20 mL/100 gram/minute, but failed to produce brain edema at 4 hours. Erythrocytes are responsible for the majority of the mass effect created by the hematoma, but packed erythrocytes alone fail to generate more brain edema than sham-treated animals at 24 hours in rats (20, 24). Furthermore, uncoagulated blood produces minimal perihematomal edema in humans (40), rats and pigs (23).

B3. Erythrocyte Lysis in Intracranial Hemorrhage

After ICH, erythrocytes within the hematoma retain their normal biconcave configuration for a while (16). Lysis of red blood cells occurs after 2-3 days in rats (our observation), 4-8 days in dogs (31) and 5-10 days in human (41). However, hemoglobin concentrations reach their peak the second day following blood injection into the subarachnoid space of dogs and then gradually disappear (42). Hemoglobin release from lysis of red blood cells (RBCs) in human intracranial hemorrhage increases during the first few days (43). Histochemically, hemoglobin and heme in the perihematoma zone can be observed at 24 hours after whole blood injection in the rabbit (44). Erythrocyte lysis appears to result from either depletion of intracellular energy reserves (1) or activation of the complement system and formation of membrane attack complex (45).

B4. Hemoglobin Toxicity in Brain Parenchyma

Our preliminary study demonstrates that intracerebral infusion of lysed erythrocytes results in marked brain edema formation by 24 hours. This edema formation appears to be hemoglobin mediated since an intracerebral infusion of rat methemoglobin at concentrations found in erythrocytes also results in marked increases in brain water content (24). Our studies also indicate that hemoglobin may have other deleterious effects on the brain. Intracortical hemoglobin injection in rats produces chronic focal spike activity, cavity lesions and gliosis at injection sites (46). Hemoglobin inhibits Na^+/K^+ ATPase activity in brain homogenates (47), activates lipid peroxidation (48), exacerbates excitotoxic injury in cortical cell culture(49) and induces depolarization in hippocampal CA1 neurons (50). Regan and Panter (51) found brief exposures (1-2 h) to hemoglobin are not toxic, but exposure of neuronal cell cultures to hemoglobin for 1 day produces concentration dependent neuronal death. Koenig and Meyerhoff also demonstrated that reconstituted rat hemoglobin, at a concentration of 25 nM, can induce the death of 50% of a primary culture of forebrain neurons within 8 hours and 72% within 24 hours (52).

The adverse effects of hemoglobin itself vary with its chemical form. Oxyhemoglobin is a spasminogen that has been implicated in cerebral vasospasm (53). In terms of ICH, however, Bradley has found only oxyhemoglobin in the hematoma for the first few hours following the hemorrhage (54). Thus, it is unlikely that oxyhemoglobin plays a role in ICH-induced edema formation, a suggestion supported by the fact that ICH does not produce marked reductions in CBF in the rat (35) and that intracerebral infusion of essentially methemaglobin can mimic the effects of erythrocytes on edema formation (24). Methemoglobin itself produces no arterial vasospasm in monkeys (53).

B5. Effects of Hemoglobin Breakdown Products and Hemeoxygenase (HO) on Brain Parenchyma

Hemoglobin breakdown products that appear as the clot lyses in the brain may play a major role in the formation of brain edema and mass effect. Heme from hemoglobin is broken down by heme oxygenase in the brain into iron, carbon monoxide and biliverdin. Biliverdin is then converted to bilirubin by biliverdin reductase (55). Carbon monoxide is a free radical that may cause tissue damage, analogous to nitric oxide-mediated damage (56, 57, 58). Iron can also stimulate the formation of free radicals leading to neuronal damage. Ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron react with lipid hydroperoxides to produce alkoxy and peroxy radicals and cause brain damage (59). Cortical injection of iron causes focal epileptiform paroxysmal discharges (60) and neuronal damage (61). Anderson and Means found iron salts inhibit spinal cord Na^+/K^+ ATPase in vivo, an effect blocked by antioxidants (62), while Willmore and Rubin found that subpial $FeCl_2$ injection induces focal brain edema and malonaldehyde formation (63). A role for iron, free radicals and lipid peroxidation in hemoglobin-induced brain injury is supported by findings that inhibition of brain Na^+/K^+ ATPase activity by hemoglobin can be blocked by desferrioxamine, an iron chelator (47), while hemoglobin-induced toxicity in neuronal cell cultures is blocked by the 21-aminosteroid U74500A, the antioxidant Trolox, and deferoxamine (51).

Bilirubin is toxic to the brain although it is considered to be an antioxidant. Fifty years ago, Jackson injected bilirubin into a dog's cisterna magna, causing severe inflammatory reactions (64). Now, various studies have demonstrated that bilirubin exerts many pernicious effects which may cause brain injury, including inhibition of the phosphorylation of the synapsic vesicle-associated protein synapsin I (65), disturbance in high energy phosphate levels (66), reduction in mitochondrial activity, disturbances in DNA synthesis, protein synthesis and ion transport (67). Amit and Brenner found that bilirubin *in vitro* is toxic to neurons and astrocytes (68). However, bilirubin, at micromolar concentration, may also act as an antioxidant (69, 70).

Hyperbilirubinemia that often occurs in newborn infants can cause severe neuronal injury, recognized clinically as bilirubin encephalopathy and kernicterus. One recent study indicated bilirubin-related encephalopathy and neuronal injury may develop through an N-methyl-D-aspartate (NMDA) receptor-mediated excitotoxic mechanism (71). Blocking hemeoxygenase, which reduces bilirubin production, has provided a protective effect against ischemic (72) and traumatic (73) brain injury.

Heme oxygenase (HO) consists of three enzymes, heme oxygenase-1 (HO-1), heme oxygenase-2 (HO-2) and heme oxygenase-3 (HO-3) (74, 75). HO-1, which has also been called heat shock protein 32 (HSP32), is induced by various stimuli (76, 77, 78). Several studies suggest that HO-1 may play an important role in cytoprotection against oxidative injury as well as against heme- and hemoglobin-induced toxicity (79, 80, 81). Induction of HO-1 by pretreating rats with hemoglobin, a potent HO-1 inducer, provides protection against lethal endotoxemia in rats. This hemoglobin-induced protective effect is blocked by tin protoporphyrin, an inhibitor of HO-1(81). Recently, Takizawa et al. reported that induction of HO-1 protects neurons from ischemic brain injury (82). HO-2, the constitutive isoform of HO, is localized mainly in brain and testes (83, 84). Total HO activity in the brain may depend on HO-2 activity because Ewing and Maines found that HO-1 induction did not change the total brain HO activity, measured by biliverdin production (85). HO-3 protein is a poor heme catalyst and is also found in the brain (75).

B6. Activation of the Complement System in Central Nervous System (CNS) Diseases

The brain is separated from the general circulation by the blood-brain barrier, a phenomenon which accounts for its relatively isolated immunological status. After BBB breakdown, plasma proteins, including complements, penetrate into brain tissue causing brain injury. Several CNS disorders show complement activation in the brain. These disorders include: CNS trauma (86, 87), multiple sclerosis (88, 89, 90), cerebral ischemia (91, 92), subarachnoid hemorrhage (92, 93), experimental allergic encephalomyelitis (94), Alzheimer's disease (95, 96) and other dementias (97).

Complement-related brain injury may be due to membrane attack complex (MAC) formation and the classic inflammatory response. The MAC consists of C5b-9 assembled following complement activation (98). Insertion of MAC into the cell membrane creates a pore which kills the cell eventually. By electron microscopy, MAC can be seen as "ring-like" structures on the erythrocyte membrane (99). Recent investigations have demonstrated that MAC not only causes cell lysis, but also modulates cellular functions such as release of cytokines, eicosanoids, oxygen radicals, and matrix proteins (100).

Anaphylatoxins C3a and C5a are generated following complement activation. C5a is also a potent chemoattractant for polymorphonuclear leukocytes and contributes to inflammatory cell injury (101). Inflammatory cells react to nanomolar concentrations of C5a with chemotaxis, upregulation of adhesion molecules and release of oxygen radicals (102). Dr. Peter Ward, consultant for this proposal, and his colleagues found that anti-rat C5a blocks C5a-mediated upregulation of ICAM-1 and P-selectin in lung (103).

Cobra venom factor (CVF) is a non-toxic protein in cobra venom. CVF is able to form a stable C3/C5 convertase which leads to complement depletion (102). Systemic complement depletion can improve blood flow and outcome following cerebral ischemia with reperfusion (104), reduce recruitment of macrophages into the nerve and subsequent macrophage-mediated demyelination (105), and reduce macrophage infiltration and activation during Wallerian degeneration (106).

B7. Significance / Approach / Innovation

Intracerebral hemorrhage is a common and often fatal subtype of stroke and produces severe neurologic deficits in survivors. Although death may occur acutely after an ICH, delayed neurologic

deterioration often occurs in patients with large hematoma, which may be related to brain edema formation. In this proposal, we will use use several well established techniques in this laboratory to determine the mechanisms involved in delayed edema formation following ICH. If our hypotheses are correct, these experiments may lead to novel methods of treating ICH by either altering erythrocyte lysis, limiting hemoglobin-induced toxicity or inhibiting complement activation.

C. PRELIMINARY STUDIES/PROGRESS REPORT

C1. Publications

C1.1. Journal Articles (* indicates included in Appendix)**:**

1.Menzies SA, Hoff JT, Betz AL: Middle cerebral artery occlusion in rats: a neurological and pathological evaluation of a reproducible model. *Neurosurgery* 31:100-107, 1992

2.Menzies SA, Betz AL, Hoff JT: Contribution of ions and albumin to the formation and resolution of ischemic brain edema. *J Neurosurg* 78:257-266, 1993

3.Yang G-Y, Betz AL, Hoff JT: The effects of blood or plasma clot on brain edema in the rat with intracerebral hemorrhage. *Acta Neurochir* 60:555-557, 1994

4.Doran SE, Roessler BJ, Hartman JW, Hoff JT, Sherrach DS, Davidson BL: Adenovirus mediated in vivo gene transfer into the CNS of a nonhuman primate. *Clin Neurosurg* 41:242-257, 1994

*5.Yang G-Y, Betz AL, Hoff JT: Experimental intracerebral hemorrhage. Relationship between brain edema, blood flow, and blood-brain barrier permeability in rats. *J Neurosurg* 81:93-102, 1994

6.Lee K, Betz AL, Keep RF, Hoff JT: Intracerebral infusion of thrombin as a cause of brain edema. *Neurosurg* 83:1045-1050, 1995

7.Lee KR, Betz AL, Keep RF, Hoff JT: The role of the coagulation cascade in brain edema formation. *Acta Neurochir* 138:396-401, 1996

*8.Lee KR, Colon GP, Betz AL, Keep RF, Kim S, Hoff JT: Edema from intracerebral hemorrhage: The role of thrombin. *J Neurosurg* 84:91-96, 1996

9.Colon GP, Lee KR, Keep RF, Chenevert T, Betz AL, Hoff JT: Thrombin-soaked gelatin sponge causes brain edema in rats. *J Neurosurg* 85:335-339, 1996

*10.Lee KR, Kawai N, Kim S, Sagher O, Hoff JT: Mechanisms of edema formation after intracerebral hemorrhage: Effects of thrombin in cerebral blood flow, blood-brain-barrier permeability, and cell survival in a rat model. *J Neurosurg* 86:272-278, 1997

11.Lee KR, Drury I, Vitarbo E, Hoff JT: Seizures induced by intracerebral injection of thrombin: A model of intracerebral hemorrhage. *J Neurosurg* 87:73-78, 1997

12.Figueroa BE, Keep RF, Betz AL, Hoff JT: Surgical management of basal ganglia hemorrhage in rat: Evidence for reduced brain edema with early hematoma evacuation. *Surg Forum* 48:539-541, 1997

*13.Figueroa BE, Keep RF, Betz AL, Hoff JT: Plasminogen activators potentiate thrombin-induced brain injury. *Stroke* 29:1202-1208, 1998

*14.Xi G, Wagner KR, Keep RF, Hua Y, deCourten-Myers GM, Broderick JP, Brott TG, Hoff JT: The role of blood clot formation on early edema development following experiemental intracerebral hemorrhage. *Stroke* 29:2580-2586, 1998

*15.Xi G, Keep RF, Hoff JT: Erythrocytes and delayed brain edema formation following intracerebral hemorrhage in rats. *J Neurosurg* 89:991-997, 1998

16.Hoff JT. Intracerebral hemorrhage: clinical and experimental aspects. *Chinese J. Nervous and Mental Diseases*. 25:3-4, 1999

17.Boulis NM, Bahtia V, Brindle TI, Holman HT, Krause DJ, Hoff JT: Adenoviral nerve growth factor and b-galactosidase transfer to spinal cord: A behavioral and histological analysis. *J Neurosurg* 90:99-108, 1999

*18.Patel TR, Shielke GP, Hoff JT, Keep RF, Betz AL: Alteration in cerebral blood flow, brain edema, and neuronal injury following intracerebral and subdural hemorrhage in the rat. *Brain Res,* in press

*19.Xi G, Keep RF, Hua Y, Xiang J, Hoff JT. Attenuation of thrombin-induced brain edema by cerebral thrombin preconditioning. *Stroke*, in press

C1.2. Books:

1.James HE, Marshall LF, Reulen H-J, Baethmann A, Marmarou A, Ito U, Hoff J, Kuroiwa T, Czernicki Z (Editors): *Brain Edema X*. Springer-Wien, New York, 1997, pp 302

2.Crockard A, Hayward R, Hoff J: *Neurosurgery: Scientific Basis of Clinical Practice, 3rd Edition*. London: Blackwell, 1999 in press

C1.3. Book Chapters:

1.Hoff J, Clarke HB: Adverse postoperative events in supratentorial surgery, in Apuzzo (ed*): Brain Surgery: Complications, Avoidance, and Management.* New York: Churchill-Livingstone, 1993, pp 99-126

2.Dickinson LD, Hoff JT: Infectious disease in neurosurgical critical care, Chapter 9, in Andrews B (ed): *Neurosurgical Intensive Care*. New York: McGraw-Hill, 1993, pp 201-226

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5.Lee K, Hoff JT: Intracranial pressure, Chapter 20, in Youmans J (ed): *Neurological Surgery,4th Ed.* New York: Saunders, 1996, pp 491-518

6.Sagher O, Hoff J: Surgical management of CNS infections, Chapter 48, in Sheld WM, Whitley RJ, Durach DT (eds): *Infection of the Central Nervous System, 2d Ed*. New York: Raven Press, 1996, pp 945-972

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11.Betz, A.L. Vasogenic edema. In: *Primer on Cerebrovascular Diseases*, M.Welch, L.Caplan, D.Reiss, B.Seisjo and B. Weir (Eds.), Academic Press, San Diego, 156-159, 1997

12.Lee KR, Hoff JT: Raised intracranial pressure and its effect on brain function, in Crockard A, Hayward R, Hoff J (eds): *Neurosurgery: Scientific Basis of Clinical Practice, 3rd ed*. Oxford: Blackwell, 1999. In press

13.Dickinson LD, Hoff JT: Infection control, in Hanley DF (ed): *Principles and Practice of Neurocritical Care*. Baltimore: Williams & Wilkins. In press

C2. PRELIMINARY DATA

The currently funded grant has explored the mechanisms of edema formation following intracerebral hemorrhage. We have established a consistent model of ICH in the rat (20, 21, 22, 23, 24, 35), determined that acute edema formation is not principally the result of ischemia (22, 35), implicated thrombin and the clotting cascade as an important element in acute edema formation following ICH (20, 21, 23) and suggested that there are different phases of edema formation with different mechanisms at different times after the ictus (24). Different work from our laboratory suggests that red blood cell lysis is important as a cause of edema that develops a few days after the initial ICH. That delayed process is the focus of this new application.

Specific data that led to the new application is outlined below. Our laboratory has expertise in a number of techniques necessary to carry out this proposal. These include a reliable model of ICH in the rat, using the stereotactic injection of blood and blood components into the caudate (20, 24, 35), as well as reproducible measurements of edema formation, blood-brain barrier disruption and cerebral blood flow (22, 35). We also are able to perform Western blots, immunohistochemistry and immuno-fluorescent double labeling (see Appendix). In addition, Peter Ward, M.D., Professor of Pathology at the University of Michigan, a consultant on this proposal, is an expert on the complement system (see Biosketch). He will provide advice and supplies necessary for this proposal.

C2.1. Intracerebral Hematoma and Brain Edema

C2.1.1. Changes in water and ion contents following ICH. Anesthetized adult rats received a sterile injection of 100 µL autologous blood into the caudate nucleus. Water and ion contents were measured immediately, at 4 and 12 hours, and daily until day 7 (10 time points, six rats at each time) after experimental ICH. The water content of the ipsilateral basal ganglia increased progressively ($p<0.002$) over the first 24 hours, then remained constant until after Day 5, when the edema began to resolve. Edema was most severe in the tissue immediately surrounding the hemorrhage; however, it was also present in the ipsilateral cortex, the contralateral cortex, and the basal ganglia. The increase in brain water content was associated with accumulation of brain sodium and a loss of brain potassium (35).

C2.1.2. Changes in cerebral blood flow following ICH. Cerebral blood flow was measured using 4-[N-methyl-^{14}C]-iodoantipyrine in groups of six rats each, at 1, 4, 24, and 48 hours after the

ICH or the sham procedure. When measured 1 hour after the ICH, CBF was reduced globally, varying from a 50% reduction (compared to sham-operated rats) in the ipsilateral basal ganglia to a 27% reduction in the contralateral cortex. CBF values returned to near those in sham-operated rats by 4 hours after ICH and remained fairly constant at 24 hours (35). By 48 hours, though, all brain regions of the experimental animals once again had significantly reduced CBF with both the ipsilateral basal ganglia and cortex levels about 48% of the corresponding levels in the sham-operated animals. The contralateral tissue was not as severely affected (35).

Although these results demonstrated significant changes in CBF following ICH, the blood flows remained above the threshold required for ischemic brain damage. However, the experimental technique employed (tissue sampling) might have missed localized areas where blood flow was reduced below the ischemic threshold. Therefore, CBF was also examined using autoradiography (Patel et al. Appendix). At 4 hours after ICH, we again found no areas where CBF fell below the ischemic threshold. This was in marked contrast to a model of subdural hematoma in the rat where CBF fell below the ischemic threshold in large areas of cortex. Thus, there appears to be no ischemic element to ICH induced brain injury in the acute phase. Whether the reduction in CBF that occurs at 48 hours (and perhaps later) is associated with localized areas where blood flow crosses the threshold for ischemic brain damage will be examined further in this proposal. A delayed effect on CBF might result from the release of hemoglobin from lysing erythrocytes, a hypothesis also examined in this proposal.

C2.1.3. Changes in blood-brain barrier permeability following ICH. BBB integrity was measured using ∗-[^{3}H]amino-isobutyric acid (AIB) (107), an amino acid not transported from blood to brain. Although the BBB was intact at 4 hours in all regions, permeability increased in the ipsilateral basal ganglia by 12 hours and to an even greater extent by 48 hours after ICH (35). Despite significant edema in the contralateral basal ganglia, BBB permeability remained normal in that region.

C2.2. Thrombin and Brain Edema

C2.2.1. Thrombin and brain water, ion contents. We use five or ten units of thrombin for infusion into the caudate because a 50-µL blood clot contains approximately 30 µl plasma; the potential amount of thrombin produced in that size clot is calculated to be ten units (22). Thrombin is infused stereotactically into the right basal ganglia of rats. Thrombin infusion results in significant increase in brain water content. Thrombin-induced brain edema is inhibited by a specific and potent thrombin inhibitor, hirudin. This edema is accompanied by increases in brain sodium and chloride contents and a decrease in brain potassium content. The time course of brain water content following infusion of rat thrombin (5 Units) is shown in **Figure 1**. Edema formation starts as early as 4 hours after thrombin infusion. The peak in edema is at 24 and 48 hours. Although water content begins to decrease after 48 hours, it is still higher than the contralateral side even at 7 days.

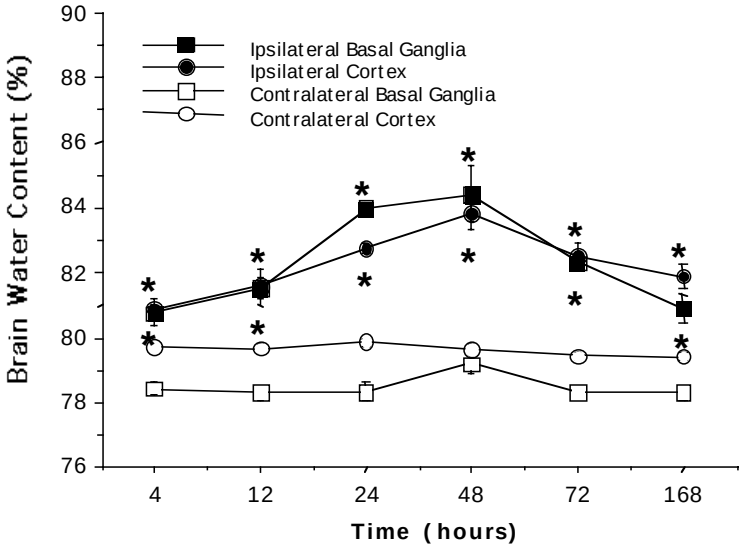

Figure 1. Brain water content 4, 12, 24, 48, 72 and 168 hours after infusion of rat thrombin (5 Units). Values are means ± SE (n=5-6). *P<0.001 versus contralateral side.

C2.2.2. Thrombin and blood-brain barrier. An intracerebral infusion of saline causes a slight increase in AIB influx (0.48±0.06 µl/g/minute), but ten units of thrombin infusion causes a much larger increase (1.26±0.19 µl/g/minute).

C2.2.3. Thrombin and rCBF. After ten units of thrombin infusion, rCBF declines over the first hour, rises to baseline or above by two hours, and then returns to baseline by 24 hours. The lowest blood flow recorded is 44.6±6.7 ml/100g/minute in the ipsilateral hemisphere at one hour. There are no significant differences between the control and thrombin-infused rats.

C2.2.4. Thrombin and brain cell toxicity. Cell culture experiments are performed to determine whether thrombin has a direct toxic effect on brain cells. Solutions containing C6 glioma cells are exposed to various doses of thrombin for 24 hours. At a dose of 100 units/ml thrombin there is an increase in lactate dehydrogenase at 24 hours, indicating significant increase in cell death rate.

C2.2.5. Thrombin and seizures. Artificial clots containing the concentration of thrombin found in a 50-∗l hematoma are infused intracerebrally in rats. Thrombin produces focal motor seizure in all animals. None of the control animals or those receiving ∗-NAPAP, a thrombin inhibitor added to the thrombin, show clinical evidence of seizure. All animals receiving thrombin alone show electrical evidence of seizure activity, whereas none of the control animals exhibit seizure activity (108).

C2.3. Thrombin Preconditioning and Thrombin-Induced Brain Edema.

Edema formation following intracerebral hemorrhage has been linked to thrombin toxicity induced by the clot. However, thrombin at low concentration actually protects neurons and astrocytes in culture from hypoglycemic and ischemic cell death. It is also known that a brief episode of brain ischemia increases neuronal tolerance to a subsequent severe ischemic episode. The objective of this study was to investigate whether pretreatment of the brain with low dose thrombin induces tolerance to a subsequent larger dose of thrombin injected into brain parenchyma.

The rat brain was preconditioned with one unit thrombin by direct infusion into the right caudate nucleus. Following thrombin pretreatment, the effects of a larger dose (five units) of thrombin on brain edema formation were studied at different intervals. We examined whether heat shock proteins (HSP) 27, HSP32 and HSP70 were induced using Western blot analysis, immunocytochemistry and immunofluoresent double staining.

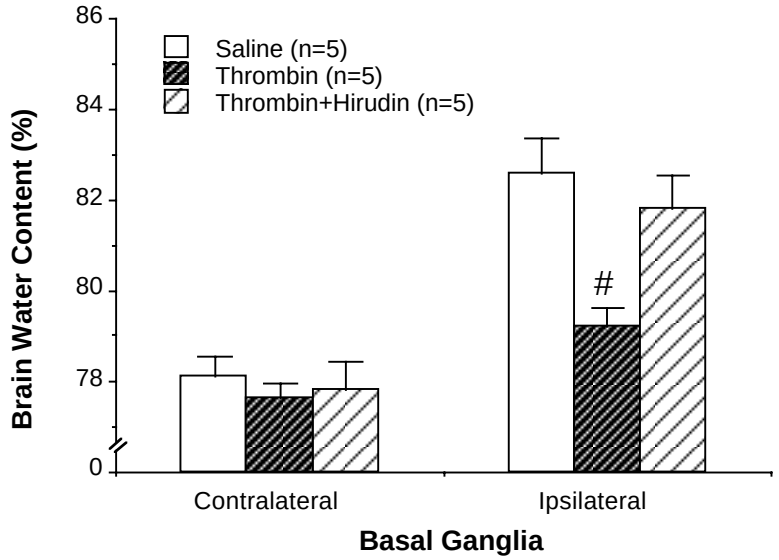

Figure 2 . Brain water contents at 24 hours after intracerebral infusion of 5 units thrombin. The brains had been infused with either saline, 1 unit thrombin or 1 unit thrombin+1 unit hirudin seven days before the large dose of thrombin. Values are expressed as means±SD, n=5. #P<0.01 versus saline or thrombin + hirudin.

Thrombin pretreatment significantly attenuated the brain edema which normally follows the infusion of a large dose of thrombin (79.2±0.4 *versus* 84.0±0.3; P<0.01). This effect was abolished by the thrombin inhibitor, hirudin (**Figure 2**). Time course studies showed the maximal effect of thrombin
preconditioning (TPC) on brain edema formation to be seven days after pretreatment (see Appendix). This time course corresponds to marked upregulation of HSP27 in the ipsilateral brain. TPC also induces HSP32, but this effect occurrs earlier than the effect on edema formation (**Figure 3**). TPC had no effect on HSP70. Immunocytochemistry and immunofluorescent double labeling showed that HSP27 and HSP32 are expressed in astrocytes after TPC. The phenomenon of thrombin-induced tolerance of the brain to edema formation may be related to HSP27 induction.

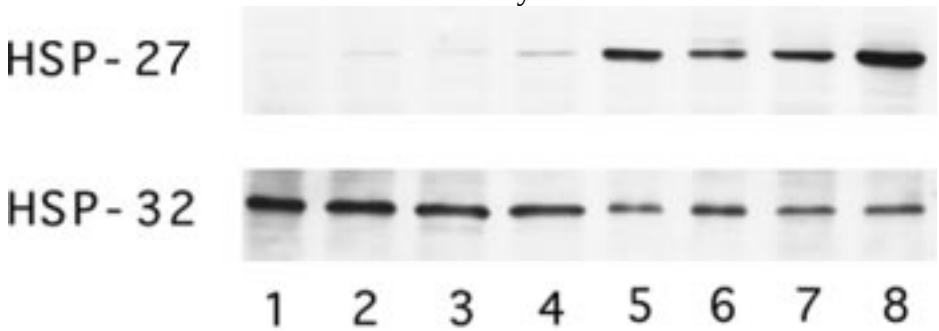

Figure 3. Time course of HSP27 and HSP32 protein levels in ipsilateral basal ganglia with thrombin preconditioning. Lanes 1 and 2 were one day following 1 unit thrombin infusion. Lanes 3 and 4 were three days following 1 unit thrombin infusion. Lanes 5 and 6 were seven days after thrombin pretreatment. Lane 7 and 8 were 14 days after thrombin preconditioning. Equal amounts (50 µg) of protein were analyzed by Western blot.

C2.4. Erythrocytes, Hemoglobin and Brain Edema

C2.4.1. Lysed erythrocytes and brain edema Lysed RBCs but not packed RBCs produce marked brain edema in the ipsilateral basal ganglia and cortex 24 hours after infusion (**Figure 4**). Compared to the packed RBCs group, brain water content of the lysed RBCs group increases

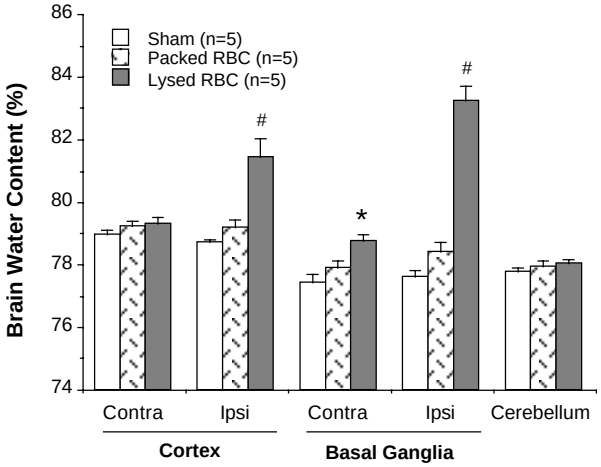

Figure 4. Brain water contents at 24 hours following sham operation or infusion of 30 µl of either packed or lysed red blood cells. Values are expressed as means ± SE, n=5. * P<0.05 versus sham and packed RBCs groups. # P<0.01 versus sham and packed RBCs groups.

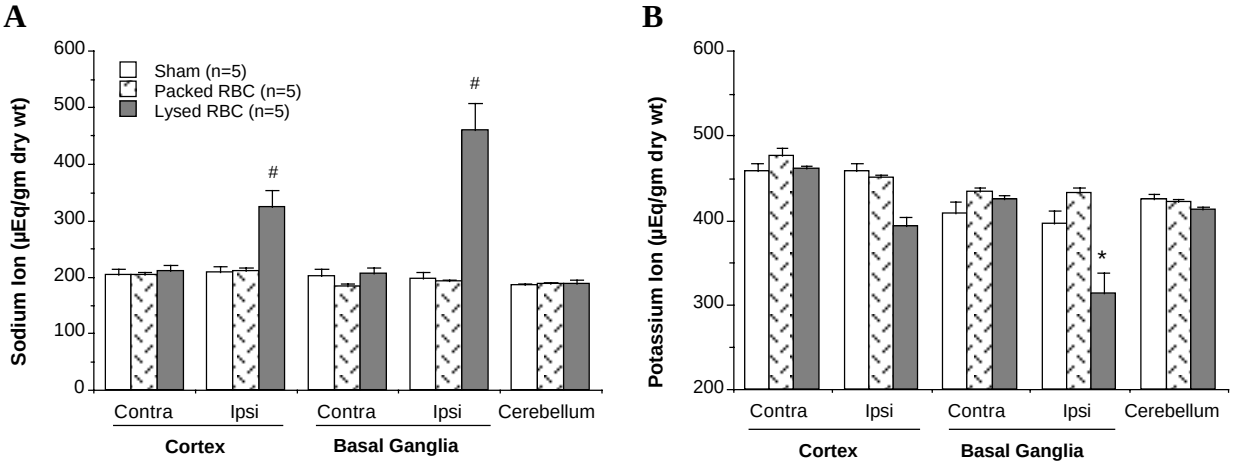

Figure 5. Brain sodium (A) and potassium ion (B) contents at 24 hours after sham operation or 30 µl either packed or lysed red blood cells. Values shown are means±SE. * P<0.05 versus sham and packed RBCs groups. # P<0.01 versus sham and packed RBCs.

significantly in the ipsilateral basal ganglia (83.2±0.5 vs. 78.4±0.3%, p<0.01). Edema formation following lysed RBCs infusion is associated with an ipsilateral accumulation of sodium and a loss of potassium (**Figure 5**).

C2.4.2. Methemoglobin and brain edema Intracerebral infusion of rat methemoglobin produces a dose-dependent increase in brain water content at 24 hours (**Figure. 6**). The increase in brain water content is also associated with accumulation of brain sodium and a loss of brain potassium.

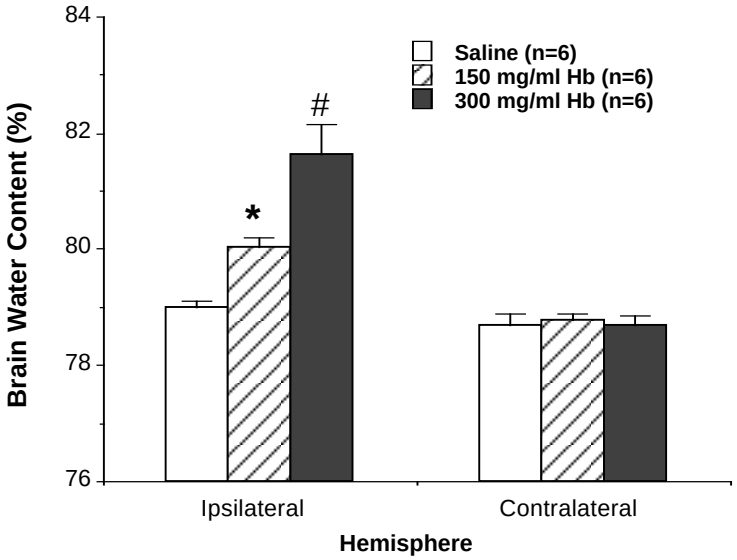

Figure 6. Brain water content at 24 hours after 20 µl hemoglobin (Hb) or saline infusion. Values shown are means ± standard error of the means. * P<0.05 versus saline group. # P<0.01 versus saline group.

C2.4.3. Packed erythrocytes and brain edema Brain water content fails to change within 24 hours after 50 µL packed RBC infusion. There is a peak increase of water content in the ipsilateral cortex and basal ganglia at 3 days after RBC infusion. At 5-7 days, ipsilateral brain water content returns to that of the contralateral tissue (**Figure 7**).

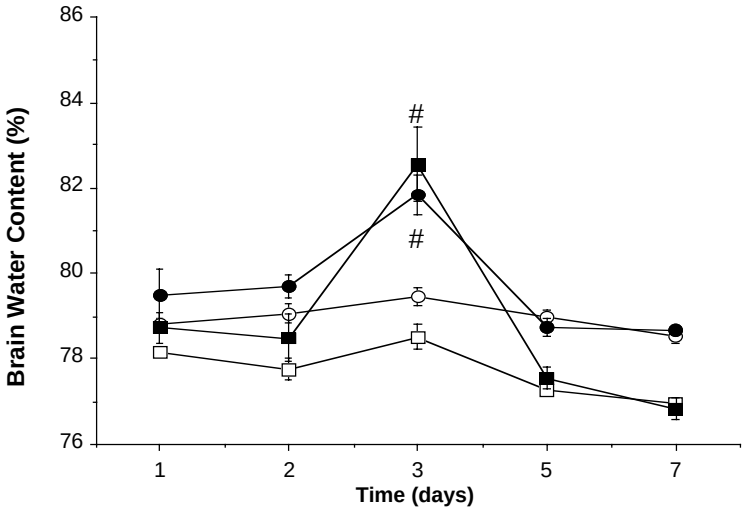

Figure 7. Brain water content at 1, 2, 3, 5 and 7 days following 50 µl packed red blood cells infusion. Measurements were made in four brain regions: ipsilateral basal ganglia (solid squares), ipsilateral cortex (solid circles), contralateral basal ganglia (open squares), and contralateral cortex (open circles). Values shown are means ± SE (n=5-6). # P<0.01 versus contralateral side.

C2.5. Complement Activation in ICH

C2.5.1. Complement C-9 accumulation and MAC formation. Western blot analysis demonstrates that C9 had a six-fold increase around the hematoma one hour after 50 µL whole blood infusion (6678±586 vs. 956±404 pixel in sham, p<0.01). Intracerebral infusion of thrombin, lysed red blood cells or hemoglobin also causes marked C9 accumulation in the ipsilateral basal ganglia (3329±433 vs. 458±395 pixel, p<0.01; 3925±516 vs. 372±103 pixel, p<0.001; 4430±433 vs. 2521±797 pixel, p<0.05), respectively. By the first 24 hours after ICH, immunocytochemistry found that C9 is accumulated around the hematoma as a ring. Three days later, however, C9 is deposited on neuronal membranes, indicating activation of the complement cascade and formation of MAC (**Figure 8**). MAC formation following activation of the complement cascade results in formation of a pore in the cell membrane, causing cell death.

Figure 8 Complement C9 immunoreactivity around hematoma at 3 days after whole blood infusion. Scale bar is 20 µm,

C2.5.2. Clusterin upregulation. Clusterin is a putative complement inhibitor which can inhibit MAC-induced cell lysis by forming clusterin-C5b-9 complex. By Western blot analysis, clusterin increases at 24 hours after 100 µL blood (3-fold increase vs. sham), five units thrombin (2-fold increase), 30 µl lysed RBCs (10-fold increase) or hemoglobin (4-fold increase) infusion and is expressed in neurons around the hematoma three days after whole blood infusion. **Figure 9** shows clusterin concentration is still increased in ipsilateral basal ganglia at three days.

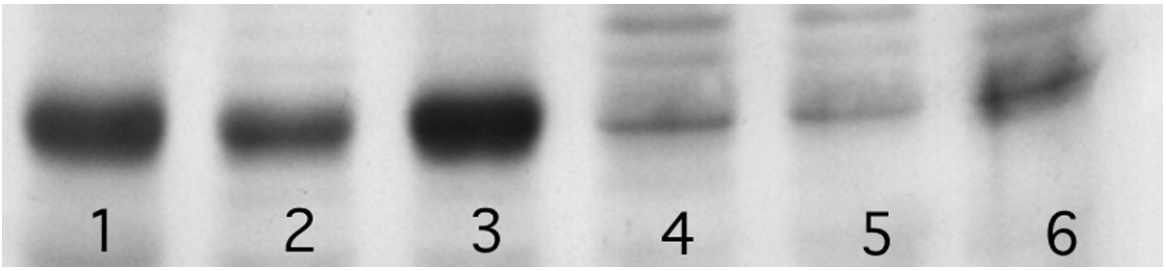

Figure 9. Clusterin protein levels in ipsilateral basal ganglia at three days after 100 µl blood infusion. Lanes 1, 2, and 3 were three days following 100 µl blood infusion. Lanes 4, 5, and 6 were sham control. Equal amounts (50 µg) of protein were analyzed by Western blot.

C2.5.3. Complement C3d. Complement C3d, a split fragment of complement factor C3, is an indicator of complement cascade activation. Our Western blot analysis shows that C3d is significantly increased in ICH (2726±847 vs. 512±218 pixel in normal controls, $p<0.05$). C3d immunoreactivity is found as fine granules near erythrocytes and neurons, which may indicate complement directly attacks erythrocytes and neurons (**Figure 10**).

Figure 10. Complement C3d immunoreactivity in the clot at 3 days after 50 µL blood infusion. Scale bar is 20 µm.

C2.5.4. Complement C5a and the inflammatory response. Complement factor C5 is one of the key factors in the complement cascade. During complement activation, the C5a fragment is selectively cleaved from the C5. C5a possesses a strong leukocyte chemotactic activity (109) and also enhances the adhesiveness of neutrophils to the endothelium through upregulation of adhesion molecules.

Figure 11. Perihematomal complement C5a immunoreactivity at 3 days after 50 μL blood infusion.

We recently initiated C5a and complement depletion studies in our rat ICH model in collaboration with Dr. Ward (consultant for this proposal). Our preliminary immunohistochemistry study showed that C5a immunoreactivity is found around the hematoma and in neutrophils (**Figure 11**)**.** Myeloperoxidase (MPO) immunostaining indicates inflammation around the ICH (accumulation of MPO positive cells following intracerebral whole blood (A), thrombin (B) and hemoglobin (C) infusion, **Figure 12**)**.**

C2.5.5. Complement depletion and brain edema following ICH. Systemic complement depletion was achieved by cobra venom factor (CVF). CVF was administered (60 units/kg, i.p., three injections at 36, 24, and 12 hours before intracerebral blood infusion, same volume saline injections as controls) and 100 μL blood was infused into basal ganglia. Brain water content was measured 24 hours later. The result showed complement depletion reduced brain edema significantly following ICH at 24 hours in the ipsilateral basal ganglia (78.8±0.6 vs. 81.6±0.8 % in control, $p<0.01$).

C2.5.6) Heparin and brain edema. Heparinizing blood prior to infusion in the caudate results in significantly less edema formation than that found with non-heparinized blood (**Figure 13 A**). This effect may result from the inhibition of the coagulation cascade by heparin. However, as with whole blood, thrombin-induced edema was significantly reduced by the co-injection of heparin.(**Figure 13B**). The mechanism behind this latter effect is still uncertain. Heparin is not only an anticoagulant but also a potent inhibitor of the complement system (110).

A

C

B

Figure 12. Myeloperoxidase immunoactivity in the basal ganglia 3 days after whole blood (A), thrombin (B), and hemoglobin (C) infusion. Scale bar is 20 µm.

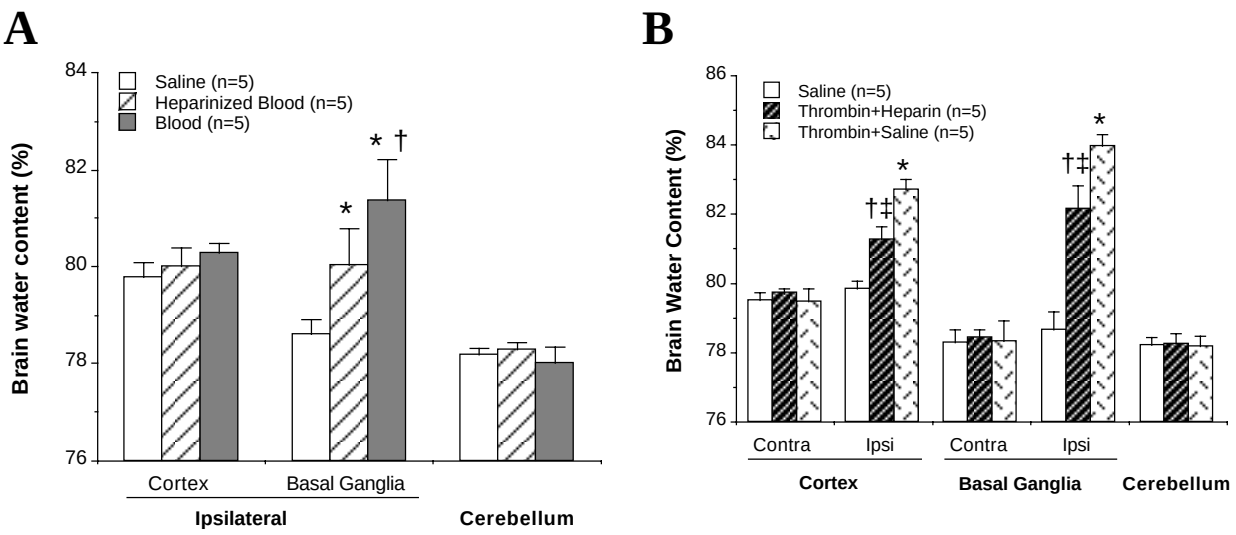

Figure 13. (A) Brain water contents at 24 hours following infusion of 50 µl of saline or heparinized blood or blood. Values are expressed as mean ± SD, n=5. *P<0.05 versus saline group. P<0.05 versus heparinized blood group. **(B)** Brain water content 24 hours after infusion of 60 ml either saline or thrombin + saline or thrombin + heparin. Values shown are mean ± SD. *P<0.01 versus saline group. P<0.01 versus saline group. ‡ P<0.01 versus thrombin + saline group.

C2.5.7. Immunofluorescent double labeling for complement C9. Morgan and collaborators reported that astrocytes and microglia produce their own complements (111). Our double staining (C9 and neuron specific enolase) shows neurons can produce C9 following ICH (**Figure 14**).

Figure 14. Double immunofluorescent labeling for neuron specific enolase (NSE) and complement C9 24 hours after 50 µL blood infusion. (A) rhodamine-labeled complement C9. (B) FITC-labeled NSE. Scale bar is 20 µm.

D. RESEARCH DESIGN AND METHODS

D.1 RATIONALE:

Our overall goal is to understand the mechanisms that lead to delayed edema formation following ICH in order to design effective treatments to prevent it. In Specific Aim 1 we seek the physical basis for delayed edema formation, i.e delayed ischemia, BBB breakdown or cytotoxicity? What components of blood induce those changes?

The study will use a rat model of ICH. Experimental models of ICH have been available since the 1960's and commonly involve the injection of autologous blood into the frontal lobe of dogs, cats or monkeys (112, 113, 114). Now, rodents have been found to provide an equally convenient and suitable model (35). Rats cost less than larger animals, are relatively homogeneous, have the cerebrovascular anatomy and physiology similar to that of higher species, and a small brain size well suited to immunohistochemical and biochemical studies. We chose to inject fresh autologous blood into the caudate nucleus at a rate of 10 µL/minute because it produces a reproducible lesion that lends itself to quantitative measurement. We found that a more rapid injection rate resulted in a variable reflux of blood along the needle track and poorly reproducible lesions. We considered developing a mouse model in order to use the transgenic strains available in that species. However, the size of the mouse caudate limits the amount of blood that can be injected to about 50 µl before it enters the ventricular system. That size of clot in the mouse produces limited edema in the adjacent brain (ipsilateral water content 79.2±0.3 %; contralateral water content 78.4±0.2%) making therapeutic studies difficult.

This study will use 50 and 100 µl blood clots in the rat. On a volume basis, such clots are equivalent to 30-60 ml clots in a human. Lisk et al. found that the mean hematoma volume in 75 patients with a supra tentorial hematoma was 33 ml (13).

The study will also use direct injection of hemoglobin (30 µl of 300 mg/ml), the amount injected being derived from the normal hemoglobin content of rat blood. However, it should be noted that although direct intracerebral injection of hemoglobin at those concentrations causes brain injury that fact does not necessarily indicate that the release of hemoglobin from an ICH will cause injury since such release will probably be gradual, in contrast to our bolus injections. Thus, it is necessary to confirm the role of hemoglobin in brain injury following ICH by other means (e.g. with inhibitors or promoters of hemoglobin breakdown; Specific Aim 2). Such confirmatory experiments are also necessary because of the possibility of impurities in hemoglobin preparations.

Much work has been done examining the role of hemoglobin in vasospasm following subarachnoid hemorrhage (53). However, it is difficult to transpose these results directly into the

problem of ICH. As described in the preliminary results section, there are marked differences in the effects of blood on the brain depending on the location of the hematoma. Thus, in the rat, a subdural hematoma causes acute cerebral ischemia whereas ICH does not. Furthermore, while injection of 200 µl of blood into the subdural space or the caudate causes marked edema formation, injection of a similar amount into the lateral ventricle does not (for the latter hippocampal water content was 79.7±0.1 and 80.1±0.3 % in saline and blood infused rats, respectively). Thus, whether there is the equivalent of vasospasm in intraparenchymal vessels after ICH is uncertain. This question will be addressed in this study using autoradiography.

There are a number of potential methods for examining whether there is delayed ischemia following a cerebral hemorrhage (or injection of blood components) in the rat. These include radioisotopes (with tissue sampling or autoradiography), microspheres, hydrogen clearance, and laser doppler flowmetry. The latter two methods have the advantage of allowing continuous or multiple measurements with time, but they are limited in that they only allow measurements in a small area of brain. Laser doppler flowmetry is also limited to the surface of the brain negating its uses in ICH. Tissue sampling, with either isotopes or microspheres, may miss focal areas of ischemia. We will, therefore, use autoradiography to monitor whether changes in CBF occur around the hematoma, this technique having the spatial resolution required to determine whether small areas of brain show reductions in CBF below the ischemic threshold.

The other two major endpoints in our study are brain edema and blood-brain barrier permeability. Edema formation is the end point we are trying to modulate since we believe it is a major determinant of survival in patients with ICH. It is also a simple, reproducible and inexpensive measure of brain injury. To assess blood-brain barrier permeability, we will measure the influx rate constant (K_i) for movement of [^{3}H]AIB from blood to brain. This amino acid is not actively transported from blood to brain (115) and thus has a low K_i (≈1.5 µl/g/min). That K_i is increased in a number of disease states where the BBB is disrupted (e.g. cerebral ischemia (107), brain tumors (115) and ICH (22)). It has the advantage over other commonly used assessors of BBB disruption, such as Evans blue, of being quantifiable, of having a very large volume of distribution within the brain so that efflux is not a concern (115), and of being a small molecular weight compound. Brain edema formation following ICH primarily results from an accumulation of Na^+ and Cl^- (35) and the atomic weight of those two elements (23 and 35.5) more closely corresponds to AIB (molecular weight 103) than albumin or inulin (MW ≈60,000 and 5,000). Currently we plan to use tissue sampling to determine changes in AIB permeability. We have used this technique previously to detect changes following ICH and intracerebral thrombin injections. If greater spatial resolution is required, AIB can also be used for autoradiography (115).

For these experiments, we will use as controls both sham-operated rats that have received no infusions into the caudate and rats that have received an intracerebral infusion of saline. The latter may cause small, transient, changes in brain water content (24) cerebral blood flow (22) and blood-brain barrier permeability (22).

In Specific Aim 2 we will examine the role of hemoglobin breakdown products in edema formation follwing ICH. This will involve intracerebral infusions of heme, biliverdin, bilirubin and Fe. As with infusions of hemoglobin, there is concern that these infusions will not mimic the gradual production of hemoglobin breakdown products during resolution of an intracerebral hematoma. This specific aim, therefore, will also examine the effect on brain injury of modulating hemoglobin breakdown follwing intracerebral infusion of blood. Heme oxygenase will be inhibited using tin protoporphyrin, tin meso-porphyrin and zinc protoporphyrin. Metalloporphyrins may inhibit enzymes other that heme oxygenase (116). A range of inhibitors will be used to elucidate whether any effects on brain injury are due to heme oxygenase inhibition. Tin chloride will be also be used as this compound can induce expression of HO1 (HSP32) in the brain ((117) and preliminary data).

An alternate approach to examining the role of hemoglobin breakdown products in ICH-induced brain edema formation is use of the transgenic mice (e.g. HO1 or HO2 knockout animals). These will not be used in the present study because of the limited size of clot that can be used in this species (see above) and because of the possibility that any change in one form of hemeoxygenase may lead to compensatory changes in the other forms (118).

Specific Aim 3 has three parts. The first (3a) examines the role of the complement cascade in ICH-induced brain edema formation (a suggestion supported by our preliminary data). The second (3b) examines whether this effect is dependent upon complement-mediated RBC lysis by examining whether the complement cascade mediates brain injury induced by lysed RBC, hemoglobin or thrombin. The third (3c) examines whether the complement system is involved in neutrophil influx into the brain following ICH, an influx that might mediate hematoma lysis but also brain injury.

This specific aim will use different ways to modulate the complement cascade. It will use two naturally occurring compounds, clusterin and vitronectin, that inhibit MAC complex insertion into biological membranes. However, these compounds have other effects that might modulate edema formation following ICH, e.g. vitronectin modulates the activity of thrombin inhibitors (119) and thrombin has been implicated in edema formation following ICH (20). Thus, results with these compounds need to be confirmed by other approaches. It should be noted, though, that an ability of these compounds to inhibit edema formation is important irrespective of the mechanism at this point since they are naturally occurring and there is evidence of clusterin upregulation following ICH (see Preliminary Data).

The three other methods that will be used to modulate the complement system are complement depletion with cobra venom factor (120), a C5a neutralizing antibody and the hereditary C6 deficient rats (PVG/c-). The C6 deficient rat (PVG/c$^-$) has great advantages in studying the role of the MAC complex in brain injury. Unlike deficiencies of the classical pathway, C1, C4 and C2, which are associated with diseases resembling systemic lupus erythematosus, C6 deficient rats are healthy when kept in the controlled environment of an animal breeding facility. This reflects the fact that the elements of the complement cascade up to C5 proceeds normally in these rats including the generation of opsonic C3b and chemotactic C5a fragments. That other elements of the complement cascade are produced in these rats limits their usefulness in determining whether the complement cascade as a whole is involved in brain injury following ICH, hence the necessity to undertake different manipulations of the complement system.

We will use a neutralizing antibody to inhibit the actions of C5a. C5a is a potent chemoattractant which might play a role in neutrophil migration into brain after ICH, the hypothesis tested in specific aim 3c. The C5a antibody has the advantage of specificity, i.e. it only inhibits one element of the complement system (121). In addition, since it will be given by intracerebral injection, it will have little systemic effect since the clearance of proteins from brain is relatively slow. A major disadvantage of the antibody is its limitations as a therapeutic approach since it requires intracerebral infusions because the antibody will not cross the intact blood-brain barrier. However, the goal of this specific aim is to implicate the complement system in brain injury following ICH and not necessarily to test the best methods for preventing such injury.

Induction of complement depletion with cobra venom factor has the advantages of depleting rats of both C3 and C5, thus preventing the generation of opsonic C3b and chemotactic C5a fragments and MAC complex formation. It will, therefore, inactivate a number of the elements that might be involved in complement-induced brain injury following ICH. It does have the disadvantage of depleting the rats of complement, making them more prone to infection. We will monitor animals closely for this possibility since such infections might modulate brain injury (e.g. by increasing body temperature). There are also concerns over other effects that cobra venom factor might have on the brain. Since CVF is a protein it is unlikely to cross the intact blood-brain barrier.

D2. RESEARCH PLAN

Hypothesis 1. Erythrocyte lysis and hemoglobin release causes delayed brain edema formation following intracerebral hemorrhage by either reducing blood flow, increasing blood-brain barrier permeability or by direct cytoxicity.

Specific Aim 1. The effect of an intraparenchymal infusion of autologous packed erythrocytes, lysed erythrocytes, and hemoglobin (oxy- and methemoglobin) will be tested. Regional cerebral blood flow and blood-brain barrier integrity will be measured at different time points.

Our preliminary studies show that intracerebral infusion of lysed RBCs results in marked brain edema formation by 24 hours. This edema formation appears to be hemoglobin mediated since intracerebral infusion of rat methemoglobin at concentrations found in RBCs also resulted in marked increases in brain water content . A comparison of edema formation produced by ICH and thrombin infusion suggests there may be delayed edema formation from RBC lysis and hemoglobin release in ICH. Data from our laboratory (24, 35) and others (31, 122, 123) indicate the perihematoma edema following ICH reaches a peak between Day 3 and Day 7. In contrast edema formation following thrombin injection peaks at 1-2 days (see Figure 1). Delayed edema formation from RBCs, peaking at 3 days (Figure 7), may explain the difference between the ICH and thrombin data. The delayed, hemoglobin-related brain edema may be due to reduction of CBF, increase of BBB permeability or direct cytoxicity.

To test hypothesis 1, lysed erythrocytes, oxyhemoglobin, methemoglobin and packed erythrocytes, will be injected into rat caudate nucleus. Regional cerebral blood flow and blood-brain barrier integrity will be measured at 1, 2, 3, 5, and 7 day (Table 1, Table 2).

Table 1. Expected regional cerebral blood flow outcomes

Time (day)	Sham	Saline control	Lysed RBCs	Packed RBCs	Oxy-hemoglobin	Met-hemoglobin
1	-	+	++++	+	++++	+
2	-	+	+++++	++	+++++	++
3	-	-	+++	++	++++	+
5	-	-	++	+	++	+
7	-	-	+	-	+	-

+++++ = Maximal rCBF reduction;
+ = Minimal rCBF reduction;
- = Normal.

Following the lead from our preliminary studies, 30 µL lysed RBCs will be infused stereotactically into the right caudate nucleus. rCBF and BBB integrity will be tested. (We use the dose of 30 µL lysed RBCs infusion since the death rate of 50 µL lysed RBCs intracerebral infusion in the rat is 100%, while the death rate is less than 10% in the 30 µL infusion group). To obtain lysed RBCs, whole blood (0.5 ml) is immeditely centrifuged at 14,000 G for 30 seconds before coagulation occurrs. The plasma and buffy coat is discarded. The RBCs are washed with five volumes of saline three times and are lysed by freezing the cells in liquid nitrogen for five minutes followed by thawing at 37 ˚C.

Table 2. Expected blood-brain barrier integrity outcomes

Time (day)	Sham	Saline control	Lysed RBCs	Packed RBCs	Oxy-hemoglobin	Met-hemoglobin
1	-	+	++++	+	++++	++
2	-	+	+++++	++	+++++	+++
3	-	-	+++	+++	++++	++
5	-	-	++	+	++	+
7	-	-	+	-	+	-

+++++ = Maximal increase of BBB permeability;
+ = Minimal increase of BBB permeability;
- = Normal.

Next, rat oxyhemoglobin and methemoglobin (30 µL, 300 mg/ml) will be injected into the right basal ganglia. (The normal concentration of hemoglobin in rat RBCs is approximately 300 mg/ml). Oxyhemoglobin and methemoglobin will be prepared from purified rat hemoglobin (Sigma) as described by Macdonald et al.(124). OxyHb is obtained from rat Hb by reduction with 10-fold molar excess of sodium dithionite followed by dialysis with 400 volumes of Elliott's solution B (mock CSF) at 4 ˚C, while MetHb is prepared by oxidation with a 1.2-fold molar excess of potassium

ferricyanide followed by dialysis. Purity will be determined spectrophotometrically. Finally, packed RBCs (30 µL) will be used at different time points. Although we may find lysed RBCs and hemoglobin cause increased BBB permeability and reduced CBF, it is still not sure that the RBCs in an intracerebral hematoma contribute to these changes. We use packed RBCs as a standard because erythrocyte lysis and hemoglobin release might occur gradually over a period of time and the hemoglobin concentration in the brain may not reach toxic levels. Packed RBCs (hematocrit level about 87%) will be obtained by centrifuging washed blood (three times in saline).

Table 3 shows the animal groups, the times for sacrifice and the animal numbers per group to test hypothesis 1.

Table 3. Animal groups and sacrifice times test hypothesis 1

Time (day)	Sham	Saline Control	Lysed RBCs	Packed RBCs	Oxy-hemoglobin	Met-hemoglobin
1	6+6	10+10	6+6	6+6	6+6	6+6
2	6+6	10+10	6+6	6+6	6+6	6+6
3	6+6	10+10	6+6	6+6	6+6	6+6
5	6+6	10+10	6+6	6+6	6+6	6+6
7	6+6	10+10	6+6	6+6	6+6	6+6

Total animals = 400 (200 for blood-brain barrier integrity tests; 200 for cerebral blood flow studies).

Hypothesis 2. Hemoglobin breakdown products play an important role in hemoglobin-induced brain edema. Blocking or inducing heme oxygenase (HO) will modify brain edema formation.

Specific Aim 2a. Brain edema, cerebral blood flow and blood-brain barrier permeability will be measured after infusing hemoglobin breakdown products (hemin, beliverdin, bilirubin and iron) into the parenchyma of brain. To test this hypothesis, we will measure brain water, ion contents, cerebral blood flow and blood-brain barrier permeability at 1, 3, and 5 day after biliverdin, bilirubin and ferrous chloride ($FeCl_2$) injection. The concentrations for intracerebral infusions are hemin (12 mg/ml), biliverdin (12 mg/ml), bilirubin (12 mg/ml) and $FeCl_2$ (300 mM) and the total infusion volume is 30 µL. We expect one or two hemoglobin breakdown products will produce brain edema, increase BBB permeability and reduce CBF at some time points. Table 4 showes the animal groups, the times for sacrifice and the animal numbers per group to test Specific Aim 2a.

Table 4. Animal groups for testing Specific Aim 2a

Time (day)	Control	Hemin	Biliverdin	Bilirubin	$FeCl_2$
1	10+10+10	6+6+6	6+6+6	6+6+6	6+6+6
3	10+10+10	6+6+6	6+6+6	6+6+6	6+6+6
5	10+10+10	6+6+6	6+6+6	6+6+6	6+6+6

Total animals = 306 (102 for brain edema studies; 102 for blood-brain barrier integrity tests; 102 for cerebral blood flow measurements)

Specific Aim 2b. To determine whether heme oxygenase (HO) with tin protoporphyrin (SnPP), tin meso-porphyrin (SnMP) or zinc protoporphyrin (ZnPP) or induction of HO-1 activity with $SnCl_2$ modifies edema formation following intraparenchymal infusion of blood or blood components.

There are two sets of studies in Specific Aim 2b. The first set examines the role of heme oxygenase inhibitors (metalloporphyrins) in brain edema formation following ICH which may relate to changes of CBF and BBB integrity. The second set examines the role of HO-1 induction in edema formation after ICH. HO activity will be assessed as the amount of bilirubin production in the presence of purified rat biliverdin reductase (125). The enzyme activity is expressed as nanomoles of bilirubin produced per hour per milligram of protein.

To test the first set of Specific Aim 2b, rats will receive different infusates with different heme oxygenase inhibitors and will be sacrificed at 24 hours after infusion (Table 5) to determine brain edema, CBF and BBB integrity.

Table 5. Animal groups for testing the first set of Specific Aim 2b

	Sham	Whole Blood 100 µL	Lysed RBCs 30 µL	Oxy-Hb 30 µL(300mg/ml)	Met-Hb 30 µL(300mg/ml)
Control	10+10+10	10+10+10	10+10+10	10+10+10	10+10+10
SnPP	6+6+6	6+6+6	6+6+6	6+6+6	6+6+6
SnMP	6+6+6	6+6+6	6+6+6	6+6+6	6+6+6
ZnPP	6+6+6	6+6+6	6+6+6	6+6+6	6+6+6

Total animals = 420 (140 for brain edema studies; 140 for blood-brain barrier integrity tests; 140 for cerebral blood flow measurements). SnPP = Tin protoporphyrins; SnMP = Tin meso-prophyrin; ZnPP = Zinc protoporphyrin.

To test the second set of Specific Aim 2b, rats will receive tin chloride ($SnCl_2$) injection subcutaneously (100 mg/kg body weight) for four days. Our preliminary results showed that Western blot can detect the HO-1 band in brain tissue after the rat receives tin chloride injection subcutaneously. (HO-1, also called heat shock protein 32, is absent from normal barin but can be induced by a number of stresses).

After HO-1 induction, rats will receive an intracerebral infusion of either 100 µL blood, 30 µL lysed RBCs, oxy-Hb or met-Hb. Animals will be sacrificed at 1 and 3 days. Brain will be sampled for edema measurements. Subcutaneous injection of saline with the same intracerebral infusion components will serve as controls. These experiments will involve 16 groups of 6 rats = 96 rats.

Hypothesis 3. Erythrocyte lysis in the hematoma following ICH is due to complement activation and membrane attack complex formation. Activation of the complement system will also exacerbate brain damage.

Specific Aim 3a. To determine whether inhibition of MAC complex formation prevents red blood cell lysis and neuron injury after ICH.

Our preliminary findings suggest that the complement cascade has been activated and the MAC has been assembled after ICH. To test this hypothesis, three sets of experiments are necessary. In the first set, clusterin or vitronectin will be infused with whole blood. Clusterin and vitronectin are designed to interact with the MAC prior to its insertion into erythrocyte membrane which may reduce brain edema following ICH. In the second set, we will use complement depleted rats to examine the role of complement on brain edema and BBB. In the third set, hereditary C6 deficiency rats will be used to test the role of MAC. The C6 deficiency (PVG/c-) rats are unable to assemble MAC and are unable to lyse activated sheep erythrocytes(126).

Specific Aim 3a.1. One, three and five days after co-injection (100 µL blood + 2 µg vitronectin, or 100 µL blood + 2 ∗g clusterin), the brain water and ion contents will be measured. Control rats will receive the same volume of blood infusion. These experiments will require 12 groups of 6 rats = 72 rats. If blood coinjections with vitronectin or clusterin result in less brain edema than that of blood injection alone, BBB permeabililty will be measured requiring another 12 groups of 6 rats = 72 rats. Immunohistochemistry will be applied to identify whether or not complement C9 deposits on neurons (three rats for each time point = 9 rats).

We expect blood + vitronectin or clusterin will produce less brain edema and markedly reduce BBB breakdown following ICH.

Specific Aim 3a.2. Animals will be complement-depleted with cobra venom factor (CVF, 60 units/kg, i.p., three times at 36, 24, and 12 hours before intracerebral infusion). Control rats will receive an equal volume of saline injection (i.p.). Both the treatment and control rats will receive 100 µL autologous blood or 50 µL packed RBCs into the right basal ganglia. Animals will be killed 1, 3, and 5 days after infusion. We will measure brain water and ion contents (12 groups of 6 animals = 72

rats); BBB permeability (12 groups of 6 animals = 72 rats); and C9 and clusterin concentrations around the hematoma (Western blot, 12 groups of 3 animals = 36 rats). Finally we will perform immunostaining for C9 and clusterin (12 groups of 2 rats = 24 rats).

We expect complement depletion will attenuate brain edema induced by blood and packed RBCs and will reduce BBB permeability. Complement depletion will also reduce complement C9 accumulation and clusterin upregulation around the hematoma.

Complement depletion will be tested with a standard CH50 (total hemolytic complement activity) assay for hemolysis of sheep erythrocytes. The CH50 in complement depleted animals should be less than 5% of the standard serum CH50 values.

Specific Aim 3a.3. Hereditary C6 deficiency rats. PVG/c- rats are deficient in complement C6 which cannot form MAC (PVG/c- and normal PVG/c rats will be available from Dr. P. Morgan, College of Medicine, University of Wales.). The experiments will examine whether brain edema is attenuated by MAC formation. Blood (100 μL) will be infused into the right basal ganglia of PVG/c- or PVG/c rats. Rats will be sacrificed one, three and five days after blood infusion. Brain water, ion contents and BBB permeability will be measured. These studies will require 12 groups of 6 rats = 72 rats (36 PVG/c- rats and 36 PVG/c rats).

Specific Aim 3b. To examine whether complement activation exacerbates neuronal injury following an intraparenchymal injection of blood components (i.e. in the absence of the red blood cell lysis effect).

Our preliminary studies showed that the complement system is activated following blood injection. The first experiment will examine which blood components cause complement system activation. We will test thrombin, lysed RBCs and methemoglobin since we think the coagulation cascade and RBC lysis play important roles in brain injury following ICH.

Thrombin (5 units in 50 μL saline), lysed RBCs (30 μL) and methemoglobin (30 μL, 300 mg/ml) will be infused into rat basal ganglia, and animals will be sacrificed at 1, 3, and 5 days later. Controls will receive the same volume of saline (30 μL or 50 μL) injection. Brain tissue will be sampled for Western blot analysis or brain will be perfused with 4% paraformaldehyde in 0.1 M pH 7.4 phosphate-buffered saline for immunohistochemistry (see specific method). C3d, C5a, C9 and clusterin will be semiquantitated with Western blots. Immunostaining and immunofluorescent double labeling will also be applied for these studies. These studies will require 10 groups of 3 rats each = 30 rats.

In the second experiment we will use complement depleted rats to test whether complement depletion attenuates neuronal injury following intraparenchymal injection of blood components. Complement depleted or normal rats will receive thrombin, lysed RBCs and methemoglobin infusion (as described above). Animals will be sacrificed 1, 3, and 5 days after infusions. Brain tissue for water, ion contents and BBB premeability will be measured. These studies will require 12 groups of 6 rats =72 rats.

We expect the complement cascade is also activated following thrombin, lysed RBCs and methemoglobin infusion. Complement depletion should attenuate thrombin, lysed RBCs and methemoglobin induced brain edema.

Specific Aim 3c. To determine whether complement activition exacerbates hemorrhagic brain injury by stimulating neutrophil influx into brain.

Preliminary studies have revealed the accumulation of myeloperoxidase (MPO) positive cells around the hematoma and in the ipsilateral basal ganglia after thrombin and hemoglobin intracerebral infusion. C5a immunoreactivities also have been found around the hematoma. This specific aim has two sets of experiments. The first, investigates whether anti-rat C5a antibody is able to block C5a-induced neutrophil infiltration and attenuate brain edema after ICH. The second examines the possible changes of C5a and neutrophil infiltration in complement depleted animals.

Anti-rat C5a antibody is available from Dr. Ward. This antibody blocks rat C5a but not C5 (121). Mulligan et al. found anti-C5a resulted in 91% reduction in chemotactic activity for neutrophils(120). In our experiment, one hundred microliters of blood will be infused into the basal ganglia and the needle will be removed. A second infusion will start 5 minutes later. This period of time will allow for clotting of infused blood. In the second infusion, rats will receive either 25-µL(50 µg/ml) of rabbit anti-rat C5a antibody (treatment) or 25-µL (50ug/ml) of normal rabbit IgG (control). Rats will be sacrificed one, three and five days later. The rat brains will be sampled for edema , MPO activity or MPO immunocyto-chemistry. The brain edema and MPO activity studies will require 12 groups of 6 rats = 72 rats. The MPO immunocytochemisty study will require 6 groups of 3 rats = 18 rats.

We expect anti-rat C5a antibody will significantly attenuate brain edema and reduce MPO activity following ICH. Intracerebral infusion of anti-rat C5a will also markedly reduce the MPO positive cell number around the hematoma.

2) Our preliminary data shows that complement depletion by CVF attenuates edema after ICH, which may be due to less neutrophil infiltration in the complement depleted rats. These experiments will examine the role of complement depletion on C5a and MPO following ICH. Animals will be divided into two groups (CVF treatment and saline control) for three sets of experiments.

In the first set, Western blot or ELISA analysis will be used to quantitate C5a following ICH. Rats will receive a 100 µl blood intracerebral injection and will be sacrificed 1 and 3 days later. Brains will be sampled for Western blot or ELISA analysis. These experiments will require 4 groups of 6 rats = 24 rats. We expect complement depletion will reduce C5a concentration around the hematoma.

In the second set, immunocytochemistry and immunofluorescent double labeling will be applied for C5a and MPO. Preliminary studies show C5a immunoreactivities around the hematoma which may be in neutrophils. We also found MPO immunoreactivities to be significantly increased after intracerebral blood, thrombin and hemoglobin infusions. We will use MPO and C5a double-staining to confirm these results. Rats will receive a 100 µL blood intracerebral injection and will be sacrificed 1 and 3 days later. Brains will be perfused with 4% paraformaldehyde in 0.1 M pH 7.4 phosphate-buffered saline (P B S) . Immunocytochemisty will be applied to identify C5a and M positive cell numbers and separate complement depletion groups and control groups. These studies will require 4 groups of 3 rats = 12 rats. We expect C5a and MPO positive cells will decrease markedly in the complement depletion ICH model.

In the third set, we will measure MPO activity around the hematoma in our complement depletion model and our control ICH model. Blood (100 µl) will be infused into rat basal ganglia and rats will be sacrificed 1 and 3 days later. Brains will be sampled for MPO activity. The method for MPO assay is similar to that described previously (127). These studies require 4 groups of 6 rats = 24 rats. Reduction of MPO activity around the hematoma in the complement depletion model is expected.

D3. TIME TABLE

	Year 1	**Year 2**	**Year 3**	**Year 4**	**Year 5**
S.A. 1 ICH and CBF/BBB breakdown	X	X			
S.A. 2a Hemoglobin breakdown and ICH	X	X			
S.A. 2b HO and ICH		X	X		
S.A. 3a MAC complex/ICH			X		
S.A. 3b Complement/blood components				X	X
S.A. 3c Complement/Neutrophils				X	X

D4. SPECIFIC METHODS

1) Animal Preparation: Adult male Sprague-Dawley rats (250-350 g) are anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg). After anesthesia is achieved, a polyethylene

catheter (PE-50) is inserted into the right femoral artery in order to monitor arterial blood pressure and to obtain blood for analysis of blood gases, blood pH, hematocrit, blood glucose concentration and for blood removal for intracerebral infusion. Body temperature is maintained at 37.5 ˚C by using a feedback-controlled heating pad.

2) Intracerebral Infusion. Before intracerebral infusion, the rats are positioned in a stereotactic frame (Kopf Instrument, Tujunga, CA), the scalp is incised along the sagittal midline using a sterile technique. A cranial burr hole (1 mm) is drilled near the right coronal suture 4.0 mm lateral to the midline. A 26-gauge needle is inserted stereotaxically into the right basal ganglia (coordinates: 0.2 mm anterior, 5.5 mm ventral, and 4.0 mm lateral to the bregma). Whole blood, packed red blood cells, lysed red blood cells or other solutions are infused into right basal ganglia using a microinfusion pump. After infusion, the needle is removed and the skin incisions are closed with sutures. Animals are allowed to recover. The seven day survival percentages are 100% in the 50 ∗l blood infusion group and >90% in the 100 ∗l blood infusion group.

3) Brain Water, Sodium, Potassium Contents. The rats are sacrificed by decapitation under deep pentobarbital anesthesia (60 mg/kg i.p.). The brains are removed immediately and a 3 mm thick coronal brain slice 4 mm from the frontal pole is cut. That slice is divided into four samples, ipsilateral and contralateral basal ganglia and ipsilateral and contralateral cortex. Tissue samples are weighed on an electronic analytical balance to the nearest 0.1 mg to obtain the wet weight (WW). The tissue is then dried in a gravity oven at 100 °C for more than 24 hours to determine the dry weight (DW). Tissue water contents (%) are calculated as ((WW-DW)/WW)*100. The dehydrated brain samples are digested in 1 ml of 1 N nitric acid for 1 week. The sodium and potassium ion contents in this solution are measured by flame photometry. Ion contents are expressed in micro equivalents per gram of dehydrated brain tissue (µEq/gm dry wt).

4) Cerebral Blood Flow. Cerebral blood flow is measured using [^{14}C]-iodoantipyrine and a modification of a previously described autoradiography method (128). Briefly, 20 µCi of radioisotope in 1.5 ml saline is administered as a ramped intravenous infusion over a period of 30 seconds. The infusion rate during the first 5 seconds is 50% of the final infusion rate. During the infusion, 14 - 18 samples of arterial blood are collected on pre-weighed filter discs. At the end of the isotope infusion, the animals are decapitated and the brains removed and frozen in isopentane at -42°C. The filter discs are re-weighed and a 30% solution of hydrogen peroxide added to bleach the blood from the discs. [^{14}C] radioactivity is determined by liquid scintillation counting. Twenty micron horizontal sections of the frozen brains are cut in a cryostat at -20°C. Serial sections at 100 µm intervals are collected onto glass slides and rapidly dried at 60°C. The slides are apposed to Kodak BMR-2 film for 7 days. Pre-calibrated [^{14}C] standards (concentration range 42 - 1610 nCi/g tissue equivalents) are placed in the film cassettes. The autoradiograms are analyzed using a computer based image analysis system (M2 MCID, Imaging Research Inc.).

The values of CBF are determined from the equation of Sakurada et al. with the knowledge of arterial blood history of [^{14}C], tissue levels of [^{14}C] and the tissue blood partition coefficient (0.79) of iodoantipyrine (128).

5) Blood-Brain Barrier Permeability. Blood-brain barrier integrity will be assessed using ∗-[^{3}H]aminoisobutyric acid (AIB). The permeability of the BBB to ^{3}H-AIB will be determined as described previously in our studies on cerebral ischemia (107) and cerebral hemorrhage (22). After an i.v. bolus injection, the amount of tracer that moves into the extravascular compartment of the brain (A_b) is divided by the integral of the plasma concentration of the tracer determined between the time of injection and killing of the rat ($\int C_p dt$). A_b is determined by subtracting the amount of tracer in the plasma compartment ($PV \times C_t$) from the total radioactivity in the brain (A_t), where PV is the plasma volume estimated with ^{14}C-inulin and C_t is the terminal plasma concentration. The integral of the plasma concentration is obtained from a continuous withdrawal of arterial blood (0.1ml/min) during the course of isotope circulation, and calculated by dividing the total counts withdrawn.

^{3}H-AIB (25 µCi) is injected 10 minutes before the end of the experiment, while ^{14}C-inulin (10 µCi) is given as a second injection 2 min before the end. Simultaneous with the ^{3}H-AIB injection, a peristaltic pump is started and blood is withdrawn from an arterial cannula. At the end of the experiment, a terminal plasma sample is obtained and the rat is killed by decapitation. The entire

contents of the arterial cannula are emptied and an aliquot pipetted for counting. Blood samples are digested in methylbenzethonium hydroxide (Sigma), bleached with H_2O_2, and counted in an aqueous-based liquid scintillation cocktail. Brain tissue is also digested in methylbenzethonium hydroxide and counted in a Beckman 3801 two-channel liquid scintillation counter. The results are expressed as a rate constant for brain uptake, which is equal to the product of the capillary permeability and the surface area of the exposed vascular bed (PS product) for a compound of low BBB permeability such as AIB.

6) Western blot analysis. Animals are anesthetized and decapitated at different time points. Brain is perfused with saline and brain tissues are sampled as described in the water content paragraph. The brain tissues are immersed in 0.5 mL Western sample buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% sodium dodecyl sulfate, 10% glycerol, and 5% ∗-mercaptoethanol) and then are sonicated for 10 seconds. Twenty-µL of the sample solution are taken for protein assay (Bio-Rad), while the rest is frozen at -20 °C for Western blot. Western blot analysis is performed as described previously from our laboratory (129). Briefly, 50 µg protein is run on 15% polyacrylamide gels with a 4% stacking gel (SDS-PAGE) after 5 minutes boiling at 95 °C. The protein is transfered to hybond-C pure nitrocellulose membrane (Amersham). The membranes are blocked in 5% Carnation non-fat dry milk in TBST (150 mM NaCl, 100 mM Tris-base, 0.1% Tween 20, pH 7.6) buffer for 1 hour at 37 °C. After washing in TBST buffer 3 times, membranes are probed with 1:2500 dilution of the primary antibody for 1.5 hours at room temperature. After washing the membranes with TBST buffer 3 times, the membranes are immunoprobed again with 1:2500 dilution of the second antibody for one hour at room temperature. Finally, membranes are washed three times in TBST buffer and the antigen-antibody complexes are visualized with the ECL chemiluminescence system (Amersham) and exposed to Kodak X-OMAT film. The relative densities of the protein bands are analyzed with a public domain NIH Image program (NIH Image Version 1.55; NTIS).

7) Immunocytochemistry. Rats are anesthetized with pentobarbital (60 mg/kg, i.p.) and perfused with 4% paraformaldehyde in 0.1 M pH 7.4 phosphate-buffered saline (PBS). Removed brains are kept in 4% paraformaldehyde for six hours, then immersed in 25% sucrose for three to four days at 4 °C. The brains are embedded in O.C.T compound (Sakura Finetek U.S.A. Inc.) and sectioned on a cryostat (18 µm thick). Sections are incubated overnight with 1:800 dilution of the primary antibody. Normal IgG (from the same species as that which produces primary antibody) is used as a negative control. After three washes in PBS, sections are incubated for 90 min with 1:1000 dilution of biotinylated second antibody. After another three PBS washes, brain sections are incubated with avidin-biotinylated horseradish peroxidase (Vector Laboratories) for 90 min. Brain sections are re-washed three times in PBS and then incubated with diaminobenzidine and hydrogen peroxide (Stable DAB, Research Genetics, Inc.). The sections are then washed in water for five minutes, dehydrated and covered with a coverslip for microphotography.

8) Immunofluoresent double labeling. Different antibody combinations will be used for immunofluresent double labeling. Each primary antibody (1:100 dilution) is incubated overnight at 4ϒC. Fluorescein isothiocyanate (FITC) or rhodamine labeled second antiboby is incubated with sections for 2 hours at room temperature. The double labeling is analyzed by a fluorescence microscope (Nikon Microphoto-SA) using a rhodamine filter and a FITC filter.

9) Myeloperoxidase activity assay. Rats are reanesthetized with pentobarbital (60 mg/kg, i.p.) and perfused transcardially with saline. Brains are sampled for water content measurement. The brain segments are then immediately frozen at -70 ϒC. Tissue segments are thawed on ice, weighed, and homogenized in 4 mL of 5 mmol/L Tris-HCl, pH 7.4, at 4 ϒC. Samples are added to 20 mL 5 mmol/L phosphate buffer, pH 6.0, at 4ϒC, homogenized and centrifuged at 30,000g for 30 minutes at 4ϒC. The supernatant is discarded and the pellet is washed again. The pellet is extracted by suspension in 0.5 % hexadecyltrimethylammonium bromide in 50 mmol/L potassium phosphate buffer at 25ϒC for approximately 2 minutes at an original tissue wet weight to volume ratio of 1:2. The samples are immediately frozen on dry ice. Freeze and thaw cycles are then performed three times with sonications for 10 seconds at 25ϒC between cycles. After sonication, the samples are incubated at 4ϒC for 20 minutes and centrifuged at 12,500g for 15 minutes at 4ϒC. The supernatants (100 µL) are mixed with 100 µL of 50 mmol/L phosphate buffer, pH 6.0, containing ∗-dianisident

dihydrochloride and hydrogen (0.005%). Absorbency is measured at 460 nm by a spectrophotometer. One unit of MPO activity is defined as the amount that degrades 1 mole of peroxide/minute at 25ϒC. MPO activity for each tissue sample is normalized on the basis of tissue wet weight in grams.

10) Statistics. Most data will be analyzed by ANOVA with a Scheffe's multiple comparisons test. Exceptions are dose responses which will employ Dunnet's multiple comparisons test and paired data (e.g. comparisons of injected and non-injected hemispheres) which will be analyzed with the paired *t*-test.

E. HUMAN SUBJECTS

None

F. VERTEBRATE ANIMALS

1. These experiments require rats. Approximately 400 male Sprague-Dawley rats, weighing 250-350 grams, will be used annually.

2. In vitro systems can't be used to study the complex interactions involving blood clot, mass effect, brain cells, and the microvasculature that follow brain hemorrhage. The rat brain is large enough to permit the injection of a reproducible volume of infusates (erythrocytes, hemoglobin and hemoglobin breakdown produces) and the measurements that we will make (i.e. water and ion contents, blood-brain barrier permeability, regional cerebral blood flow, Western blot analysis, immunocytochemistry, and immunofluresent double labeling). Based upon our studies with these models, group sizes of 6 animals are required.

3. Animal husbandry will be provided by the Unit for Laboratory Animal Medicine (ULAM) under the guidance of supervisors who are certified Animal Technologists. Veterinary care will be provided by ULAM faculty members and veterinary residents. The University of Michigan has an Animal Welfare Assurance (A3114-01) on file with the Office for Protection from Research Risks and is fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

4. All invasive procedures will be conducted under anesthesia using sterile technique. Animals that are allowed to recover after the injection of erythrocytes, hemoglobin or hemoglobin breakdown products are kept warm until awake and then watched carefully every 6-12 hours. Our model of intracerebral hemorrhage allows the rats to survive for at least a week with less than 10% mortality. The majority of the rats begin eating and drinking water within 6 hours of the operation. Some have a mild unilateral weakness that does not interfere with their ability to move around the cage and to feed and clean themselves. Any animal which appears sick, has obvious infection at the site of surgery, or does not drink following the procedure will be euthanized.

5. All animals will be sacrificed within 7 days of the operation. They will be reanesthetized and euthanized by decapitation without awakening from the anesthetic.

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H. CONSORTIUM/CONTRACTUAL ARRANGEMENTS

None

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