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Cerebral Vasospasm after Experimental Subarachnoid Hemorrhage in the Canine Model: Evidence for the Role of Immunological Reactivity

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I. INTRODUCTION

Recent studies[1,2] have demonstrated that perivascular inflammation and hemolysis of subarachnoid erythrocytes can each independently provide sufficiently large vasoactive stimuli to cerebral vessels to account for the constriction characteristic of cerebral vasospasm after subarachnoid hemorrhage(SAH). Further experiments suggested that hemolysis of subarachnoid erythocytes may involve not simply a spontaneous degradation of cellular integrity, but rather an active hemolysis induced by cytolytic factors present in plasma. This cytolytic reaction was demonstrated in vitro with canine[2] and human[3] erythrocytes which had incubated 72 hours ex vivo in an environment intended to resemble that of the subarachnoid blood clot in vivo.

When such intact but prematurely "aged" autologous erythrocytes were introduced to the subarachnoid space of dogs at a hematocrit greater than 10% in a plasma-contraining medium, rapid hemolysis of those cells provoked severe and immediate vasoconstriction, which resolved slowly and incompletely over 72 hours[2]. Interpretation of those experiments was complicated however by the intense early phase of erythrocyte hemolysis during the first 2-5 hours after experimental SAH. It was unclear whether the more moderate sustained vascular reaction(that is, beyond 12 hours) represented continued hemolysis of subarachnoid erythrocytes or very slow recovery from earlier exposure to high concentrations of vasoactive hemolysate. It has been reported, for example, that exposure for 24 hours to oxyhemoglobin leads to essentially irreversible constriction in isolated vascular smooth muscle cells[4].

The experiments presented below intended to clarify that issue by using a subarachnoid injectate containing erythrocytes subject to cytolytic attack by canine plasma factors, but which would not undergo rapid initial hemolysis. Subarachnoid injections of cross-species blood were studied in the canine model of cerebral vasospasm, since such material would not be subject to immediate hemolysis by its own plasma during clot placement in the basal cistern, but could provoke an immunological cytolytic reaction against subarachnoid erythrocytes which would be subject to rates of extravasation of canine plasma factors. These studies were further prompted by recent reports in the literature indicating that serum

immunocomplex levels may be predictive for the occurrence of cerebral vasospasm in SAH patients[5,6] and an earlier study[7] showing deposition of IgG and complement C3 in the affected arteries of SAH patients with chronic vasospam.

II. METHODS AND MATERIALS

Subarachnoid Injections in the Canine Model

Cerebral angiography and subarachnoid injections were performed in dogs as described elsewhere[1], except that in some experiments the indwelling vertebral artery catheter was not used. Instead, the left vertebral artery was catheterized by placement under fluoroscopy of a transfemoral angiographic catheter (100 cm Torcon Blue, 6.5 F, Cook Co.) which was removed post-operatively and reinserted for angiography at later times. Sterile xenologous whole blood samples were drawn shortly before use. Human blood was drawn by venipuncture from 3 different volunteers, rabbit blood from a femoral artery catheter, and rat blood by cardiac puncture after thoracotomy.

Vasospasm was measured as basilar artery constriction and data analysis was conducted as described elsewhere[1]. The cerebral vasculature was fixed in situ prior to excision of the brain. The animal was exsanguinated from a femoral artery catheter while saline was pressure-infused into the vertebral artery. When effluent hematocrit was less than 5%, the vertebral artery was infused with 250 ml saline containing 10% glutaraldehyde or formalin for 15 min. The brain was then carefully excised and immersed in 2.5% formalin for 24 hours. The brain stem ventreal surface with subarachnoid membrane, clot, and underlying basilar artery intact, was then removed to a thickness of ~5 mm and placed in fresh fixative until final preparation for histology.

In Vitro Measurements of Erythrocyte Lysis by Canine Plasma

Canine plasma samples were prepared from freshly drawn heparinized blood(12-20 U/ml) by centrifugation(2000xg, 8-12 min), pooled in sterile 10 ml siliconized glass tubes, and recentrifuged. No cell counts were detectable in the resultant plasma. Erythrocytes from human, rabbit, and rat blood were prepared from freshly drawn anticoagulated blood by centrifugation at 150xg for 5-8 min. Supernatant plasma and buffy coat was aspirated and cells washed 2x with warm sterile saline by resuspension and low-speed centrifugation. No white cells or platelets were countable in the final erythrocyte preparation.

Xenologous erythrocytes were quickly resuspended in canine plasma at ~10% hematocrit and divided into multiple aliquots (0.8-1.0 ml) in sterile 1.5 ml plastic microcapusles for incubation at 37℃. At various times up to 2 hours after addition of erythrocytes (including one sample processed immediately), the samples were briefly resuspended and centrifuged at 15,000xg for 3-4 min to pellet unlysed cells. Supernatant was diluted for spectrophotometric assay of the 575 nm hemoglobin peak as an index of hemolysis. In addition, one aliquot was ultrasonicated to induce complete hemolysis so that samples could be normalized to % hemolysis of total erythrocytes present. In several cases, in order to verify the absence of artifact due to manipulation of the cells, isolated erythrocytes were returned to their autologous plasma and incubated in parallel with other samples. Lysis in such control measurements averaged 0.4% of total erythrocytes in 2-3 hours. To estimate the involvement of non-specific cytolysis, control measurements were also performed with xenologous cells in canine plasma which had all divalent metal cation chelated by the addition of 7.5 mM K3EDTA, since divalent cations are required for activation of cytolytic complement proteins[8,3]. Hemolysis by canine plasma in those control measurements averaged also 0.4 % of total xenologous erythrocytes in 2 hours.

Measurements of Bulk Protein Extravasation into CSF

Control cerebral angiography and subarachnoid injections of various materials were performed in a number of animals, as described. At 48 hours after subarachnoid injection, cerebral angiography was repeated and 50 mg/kg Evans Blue(Sigma Chemical Co.) infused intravenously in 100 ml saline over 1 hour. 24 hours later(72 hours after subarachnoid injection), a heparinized blood sample was centrifuged to cell−free plasma and a sample of CSF withdrawn by needle puncture of the cisterna magna. Great care was taken that the cisternal puncture be blood−free. The CSF sample was briefly centrifuged to remove any traces of particulate or cellular matter. The concentration of Evans Blue in plasma and CSF was determined spectrophotometrically at the 625 nm peak against standardized concentrations of Evans Blue in saline or plasma.

III. RESULTS

Reaction to Subarachnoid Clots of Human, Rabbit, and Rat Blood

Fresh human blood(3.0 ml) was injected over 1 minute into the basal cistern of 7 dogs and cerebral angiography repeated at various intervals to 72 hours post−injection. All animals showed degrees of neurological deficit (paresis and lethargy) and one animal died overnight. The mean(±SEM) angiographic results, where basilar artery diameter is expressed as % of control diameter before experimental SAH, are shown by the filled circles and solid line in Figure 1 in comparison to control data1 for basilar artery reaction to a single subarachnoid(SA) injection of 3.0 ml autologous whole blood. Individual angiographic results for 2 animals injected with rabbit blood(1 animal died overnight) are shown by the filled squares. To further verify the absence of species−specific response, 5 animals received 3.0 ml SA injections of freshly−drawn rat blood(1 animal died almost immediately after injection). Those mean(±SEM) angiographic results are shown by the filled triangles. No substantive differences were found in vascular reaction to the 3 xenologous blood types, which provoked an initial moderate vascular reaction progressing, after some delay, to severe chronic constriction at 72 hours.

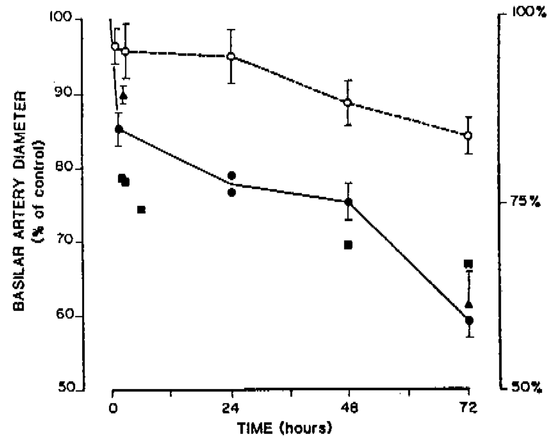

Figure 1. The mean(±SEM) basilar artery diameter is shown as % of control diameter for the 72 hour period following subarachnoid injections in dogs of autologous whole blood(open circles) and xenologous whole blood: human(filled circles, n=6), rat(filled triangles, n=4), and rabbit(filled squares, n=2). Symbols without error bars indicate single determinations and the solid line is drawn through the mean response to human blood.

In Vitro Lysis of Xenologous Erythrocytes by Canine Plasma

Preparations of human(n=4), rat(n=2), and rabbit(n=2) erythrocytes resuspended to 10% hematocrit in fresh heparinized canine plasma were rapidly hemolysed during incubation at 37℃, hemolysis being virtually complete by 2 hours(Figure 2). Control measurements of hemolysis of those cells upon resuspension in autologous plasma indicated no significant artifact due solely to handling of the cells. Complete inhibition of hemolysis of xenologous erythrocytes by canine plasma after chelation of ionized Ca^{++} and Mg^{++} with EDTA was observed, consistent with involvement of complement protein cytolytic mechanisms[8,3].

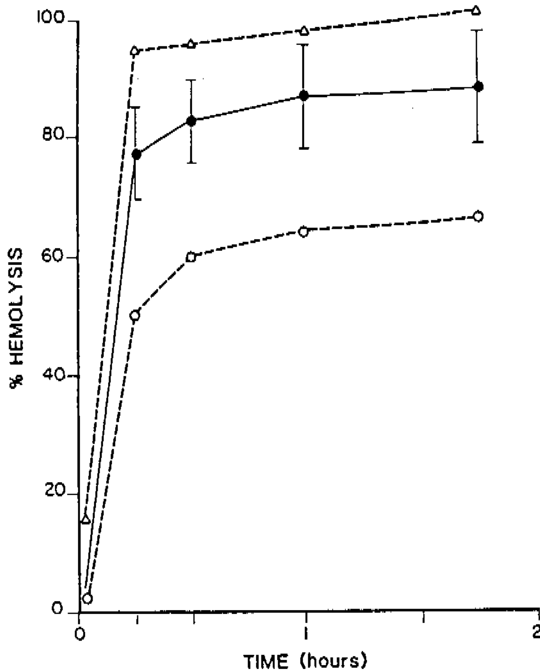

Figure 2. The time course of relative hemolysis in samples of xenologous erythrocytes incubated 2 hours in cell−free canine plasma are shown. The filled circles show mean(±SEM) hemolysis in 4 samples of human erythrocytes. The open symbols show the average hemolysis in 2 samples each of rabbit(triangles) and rat (circles) erythrocytes.

Attempts to Inhibt Hemolytic Reactions In Vivo

Preliminary studies with immunosuppression further suggest, more specifically, involvement of the cell−free pathways of complement activation[9]. Three animals each received two slow(2−3 hr) IV infusions 6−10 hours apart of cyclosporine A (Sandimmune, Sandoz Pharmaceutical Corp.) at 6 mg/kg within the 24 hour period before SA injection of 3.0 ml human blood, and daily maintenance by one slow infusion of 12 mg/kg. Cyclosporine A is believed to suppress cell−mediated mechanisms of immunological recognition and is used clinically with similar administration schedule to prevent organ transplant rejection[10,11]. The data of Table I in comparison to Figure 1, while limited, nonetheless suggest that suppression of cell−mediated immunological reactivity did not significantly alter the reaction to xenologous SA erythrocytes.

Table 1. Angiographic Results in Cyclosporine Treated Dogs.

Animal	Basilar 1−2hr	Artery 24hr	Diameter as % 48hr	of Control 72 hr
#52	93.8	74.9	died	−
#54	85.5	−	87.1	74.4
#55	−	81.5	79.5	60.2

In one trial experiment (limited by expense), the effect of in vivo "decomplementation" on chronic cerebral vasospasm in the "double−SAH" canine model[12] was examined using systemic cobra venom factor(CoVF, Cordis Laboratories), which depletes complement protein[13] by hydrolytic attack on C3. After a priming dose of 120 U/kg and daily maintenance at 500 U, periodic serum samples were assayed for [C3] and hemolytic activity using commercial kits(Diamedix Corp.). A second large priming dose 4 hours before the second experimental SAH was followed by maintenance at 500 U/day until final cerebral angiography. As seen in Table II, a high degree of C3−depletion(≥ 98%) was not maintained for more than 24−48 hours, leaving a remnant hemolytic activity of 20−25% of control value. Control and final cerebral angiography(Figure 3) indicates that reduction of serum hemolytic activity to 20−40% of control does not impair the development of moderate cerebral vasospasm.

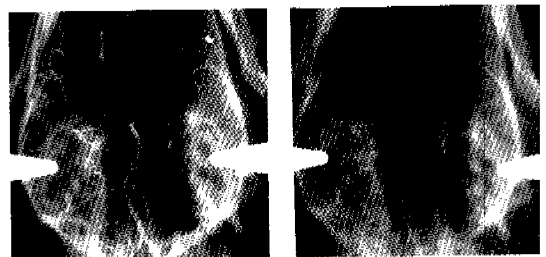

Figure 3 Angiograms taken before experimental SAH (A) and on Day 7 of the "double−SAH" protocol(B)

are shown for the CoVF-treated animal described in text. Reduction of total hemolytic activity in serum to 23-40% of control value during the final 72 hours did not prevent the development of cerebral vasospasm.

Table 2. Decomplementation by IV Cobra Venom Factor in Double-SAH Canine Model of Cerebral Vasospasm.

Day of Model	Procedure	Serum [C3]	Total Hemolytic Activity
0	Angiography		
	Serum	100%	100%
	2000 U CoVF		
1	500 U CoVF		
	1° SAH		
	Serum	2%	21%
2	500 I CoVF	–	–
3	Serum	9%	38%
	500 U CoVF		
4	Serum	8%	33%
	1500 U CoVF		
	2° SAH		23%
	Serum	1%	
5	500 U CoVF		–
	Serum	2%	
6	500 U CoVF		36%
	Serum	9%	
7	500 U CoVF		40%
	Serum	5%	
	Angiography		
Mean Hemolytic Activity after SAH (± SE)			32% ±8

Plasma Protein Extravasation for Various Subarachnoid Media

Evans Blue dye(EB) binds tightly to plasma protein, presumably primarily albumin[14]. Appearance of the dyestuff in samples of bulk CSF reflects then movement of plasma protein into CSF. After control angiography, dogs received SA injections of platelet-rich plasma(n=2) as control injectate[2], autologous whole blood(n=2), and human blood(n=2). At 48 hours after SA injection, cerebral angiography was repeated and EB slowly infused 2 hours later at 50mg/kg IV. CSF and plasma samples were taken 24 hours later and EB concentration determined spectrophotometrically against control samples of CSF and plasma. The results for each animal are shown in Figure 4, were EB concentration in CSF is expressed as % of plasma EB concentration, along with average angiographic reaction determined at 48 hours post-injection. Xenologous erythrocytes in the SA space provoked a substantial increase in the entry of plasma protein into CSF, as well as inducing severe vasospasm.

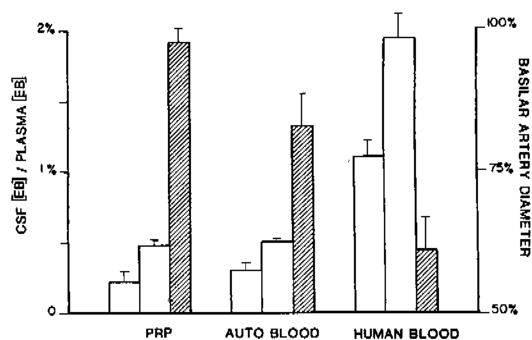

Figure 4. The bar graph shows bulk CSF concentration of Evans Blue measured 24 hours after administration of dye and 72 hours after subarachnoid injections of PRP, autologous whole blood, and human blood in each of 2 animals. Dye concentration is expressed as % of measured plasma concentration in each animal. The error bars are based on duplicate measurements of CSF and plasma samples. The cross-hatched bars show average basilar artery diameter as % of control diameter for each subarachnoid injectate(±range).

In order to verify that there was no movement of free dye(MW 960.8) from plasma to CSF, which would then not reflect possible movement of serum complement protein, samples of CSF and plasma from animals with EB were subjected to molecular ultrafiltration(MWCO 10,000, Amicon Corp.). Detectable EB in the ultrafiltrate was absent, indicating

that all EB measured in CSF and plasma samples was tightly bound to protein.

Histological Examination of Subarachnoid Materials

It was evident from gross examination of the ventral surfaces of brain stems from animals receiving SA injections of xenologous whole blood that substantial hemolysis had occurred in the SA clot. Large areas showed an overlay of material from which most hemoglobin color had been eluted in comparison to the usual coverage obtained with SA injections of autologous whole blood in the same time period. Figure 5 illustrates this finding, comparing the brain stem of an animal injected with autologous whole blood(A) at 72 hours with the brain stems of animals at 48(B) and 72(C) hours after SA injection of human blood.

Figure 5. Typical coverage of the ventral brain stem surface is shown in A at 72 hours after SA injection of autologous whole blood. Increased hemolysis in the xenologous SA clot is shown at 48 hours(B) and 72 hours(C) after SA injection of human whole blood into the canine basal cistern.

In addition to macroscopic evidence, there was, upon microscopic examination, indications of increased hemolysis and infiltration of the xenologous SA clot by inflammatory cell types, (Figure 6). In panel B, there is a somewhat increased population of dark staining cells in the perivascular space surrounding smaller vessels filled with autologous whole blood at 72 hours post−SA injection ; whereas very dense populations of such cells were seen in the perivascular space after xenologous SA blood injection, with evident reduction in erythrocyte numbers.

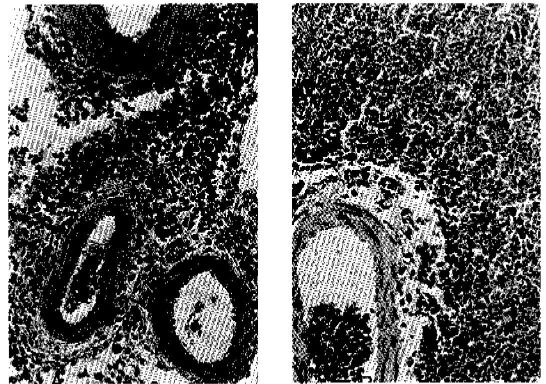

Figure 6. Light microscopy of the perivascular space of small vessels in the basal cistern surrounded by autologous whole blood clot is shown in panel B (right), indicating some degree of infiltration by inflammatory cells types. A similar micrograph of vessels surrounded by human whole blood clot shows much increased inflammatory infiltration and reduced numbers of erythrocytes in panel A(left).

Ⅳ. DISCUSSION

The experiments presented above demonstrate that while a single subarachnoid injection of autologous whole blood in dogs produces, on average, only a moderate vascular reaction in 72 hours ; a similar injection of "foreign" whole blood leads to severe cerebral vasoconstriction. Parallel in vitro experiments demonstrated that canine plasma, given access to those xenologous subarachnoid erythrocytes, would avidly lyse them. Considering the known potent vasoactivity of erythrocyte lysate(cf ref. 2), the vascular reaction observed likely arose then from hemolysis of the "foreign" subarachnoid erythrocytes as cytolytic plasma factors achieved access to those cells. This interpretation is supported by preliminary studies(Figures 4) showing that between 48 and 72 hours, which is just when vascular reaction to xenologous subarachnoid blood clot shows a dramatic

increase(Figure 1), extravasation of plasma protein into the CSF is also greatly increased relative to autologous blood. This interpretation is also in line with other experimental work showing that subarachnoid blood increases protein extravasation relative to the control condition.

It is possible that a similar immunologically−based cytolytic reaction against "aging" autologous canine erythrocytes in the subarachnoid clot plays a role in the onset of cerebral vasospasm after SAH in our canine model, as recently hypothesized for human erythrocytes[3]. The delayed onset of cerebral vasospasm of varying severity after SAH reflects then a number of competing processes. Initially after SAH, an influx of plasma protein into the subarachnoid clot provokes no major episode of hemolysis or chemotaxis of inflammatory cells because the erythrocytes are still sufficiently normal to be recognizable as an autologous tissue. After several days incubation in the non−supportive environment of the dense subarachnoid clot, however, the cells become sufficiently denatured to be lysed quantitatively by extravasated cytolytic plasma factors. Acting in the opposite direction however, is the fact that during this time period needed for subarachnoid cells to become so denatured, the mass of subarachnoid clot is continuously declining due to slow spontaneous hemolysis, breakdown of the fibrin network, and sloughing off of intact erythrocytes into the bulk CSF circulation. Ultimately, the timing and severity of cerebral vasospasm will depend upon a sufficient mass of sufficiently denatured erythrocytes surrounding the vessel at a time when blood−brain−barrier permeability is sufficiently high to admit large quantities of plasma cytolytic factors. This hypothesis is illustrated schematically in Table III below, where "+'s" give a quantitative indication of the various components in the subarachnoid blood clot as a function of time after SAH.

Table 3. Schematic of Hypothesis

	Perivascular Subarachnoid Components			
Days p SAH	Fresh RBC's	"Aged" RBC's	Cytolytic Plasma	Medial Lysate
0	+++++ +++++ +++++			
1	+++++ +++++ +++++		+	
2	+++ +++++ +++	+++	++	+
3	+++	+ +++ +++	+ +++ +	++
4		+ +++ +++	+ +++ +	+ +++ +
5		+ + +	+ + +	+ +++ +
6		+	+	+ + +
7	Resolved			

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국문초록＝

개의 실험적 지주막하 출혈 실험모델에서의 면역학적 반응에 대한 연구

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개의 혈관연축 실험모델에서 자가혈액과 이종혈액을 주입하여 단기간내의 혈관조영상의 반응을 검사하였다. 자가혈액 주입 동물에서는 72시간내에 약 15% 정도의 미약한 혈관연축을 보였으나 이종혈액을 주입한 경우에는 40% 이상의 심한 수축을 보였다.
혈액용해의 정도에 대한 실험관 측정과 개의 연구 부위에 형성된 혈종의 모양을 살펴보았을때 혈관연축의 증가는 용혈산물의 유출과 직접적인 관련이 있을 것으로 보인다.
심한 혈관연축의 발현시각과 척수액내에 혈장단백의 유출의 실험적 상관관계는 BBB의 파괴와 혈관주위의 세포분해인자의 유입을 시사하며 이러한 면역학적 반응이 지주막하 출혈에 있어서 혈관연축의 지연성 발현에 한 몫을 하는 것이다.

Keywords : Cerebral vasospasm, Subarachnoid hemorrhage, Xenologus erythrocytes,
Immunological reaction, Complement protein.