Immunological reaction against the aging human subarachnoid erythrocyte

A model for the onset of cerebral vasospasm after subarachnoid hemorrhage

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The role of the aging human erythrocyte in the mechanisms leading to cerebral vasospasm after subarachnoid hemorrhage was investigated using an in vitro model for the environment of the erythrocyte in a subarachnoid blood clot. It has long been suspected that, due to its potent vasoactivity, erythrocyte lysate provides the major vasoconstrictive input to cerebral arteries during vasospasm. Under the model conditions (incubation at 37°C in an artificial cerebrospinal fluid), however, the rate of spontaneous hemolysis was quite slow (about 1%/day), becoming only somewhat more rapid after 4 days' incubation. The rate of hemolysis of aging erythrocytes was dramatically increased (500- to 1000-fold) by the addition of plasma proteins, but only after the erythrocytes had aged 2 to 3 days, or more. The mechanism of age-dependent, plasma-induced hemolysis of originally autologous erythrocytes is shown to involve activation of the plasma complement protein pathway, analogous to the mechanisms of innate immunity which lead to lysis of nonautologous cell types and activate the inflammatory response.

KEY WORDS: erythrocyte • hemolysis • subarachnoid hemorrhage • cerebral vasospasm

While the studies reported below do not yet suggest any way to prevent cerebrovascular reaction to erythrocyte lysate, a controlling process against which intervention may be possible is identified.

Experiments in the canine model of cerebral vasospasm after SAH suggested that an immunological component plays an important role in the mechanism of cerebral vasospasm. The presence of foreign bodies alone (dextran or latex beads) in the canine subarachnoid space provoked chronic vasoconstriction which was more severe and more rapid in its onset than that produced by autologous whole blood. Histological examination of the subarachnoid space showed massive infiltration by inflammatory and immunoreactive cells. A similar, even more potent reaction in terms of vasoconstriction was found when a more physiological, but nonetheless immunoreactive, subarachnoid injectate was studied: cross-species blood. In light of the highly...
variable delay (4 to 14 days) observed clinically in the onset of cerebral vasospasm after SAH, the experiments presented here were initiated to determine whether the human erythrocyte, aging in the subarachnoid space, might lose its identity as autologous material, thereby provoking a reaction much like that seen in animal studies with subarachnoid injections of foreign bodies or cross-species blood.

Materials and Methods

Preparation and Aging of Human Erythrocytes

Approximately 10 ml venous blood was drawn from volunteers into evacuated siliconized glass tubes using a sterile technique and either of two methods of anticoagulation: heparin at 12 to 20 U/ml whole blood or tripotassium (K₃) ethylenediaminetetra-acetic acid (EDTA) at approximately 4 µmol/ml whole blood. Erythrocytes were separated by either low-speed centrifugation (500 to 800 G for 12 to 15 minutes) or passive sedimentation at 37°C for 60 to 90 minutes. Buffy coat and plasma were aspirated by sterile pipette and the erythrocytes were gently resuspended in warm sterile saline (0.9% NaCl) and centrifuged at 500 G for 7 minutes. The supernatant was removed and the saline wash step repeated. At each washing, the uppermost 1 to 2 mm of the erythrocyte pellet was aspirated to remove any traces of white cells. The erythrocytes were then washed once by resuspension and centrifugation in the sterile-filtered solution in which they would incubate for time periods up to 7 days.

The media used in this study were normal saline, bicarbonate/CO₂-buffered Krebs-Henseleit physiological saline solution (PSS), or a solution with the electrolyte content of cerebrospinal fluid (CSF). For better pH maintenance, a nonvolatile hydrogen ion buffer was used. Artificial CSF contained (in mM): NaCl, 129; KCl, 4.1; Na₂HPO₄, 1.54; HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid), 24.9; MgCl₂, 1.19; CaCl₂, 1.0; glucose, 5 or 10 in various cases; and NaOH, 14, to bring the final pH to a mean level of 7.35 (± 0.05, standard error of the mean) at 37°C. Measured osmolality without glucose was 299 mOsm. In particular experiments, other added materials were: lyophilized dog albumin; lyophilized naja haje cobra venom factor; and ethyleneglycol-bis (β-aminoethyl ether)-N, N’-tetra-acetic acid (EGTA).

The cells were then finally resuspended in the chosen medium and allowed to settle passively, incubating at 37°C for various times without agitation or opening of the tubes. The final hematocrit of the complete cell suspension was typically 15% to 20%, while hematocrit in the settled erythrocyte pellet was about 60% to 70%. Samples of these erythrocyte preparations gave no detectable white blood cell counts. These conditions of a dense pellet of erythrocytes in a diffusion-limited environment of artificial CSF resemble those of erythrocytes in a subarachnoid blood clot after clot retraction and bulk CSF circulation have removed the great majority of serum protein and other serum factors.

Determination of Spontaneous Erythrocyte Lysis

Erythrocyte lysis under these conditions was determined as a function of time by scanning absorption spectrophotometry (575 nm peak) of hemoglobin released into the incubation medium. To determine spontaneous erythrocyte lysis (that is, the release of erythrocyte contents into the stagnant pellet during incubation in the absence of added nonerythrocyte factors), the tubes were gently mixed at a chosen time of aging and two aliquots were taken into 1.5 ml plastic micro-capsules. One was subjected to eight to ten 0.5-second bursts of ultrasound on a Branson cell disruptor* for complete hemolysis and both were centrifuged for 4 minutes at 15,000 G. The absorbance spectra of the spontaneous and sonicated supernatants were measured after suitable dilution. The ratio of absorbances was taken as a measure of the fraction of erythrocytes which had lysed during the incubation period and acted as an internal correction for any changes in the shape of the absorption spectra with aging.

Analysis of Plasma-Induced Erythrocyte Lysis

Rates of hemolysis of erythrocytes incubated in this environment for various times were measured during several hours' exposure to various media (saline, artificial CSF, or autologous plasma). After sampling for determination of spontaneous lysis, the cell suspension was centrifuged at 500 G for 7 minutes and the supernatant was aspirated. The cells were then resuspended in warm sterile saline and recentrifuged. The saline wash steps were repeated (usually three times) until supernatant color indicated that lysate from less than 0.01% of cells remained.

During these steps, 30 to 40 ml fresh venous blood autologous to the incubated erythrocytes was collected by sterile venipuncture into siliconized evacuated tubes. Plasma was harvested after centrifugation at 1600 G for 12 to 15 minutes, pooled together, and recentrifuged for 10 to 12 minutes. Cell counts in plasma were below detection level. Since preliminary studies showed that ionized Ca ++ in the test medium affected erythrocyte lysis, the method of anticoagulation for preparation of plasma was important. In some cases, heparin at 12 U/ml whole blood was used, in which case ionized Ca ++ was at the normal physiological value (0.9 to 1.1 mM). In other cases, blood was anticoagulated with K₂EDTA at 7.5 ± 0.5 mM plasma concentration, so that plasma ionized Ca ++ was less than 10⁻⁷ M. Some aged erythrocytes were used directly after the last saline wash; however, in most instances the erythrocyte pellet, after being washed three times in saline, was resuspended in two to three volumes of EDTA-anticoagulated autologous plasma and centrifuged at 500 G for 5 minutes

*Branson cell disruptor, Model 350, manufactured by Branson Ultrasonics Co., Danbury, Connecticut.
FIG. 1. Spontaneous hemolysis of human erythrocytes aging under the conditions described is expressed as a fraction of total erythrocytes and plotted against time. The 24-hour intervals shown are typically ± 2 hours range for any data point. Open circles indicate preparations incubated in artificial cerebrospinal fluid, while closed circles indicate preparations in other incubation media. The solid line shows the mean values at each 24-hour interval.

Results

Rate of Spontaneous Lysis of Aging Human Erythrocytes

Erythrocytes from four volunteer donors were aged at 37°C in either normal saline, Krebs-Henseleit PSS, or, in most cases, artificial CSF. Spontaneous hemolysis was determined at times from 0 to 7 days. No consistent dependence on choice of incubation medium was found. The unreduced data are shown in Fig. 1.

The rate of spontaneous hemolysis during the first 72 hours is comparatively constant, averaging by linear regression analysis 1.01% ± 0.14%/day with a regression coefficient of 0.73. In adult humans, the erythrocyte lifetime ranges from 110 to 135 days in situ; thus, the rate of erythrocyte clearance from the systemic circulation (0.75% to 0.90%/day) does not differ substantially from our observed rate of spontaneous lysis.

Beginning at around Day 3, however, the rate of spontaneous hemolysis increases. Linear regression analysis of the data from Days 3 to 7 gives an average rate of lysis of 3.54% ± 0.38%/day over that time period (r = 0.71). Nonlinear least-squares fitting to a continuous exponential process for all data for Days 0 to 7 marginally improves the regression coefficient to 0.77 but the fit of this function to the first 3 days, where the data seem most reproducible, is poor. The data seem best represented by a two-component linear process. Further experiments regarding this characteristic "break-point" in the time-course of spontaneous lysis with aging are presented below under the heading "Effects of pH and Glucose".

Plasma-Factor Induced Lysis of Aging Human Erythrocytes

Experiments were undertaken to determine as a function of time the extent to which the human erythrocyte, aged under conditions resembling those of the subarachnoid blood clot, lost its identity as autologous tissue. Loss of autologous identity was measured as lysis of erythrocytes upon addition to autologous plasma, in the sense that cytolytic activity in human blood is activated by the presence of virtually any foreign cell type. For internal consistency, considering the variability indicated in Fig. 1, these measurements were performed with erythrocytes and plasma from a single donor. A brief confirmatory set of identical measurements was made using blood from a second donor.

After sampling the resuspended aged erythrocytes to measure spontaneous hemolysis, cells were washed three times with warm sterile saline and once with EDTA-anticoagulated autologous plasma. Aliquots of packed erythrocyte pellets were suspended in various test media and hemolysis, expressed as the percent of total erythrocytes, was determined over a 3-hour incu-
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**Figure 2.** Mean values (± standard error of the mean) of relative hemolysis (% of total erythrocytes) in erythrocyte preparations aged *ex vivo* for various time periods during a 3-hour period following addition of the cells to autologous plasma containing normal levels of ionized Ca²⁺ (*open circles*). The *filled circles* show the mean values of "background" lysis, that is, hemolysis measured in autologous plasma with zero ionized Ca²⁺ and in artificial cerebrospinal fluid (CSF) with or without ionized Ca²⁺; *n* indicates the number of data sets comprising the means for hemolysis in autologous plasma with normal Ca²⁺, while background lysis was determined in a larger number of data sets. In Panel A, the ordinate differs from that in other panels by a factor of 100. In Panel D, hemolysis in autologous plasma with added EDTA was measured in eight data sets and was significantly greater (mean ± standard error of the mean: 13.5% ± 2.6% at 3 hours) than the spontaneous background lysis observed in artificial CSF with or without ionized Ca²⁺, which averaged 4.0% ± 2.7% at 3 hours. No such difference was noted in the other panels.

Figure 2 shows the time-courses of "plasma-induced lysis" of erythrocytes aged 0 and 1 day, 2 days, 3 days, and 5 days, as determined in autologous plasma in the presence or absence of divalent metal cations. Stagnant aging of human erythrocytes up to 24 hours does not significantly impair recognition of these cells as autologous tissue (that is, there was no significant hemolysis beyond control levels). By 3 days of aging, however, the loss of autologous recognition is virtually complete. In autologous plasma at normal or reconstituted concentrations of divalent metal cations, a 5% to 10% hematocrit of human erythrocytes aged 3 to 5 days is about 60% lysed in 1 hour and 80% to 90% lysed within 3 hours. The time courses of hemolysis in Panels C and D of Fig. 2 are not statistically different. Clearly, there is a rapid and progressive loss of autologous identity between 24 and 72 hours of incubation, even though the rate of spontaneous lysis of such cells remains low (about 1%/day).

A direct comparison between the rate at which aging
human erythrocytes spontaneously lyse under these conditions and the rate at which identically aged cells develop an autoimmunogenicity which activates their lysis by plasma factors is shown in Fig. 3. The lower panel shows mean values of spontaneous lysis, fit by linear regression in the time ranges 0 to 3 days and 3 to 7 days. The upper panel shows mean values of plasma-induced lysis measured after 3 hours of exposure to autologous plasma with normal or essentially zero divalent metal cation concentration. Lysis measured in control media with or without added EDTA was less than 1% at 3 hours treatment in all cases except Day 5, which averaged 4.0% ± 2.7%. Hemolysis measured in normal autologous plasma for erythrocytes aged 3 and 5 days was not statistically different.

It should be emphasized that the above results pertain to erythrocytes incubated at 37°C. In two cases, fresh human erythrocytes were prepared exactly as above and incubated for 5 days at 4°C. During 3 hours of exposure to autologous plasma, erythrocyte lysis was less than 0.01% in both cases.

**Effects of pH and Glucose**

Experiments were performed to determine changes in certain readily accessible parameters and whether altering those parameters affected observed rates of spontaneous and induced lysis. The simultaneous time courses of pH, glucose, and spontaneous lysis in the incubation medium were determined in multiple preparations of erythrocytes from two different donors at times between 0 and 7 days (Fig. 4). While there is some consistent difference in the data for the two donors, similar trends are evident. The average time-

### Table 1

**Comparison of spontaneous erythrocyte lysis in perfused and nonperfused aged preparations**

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Perfused Sample</th>
<th>Non-Perfused Sample</th>
<th>% Lysis</th>
<th>Difference</th>
</tr>
</thead>
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<td></td>
<td>Glucose pH</td>
<td>Glucose pH</td>
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<tr>
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<tr>
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<td>60 6.01 14.50</td>
<td>0 6.25 14.20</td>
<td>+0.30</td>
<td></td>
</tr>
</tbody>
</table>

*Glucose is expressed in mg/dl. The mean change in % lysis is not statistically significant at the p = 0.01 level.*

![Fig. 3](image)

**Fig. 3.** Lower: The spontaneous hemolysis of aging human erythrocytes is shown as a function of incubation time in the lower panel. Mean values (± standard error of the mean (SEM), when larger than the symbol) were calculated from the data of Fig. 1, while the solid line represents linear regression results. Broken line indicates occurrence of unidentified processes which result in higher rates of spontaneous lysis in erythrocytes aged more than 3 days. Upper: The mean relative hemolysis measured in similarly aged erythrocyte preparations after a 3-hour exposure to normal autologous plasma (filled circles) and to plasma with zero ionized Ca**++**(open circles). For various incubation periods, the average spontaneous hemolysis values (± SEM) observed in the data sets of the upper panel were 0.22% ± 0.5% for 0 days (three data sets), 0.68% ± 0.12% for 1 day (three data sets), 3.22% ± 0.30% for 2 days (four data sets), 2.75% ± 1.23% for 3 days (five data sets), and 6.80% ± 1.60% for 5 days (five data sets). These values differ slightly (but in most cases not significantly) from the mean values observed in the larger data sets of the lower panel.

![Fig. 4](image)

**Fig. 4.** A and B: The measured values of pH (electrode) and glucose (reagent test strip) in the incubation medium of erythrocytes aged for various times are shown. Open circles and filled circles represent data for two different donors. Solid lines are drawn through the mean values (± standard error of the mean (SEM)) at each 24-hour interval. C: Mean (± SEM) spontaneous hemolysis measured in the same data sets as above on each day. The numbers denote the number of data sets.
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course of spontaneous lysis for this data subset is essentially the same as that presented in Figs. 1 and 3, except that the initial low rate of lysis (1%/day) appears to extend fully to Day 4. The percentage of lysis on Day 7, however, does not differ statistically from the data of Fig. 1.

The data for pH and glucose show a clear parallel trend, each falling abruptly for the first 2 to 3 days, then becoming nearly constant for the rest of the incubation periods (Fig. 4A and B). During the latter time, the rate of spontaneous lysis, after some delay, increased significantly (Fig. 4C). While the parallel decline in and eventual leveling off of pH and glucose seem simply explained on the basis of lactic acid production and eventual substrate depletion, it is not obvious that cessation of energy metabolism is directly responsible for the increased rate of spontaneous erythrocyte lysis, since a significant increase is not seen until fully 24 to 36 hours after pH and glucose have attained minimum values.

To further examine this question, samples of washed erythrocytes were prepared in duplicate. One tube was aged exactly as before. The second tube was allowed 2 hours for erythrocyte sedimentation, then was continuously perfused from a motor-derived syringe fitted with a sterilizing filter through a No. 22 needle which reached the bottom of the tube. Overflow through a needle which just reached the upper surface of the incubation medium was collected for analysis of spontaneous lysis. The comparison was performed once with cells aged 7 days and twice with cells aged 5 days. The perfusion rate into the 12-ml tubes was 5.6 to 6.9 ml/day with artificial CSF containing 10 mM glucose. In one case (7-day incubation), 300 µg/ml canine albumin was added to the incubation and perfusion media to resemble the protein content of CSF and possibly enhance erythrocyte stability. The individual results of the paired comparisons are given in Table 1. The data indicate that continuous perfusion with glucose-containing medium, while adequate to prevent substrate depletion, did not significantly reduce spontaneous lysis. These preparations were further used to show that development of lysis induced by plasma factors, as seen in Figs. 2 and 3, is not an artifact of substrate depletion. Both sets of continuously perfused and nonperfused erythrocytes aged 5 days were tested for plasma-induced hemolysis in autologous plasma. As shown in Fig. 5, continuous provision of substrate for energy metabolism does not reduce the rate of hemolysis observed upon adding the cells to autologous plasma relative to the rates observed with stagnant aging of the same cells.

Role of Plasma Complement Protein and Divalent Metal Cation

Plasma, autologous to erythrocytes aged 72 hours and anticoagulated with heparin, was incubated at 37°C for 60 or 90 minutes after the addition of cobra venom factor, 10 or 20 U/ml respectively (amounts adequate to effectively decomplement the plasma). To one-half of this plasma, 7 mM EDTA was added to chelate Ca++ and Mg++. Another tube of identically prepared heparinized plasma was incubated simultaneously without adding cobra venom factor. An aliquot of aged erythrocytes was added to each of the three tubes, and hemolysis was determined over a 3-hour incubation period at 37°C. Compared to normal plasma, the hemolytic activity of decomplemented plasma, after subtracting background lysis, was inhibited by 72% and 76%, respectively, in the two experiments (Fig. 6).

It is known that the formation of activated complement protein complexes requires Mg++ ions. Preliminary studies indicate that the full expression of hemolytic activity shows a high-affinity requirement for Ca++
as well (Fig. 7). Various concentrations of EGTA, which chelates Ca++ with much greater affinity than Mg++, were added to volumes of heparinized plasma. With EGTA up to 2.5 mM, the calculated values for ionized Mg++ remained virtually constant. Aliquots of erythrocytes aged 72 hours were added and the time course of hemolysis was measured over 3 hours. Only when ionized Ca++ was reduced to 10 μM was a measurable reduction (about 25%) in hemolysis observed. In the presence of 0.9 to 1.1 mM Mg++, reduction of ionized Ca++ to 1 μM inhibited hemolysis as effectively as did the removal of both divalent metal cations by the addition of EDTA. We have not yet identified conditions which remove Mg++ in the presence of nearly constant Ca++, so the extent to which Ca++ ions may substitute for Mg++ in this process remains unknown.

**Discussion**

As shown in Figs. 1 and 3, the human erythrocyte incubating ex vivo under conditions resembling the environment of the subarachnoid blood clot after SAH lysed only very slowly (at about 1%/day). While hemolysate is strongly vasoactive, the products of hemolysis may diffuse into the bulk CSF circulation as well as the media of nearby cerebral arteries. For rates of spontaneous hemolysis as determined here, the concentrations of hemolysate reaching vascular smooth-muscle cells may be insufficient to provoke more than a minimal reaction.

As suggested in the lower panel of Fig. 3, the unidentified processes which lead to somewhat higher rates of spontaneous lysis in erythrocytes aged more than 3 days probably begin around Day 2, which is also the earliest time at which exposure to plasma factors induces significant hemolysis of aged intact erythrocytes. This coincidence in time is suggestive that both processes involve some common step in their mechanisms, such as denaturation of important membrane-associated proteins. Such proteins could include not only structural factors, but immunological recognition factors as well.

In vivo, nonautologous cells can activate the complement protein cascade, leading to the formation of terminal membrane attack complex and subsequent lysis of foreign cells. As shown in Figs. 2 and 3, human erythrocytes incubated 72 hours or more under conditions like those of the subarachnoid clot are rapidly lysed upon exposure to autologous plasma. It was further shown that this aging-dependent hemolysis involves activation of the complement protein system via the pathways of innate immunity.

Plasma complement protein can be reduced to less than 5% of control value by brief treatment with purified cobra venom factor, which massively activates the complement cascade by direct enzymatic action on C3. Activated complement factors are then destroyed by endogenous processes, effectively decomponenting the plasma. As seen in Fig. 6, hemolysis of aged erythrocytes by plasma decomplemented by cobra venom factor was inhibited by approximately 75%. The 25% hemolytic activity remaining was likely due to incomplete decomponenting. Since the complement cascade involves great amplification at successive steps, a remnant 5% of C3 activity after treatment with cobra venom factor may express much more than 5% endpoint activity (hemolysis, in this case). In one in vivo trial of decomponenting as prophylaxis against cerebral vasospasm in the double-SAH canine model, a 7-day regimen of intravenous cobra venom factor (6000 U total) reduced serum C3 to 3% to 5% of control value, but total serum hemolytic activity remained at 40% of control. This in vivo treatment, which failed to prevent vasospasm as well, indicates that complete blockade of plasma hemolytic activity by decomponenting is likely unattainable.

In vivo studies of the cerebrovascular response to nonautologous (cross-species) erythrocytes in the canine subarachnoid space suggest that immunoreaction against the subarachnoid clot and subsequent hemolysis may play a role in the onset of cerebral vasospasm after SAH. The results presented above demonstrate that human erythrocytes embedded in subarachnoid blood clot may well lose their identity as autologous tissue with time and provoke a similar immunoreaction. If, in vessels surrounded by adventitial subarachnoid blood clot, the blood-brain barrier becomes sufficiently impaired that plasma complement proteins are extravasated, then erythrocyte lysis would proceed rapidly in a fashion quantitatively related to vessel permeability. That same process would lead to the formation of activated complement factors which are chemotactic for inflammatory and immunoreactive cell types, possess some inherent vasoactivity, and can further increase vessel-wall permeability (such as C5a and histamine); in short, the process is subject to positive feedback. Under the appropriate conditions, this cy-
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...icle of events may lead to an explosive release of erythrocyte lysate and consequent severe vasospasm with rapid onset.

The results presented here lead us to propose a multifactorial model for human cerebral vasospasm after SAH which involves at least three contributory and interrelated factors: 1) an inflammatory/immunological reaction against erythrocytes progressively rendered functionally nonautologous by aging in the subarachnoid space; 2) increased local blood-brain barrier permeability in vessels underlying the subarachnoid clot and extravasation of plasma complement proteins, with consequent chemotactic, hemolytic, and permeability increasing activities; and 3) rapid release of erythrocyte lysate induced by hemolytic plasma complement proteins. This model is consistent with two recent clinical studies reporting that increased serum immunocomplexes correlate closely with the occurrence of cerebral vasospasm in patients with SAH. The importance of inflammatory processes was previously considered in the canine model of cerebral vasospasm. Furthermore, it has been shown that the endothelium of canine basilar artery, while appearing morphologically intact, is functionally impaired after SAH; this finding has been confirmed by others. Several laboratories have reported increases in blood-brain barrier permeability in cerebral arteries after exposure to subarachnoid blood or other insults. Successful therapy against cerebral vasospasm after SAH may well require simultaneous management of all three contributory factors described above.

References

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