Acute intravascular hemolytic anemia in the black rhinoceros: Hematologic and immunohematologic observations

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SUMMARY

To investigate the syndrome of acute intravascular hemolytic anemia in the black rhinoceros (*Diceros bicornis*), laboratory techniques used in the differential diagnosis of hemolytic anemias were performed on blood samples from 6 black rhinoceroses: 3 nonrelated healthy rhinoceroses, 1 rhinoceros with iron deficiency anemia, and 2 rhinoceroses with intravascular hemolysis. Osmotic fragility, erythrocyte membrane protein composition, hemoglobin electrophoresis, and hemoglobin stability did not distinguish between healthy and affected (anemia or hemolysis) rhinoceroses. Polyclonal antiglobulin reagents were prepared in rabbits, using whole rhinoceros serum and purified rhinoceros immunoglobulin G. These reagents were nonreactive against erythrocytes of the healthy and iron-deficient rhinoceroses. Reactions with RBC from the rhinoceros with fatal hemolytic anemia indicated increased membrane coating by the third component of complement; this was not evident in a second rhinoceros that survived a hemolytic event.

Hemolytic anemia is an important disease in captive black rhinoceroses (*Diceros bicornis*).[1-4] In one survey, 27 episodes of hemolysis in 20 black rhinoceroses were identified, 16 episodes were fatal, and 40% of mortalities of adult black rhinoceroses were associated with hemolytic anemia. Although findings in several rhinoceroses were consistent with leptospirosis, findings in many of the others were not.

Severe, occasionally fatal, massive intravascular hemolysis has been described in dogs, cats, horses, children, and human adults.[5,6] The underlying pathogenesis reflects an intrinsic erythrocyte defect (generally, deficiency of an enzyme essential to cellular metabolism or a hemoglobinopathy) or an extrinsic process (such as infection, exposure to chemicals directly toxic to RBC, or an immunologically mediated process, such as autoimmunization or sensitization to drugs or chemicals, resulting in deposition of antibody and/or complement on the erythrocyte membrane).[6] In man, intrinsic defects are usually heritable and characteristically have a familial incidence. Human autoimmune hemolytic anemia and drug/chemical immunologically mediated hemolysis generally do not follow a familial pattern, although rare examples of familial human autoimmune hemolytic anemia have been reported.[7]

The purpose of the present study was to evaluate blood samples from healthy black rhinoceroses and black rhinoceroses with hemolytic anemia, using general laboratory techniques used in the differential diagnosis of human hemolytic anemias and antiglobulin reagents prepared against rhinoceros serum proteins.

Materials and Methods

Blood samples—Blood samples from 6 rhinoceroses were evaluated. Two of the rhinoceroses had histories of intravascular hemolysis of unknown cause: a 20-year-old male (rhino 1, studbook No. 155)[8] that died during a hemolytic crisis at Busch Gardens in Tampa, Fla, and a 12-year-old male (rhino 2, studbook No. 161) that had a mild episode of hemolysis/hemoglobinuria at the Denver Zoological Park. The 3 healthy rhinoceroses evaluated were a 23-year-old female from the St Louis Zoo (rhino 3, studbook No. 121), an 8-year-old male from the St Louis Zoo (rhino 4, studbook No. 251), and a 9-year-old female from the Detroit Zoological Park (rhino 5, studbook No. 212). Rhino 5 had given birth to 4 offspring by a previous male; 3 of the offspring were affected by hemolytic crises and 2 died during their episodes. Samples were also obtained from a female black rhinoceros (rhino 6, no studbook No.) at a Texas ranch,[a] with a presumed blood loss anemia secondary to a chronic gingivitis; this rhinoceros was the only animal of South African origin evaluated (the other 5 rhinoceroses were East African in origin or parentage.

Hematologic evaluations—Using a Coulter counter,[b] complete blood cell count and RBC indices[9] were performed on blood collected into EDTA anticoagulant. Immediate and incubated osmotic fragilities were performed essentially as described by

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[a] Mantzell T, Waterfall Ranch, Fort Worth, Tex.

[b] Model S Plus III, Coulter Electronics Inc, Hialeah, Fla.

Dacie,[10] using a commercial test kit.[c] The sucrose hemolysis test was performed according to the method of Hartman and Jenkins.[11] Erythrocyte membrane proteins were examined after processing, using the method of Laemmli.[12] Hemoglobin electrophoresis was performed on cellulose acetate membranes at pH 8.6, according to the method of Briere et al,[13] and on acid citrate agar at pH 6.2, as described by Robinson et al.[14] The isopropanol test for unstable hemoglobins was performed according to the method of Carrell and Kay.[15] Rhinoceros serum proteins and purified protein fractions were evaluated according to Johansson's method, using agarose-gel electrophoresis,[16] and Kohn's method for cellulose acetate electrophoresis.[17] Immunofixation was performed according to the method of Richie and Smith.[18] Immunoelectrophoresis was performed as described by Scheidegger.[19] Washed rhinoceros RBC were suspended in a solution containing sucrose 15.4%, dextrose 5.9%, and NaCl 0.29%, and were droplet frozen in liquid nitrogen, and stored in liquid nitrogen (−196 C). When needed, droplets were added to 0.9% NaCl at 37 C and washed with saline solution at room temperature (~ 22 C) until free of hemolysis.

Preparation of immunoglobulin (Ig)G-coated and C3-coated RBC—Human Rh (D)-positive RBC (Hu RBC) were coated with human IgG anti-D (Hu IgG), by incubation at 37 C for 30 minutes (Hu RBC-Hu IgG); these RBC were not coated by human complement components. Human RBC were coated with rhinoceros IgG (Hu RBC-rhino IgG) and rhinoceros RBC (rhino RBC) were coated with rhino IgG (rhino RBC-rhino IgG), using the chromic chloride method.[20] Human RBC were coated with human C3b (Hu RBC-Hu C3b) by activation of the alternative pathway as previously described.[21] Human RBC were coated with human C3d (Hu RBC-Hu C3d) by incubation of Hu RBC-Hu C3b with 0.1% trypsin in 0.1*M* phosphate buffer (pH 7.7) at 37 C for 30 minutes. Human RBC were coated with rhinoceros C3b (Hu RBC-rhino C3b) and with rhinoceros C3d (Hu RBC-rhino C3d) exactly as previously described,[21] except that washed human RBC suspended in thawed rhinoceros acid-citrate dextrose (ACD) preservative plasma to a PCV of 40% were substituted for whole human blood prior to complement activation. Rhinoceros and human blood had been collected into ACD solution. Such RBC were nonreactive with anti-rhino IgG reagent. Rhinoceros RBC were coated with rhino C3b (rhino RBC-rhino C3b) by activation of the classical complement pathway as described by Freedman and Mollison,[22] using whole rhinoceros ACD blood. To produce rhino RBC-rhino C3d, the rhino RBC-rhino C3b were trypsinized as previously described, except that incubation at 37 C was for only 15 minutes.

Preparation of antisera to rhinoceros serum proteins—To prepare anti-whole rhinoceros serum (anti-WRS) reagent, New Zealand White rabbits were given 4 intraperitoneal injections of healthy black rhinoceros serum suspended in colloidal aluminum hydroxide, as previously described.[23] Blood was collected from these hyperimmunized rabbits beginning 1 week after the 4th injection. Their serum was separated and was heat inactivated for 30 minutes at 56 C.

When anti-WRS was reacted against whole rhinoceros serum that had been electrophoresed in 1.5% agar at pH 8.6, > 20 distinct precipitin arcs were observed, in a pattern generally similar to that seen with anti-whole human serum reacted against human serum. A broad cathodal precipitin arc was seen closely resembling human IgG in position, staining, and intensity.

Serial dilutions of the anti-WRS caused agglutination of healthy rhinoceros RBC to a titer end point of 512. Multiple absorptions of the antiserum with human RBC (washed 10 times with isotonic saline solution) did not affect agglutination of rhinoceros RBC. However, 2 absorptions with equal volumes of washed healthy rhinoceros RBC completely removed agglutinating antibody for the rhinoceros RBC used for absorption and for RBC from 2 other healthy rhinoceroses. Antiserum absorbed only with rhinoceros RBC reacted weak to trace with normal group O human RBC to a titer of 4.

To prepare anti-rhino IgG reagent, rhino IgG was purified, using anion exchange chromatography.[24] Rhinoceros serum was equilibrated against 0.0175*M* phosphate buffer (pH 6.8) and was applied to a DEAE-cellulose column equilibrated and eluted with the same buffer. A large, almost symmetrical, colorless protein peak emerged from the column; a variety of colored serum components remained tightly bound at the top of the column. The protein peak was concentrated, using positive-pressure ultrafiltration to a final protein concentration of 20 mg/ml, as determined by UV absorption at 280 nm, using an extinction coefficient E $(\frac{1\%}{1\ cm})$ of 13.5. Microzone cellulose acetate paper electrophoresis and agarose-gel electrophoresis confirmed a single heterogeneous protein band in the position expected for human IgG.[25] Immunofixation indicated cross-reactivity with anti-human IgG.

Rabbits were given 4 intraperitoneal injections of rhino IgG (1 mg/injection) suspended in colloidal aluminum hydroxide, as previously described.[23] Antisera were harvested, heat inactivated, and tested against electrophoresed rhinoceros serum and against purified rhino IgG. Broad mirror-image, intensely stained cathodal precipitin arcs were observed against both electrophoresed products. The absence of additional arcs with rhinoceros serum supports the monospecificity of the anti-rhino IgG sera.

The anti-rhino IgG serum was tested in 10 serial dilutions against healthy rhinoceros RBC. Agglutination was not observed, obviating the necessity for absorption of the reagent before use with rhino RBC. The nonabsorbed serum gave only trace agglutination of human RBC, with a titer of 2.

Performance of antiglobulin tests—Antiglobulin sera were serially diluted from 1-in-2 to 1-in-1,024 in isotonic saline solution. Red blood cells were washed 4 times in isotonic saline solution, and diluted in saline solution to a 5% suspension. One drop of each serum dilution was mixed with 1 drop of cell suspension on an opaque glass tile, and gently rocked at room temperature. Reactions were visually examined after 5 and 10 minutes and were graded from ± to 4 (± = rare 2- to 4-cell agglutinates, mostly free cells; 1 = many 2- to 8-cell agglutinates, many free cells; 2 = many 4- to 20-cell agglutinates, few free cells; 3 = numerous 10- to 20-cell agglutinates, numerous multicell masses, rare free cells; 4 = large masses of agglutinated cells, 0 to rare free cells).

Results

Hematologic values—Blood was collected from rhino 2 during an episode of moderate intravascular hemolysis. Rhino 6 had severe hemorrhagic gingivitis and associated blood loss anemia when blood was collected (Table 1). Unlike the 1% to 2% of reticulocytes normally seen in man,[26] reticulocytes rarely were seen in the 2 healthy rhinoceroses. Rhinoceros platelets appeared small and remarkably uniform in size, compared with normal human platelets.[26] This observation was confirmed by the low mean platelet volumes of 4.3 and 5.3 fl, compared with the normal human values (7.4 to 10.4 fl).[26]

Reliable evaluation of hematologic values could not be determined for blood samples collected from rhino 1, which died of massive hemolysis; its blood was almost totally hemolyzed (PCV = < 5%). The Denver Zoological Park reported that for rhino 2 (with relatively mild hemolytic anemia), the hemoglobin concentration decreased from 12.4 g/dl to 9.5 g/dl, the PCV decreased from 37% to 28%, and the RBC concentration decreased from $5.52 \times 10^6/\mu l$

[c] Unopette Test 5830, Becton, Dickinson & Co, Rutherford, NJ.

TABLE 1—Hematologic values* of two healthy black rhinoceroses, one black rhinoceros with intravascular hemolysis, and one black rhinoceros with presumed blood-loss anemia

Health status	Rhino No.	Blood collected (mo/day/yr)	Blood evaluated (mo/day/yr)	Hb (g/dl)	RBC ($\times 10^6/\mu l$)	PCV (%)	Retic (%)	WBC ($\times 10^3/\mu l$)	MCV (μm^3)	MCH (pg)	MCHC (g/dl)	Platelets (No./μl)	MPV (fl)
Intravascular hemolysis	2	9/21/84	9/22/84	12	3.64	35.7	0	5.4	98.2	33	33.6	330,000	
Healthy	3	4/24/84	4/24/84	14.3	4.52	43.5	0.2	8	96.3	31.6	32.8	148,000	5.3
	4	11/29/83	11/29/83	15.2	4.93	46.4	0	9.3	94.2	31	32.9	282,000	4.3
Blood-loss anemia	6	2/26/85	2/27/85	8.1	3.17	24.6		6.9	77.6	25.7	33.0	239,000	

* Normal rhinoceros values (n = 6) for Hb = 16.6 g/dl, RBC = 5.2 × $10^6/\mu l$, PCV = 47.9%, WBC = 10.7 × $10^3/\mu l$, MCV = 31.6 pg, and MCHC = 34.6 g/dl.
Hb = hemoglobin, Retic = reticulocytes, MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, MPV = mean platelet volume, NA = not applicable.

to 3.35 × $10^6/\mu l$ in the 20 days after hemoglobinuria was noticed. There was no change in mean corpuscular volume or mean corpuscular hemoglobin concentration. Morphologically, RBC from rhinos 1 and 2 were not different from those of the healthy rhinoceroses (rhinos 3 and 4). Reticulocytes were not seen in the blood of rhinos 1 and 2, although 1 nucleated RBC/100 RBC was noticed in the first sample from rhino 2 and rare nucleated RBC were noticed on the smear from severely affected rhino 1. The abnormal findings for rhino 6 (Table 1) were typical for iron deficiency secondary to chronic blood loss anemia, ie, microcytosis (mean corpuscular volume was 77.6 fl) and hypochromia (mean corpuscular hemoglobin concentration was 25.7 pg), clearly distinguishing the pathogenesis of the anemia of rhino 6 from that of the moderate intravascular hemolytic process of rhino 2.

Immediate and incubated osmotic fragility of rhinos 3 and 4 (healthy rhinoceroses) and of rhino 2 (moderate hemolytic anemia) were within normal limits for those of man.[10] Sucrose hemolysis tests (used to detect the human erythrocyte abnormality in patients with paroxysmal nocturnal hemoglobinuria[11]) could be performed reliably only on blood samples from rhinos 3 and 4; the results were within the normal range for man.[11]

Rhinoceros RBC antigenic reactivity—Red blood cells from 2 healthy adult black rhinoceroses (rhinos 3 and 4) were agglutinated by sera from pigs, chickens, goats, man, monkeys, sheep, dogs, and cattle. The same RBC were not agglutinated by sera from rabbits, guinea pigs, horses, tapirs, or mice. Antisera specific for 15 human blood group antibodies (A, B, A-B, D, C, E, c, e, Le^a, M, P_1, Kell, Fy^a, S, and Jk^a) did not agglutinate rhino RBC. However, positive reactions with 3 of 4 lectins indicated the presence of membrane carbohydrate residues commonly related to human blood group systems.

Rhinoceros RBC membrane proteins—Isolated RBC membrane proteins of rhinos 3 and 4 (healthy rhinoceroses) and from rhino 2 were evaluated. Cell suspensions were depleted of leukocytes, and RBC stroma (prepared by hypotonic lysis) were solubilized and subjected to sodium dodecyl sulfate polyacrylamide-gel electrophoresis. Rhinoceros protein bands were visualized (silver staining) and compared with identically prepared human RBC-membrane proteins.

Rhinoceros samples contained a deeply staining band at approximately 90 kD, corresponding to human band 3 protein.[27] Rhinoceros samples also had a deeply staining band in the band 4.5 region of human RBC membranes.[27] The rhinoceros band was relatively condensed in comparison to the multiple components evident in the human band 4.5 region. A rhinoceros band corresponding to human band 6[27] also was seen.

Comparisons of RBC membrane bands from the 3 rhinoceros samples did not indicate a definitive difference among them. However, comparisons were not optimal because the rhinoceros samples became available at different times, and thus could not be electrophoresed simultaneously on a single gel. Nonetheless, highly similar patterns were obtained for RBC from the 2 healthy rhinoceroses and from the mildly affected rhinoceros (rhino 2), using RBC coincident with active hemolysis and RBC obtained 1 month later, when the rhinoceros was convalescent, without evidence of hemolysis.

Rhinoceros hemoglobin electrophoretic mobility—Hemoglobin from rhinos 3 and 4 was electrophoresed under standard conditions on cellulose acetate paper at pH 8.6 and on citrate agar at pH 6.2. An example and a mixture of human A, F, S and C hemoglobins are compared in Figure 1. At pH 8.6, rhinoceros hemoglobin separated into 2 bands, both of which migrated further anodally than did human A hemoglobin. The major rhinoceros hemoglobin band (accounting for approx 80% of the total hemoglobin) migrated distal to the region of the abnormal unstable human hemoglobin H. At pH 6.2, rhinoceros hemoglobin migrated cathodally as a single band (with possibly a faint slower-migrating component); its position corresponded approximately to that of human F hemoglobin. Blood was collected from rhino 2 (the moderately affected rhinoceros) during the episode of hemolysis and after the hemoglobin concentration had returned to normal; both electrophoretic patterns were indistinguishable from those of the 3 unrelated healthy rhinoceroses.

Rhinoceros hemoglobin stability—The isopropanol test for unstable human hemoglobins was applied to one hemolysate prepared from healthy rhinoceros RBC (rhino 3) that had been stored frozen for 11 months at −80 C and to another hemolysate prepared from an EDTA-blood sample from rhino 6 that had been airmailed overnight (without refrigeration) and subsequently stored for 7 days at 4 C before evaluation. Both samples had moderately decreased stability, compared with hemoglobin from a freshly collected blood sample from a healthy human volunteer. However, because of the unusual storage conditions of both rhinoceros samples, definite conclusions could not be made about the stability of rhinoceros hemoglobin relative to normal human hemoglobin.

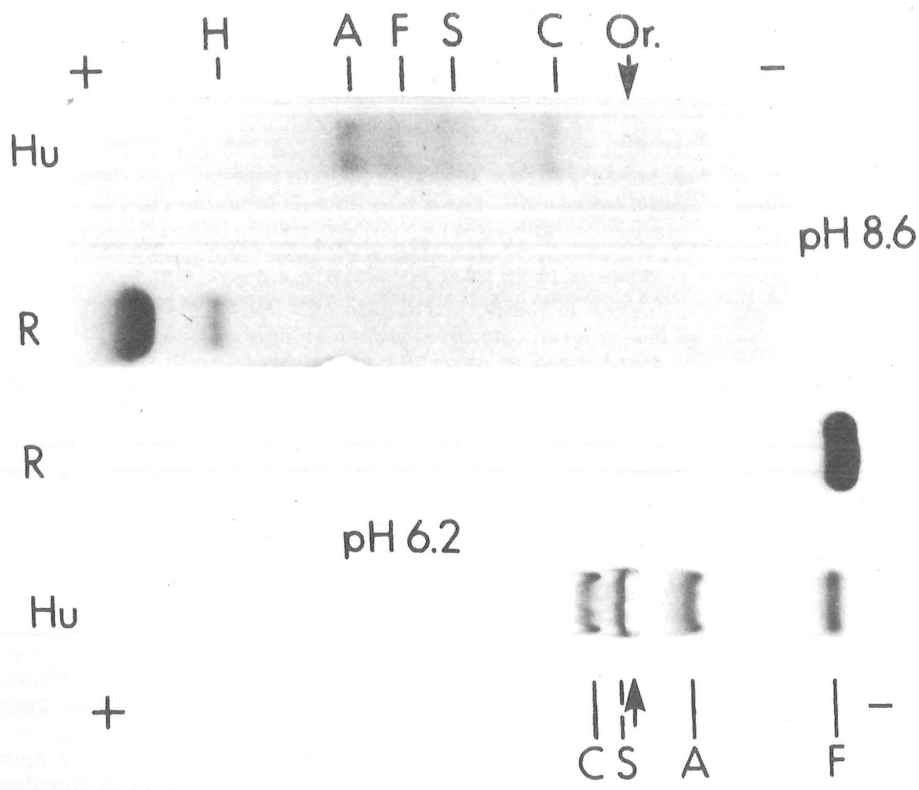

Fig 1—Electrophoresis of hemoglobin from a healthy black rhinoceros (R = rhino 4), using cellulose acetate paper at pH 8.6 and acid citrate agar at pH 6.2. A mixture of human (Hu) hemoglobins is shown for comparison (identified are bands for hemoglobins A, F, S, and C). The approximate position of human hemoglobin H (not included in the mixture) also is indicated. Points of application (Or.) are indicated by arrows.

Electrophoretic comparison of human and rhinoceros serum proteins—In preparation for making broad-spectrum and monospecific anti-IgG antisera to black rhinoceros serum proteins, agarose-gel electrophoresis at pH 8.4 of nonhemolyzed serum from a healthy black rhinoceros (rhino 4) was compared with normal human serum (Fig 2). Several similarities were apparent in the 2 specimens stained with amido black for protein. The densely stained anodal homogeneous band was analogous to human serum albumin, and the heterogeneous smudge on either side of the origin was in the position of human IgG. Using immunofixation and antisera monospecific for well characterized human serum proteins, cross-reacting precipitation permitted localization of the following rhinoceros serum proteins: IgG, IgM, C3 (2 bands), and transferrin. Cross-reactions did not occur with antisera to human IgA, κ and λ immunoglobulin light chains, β-lipoprotein, β 2-glycoprotein III, plasminogen, and properdin factor B. Identical results were obtained with serum from a second normal black rhinoceros. Precipitin bands were relatively weak (compared with what would be expected with the corresponding human proteins, except for precipitin bands of rhinoceros C3, which reacted strongly).

Reactivity of antisera to rhinoceros serum proteins—Rabbit serum raised against purified rhino IgG strongly agglutinated human RBC coated with rhino IgG (Table 2). Agglutination was totally inhibited by prior neutralization of the serum with an excess of rhino IgG; inhibition did not occur following attempted neutralization with human IgG. The anti-rhino IgG reagent induced moderately strong cross-reactive agglutination of Hu RBC-Hu IgG. Minimal cross-reactive agglutination was manifested by a potent monospecific anti-Hu IgG reagent against the Hu RBC coated by rhino IgG. These results (Table 2) establish that the anti-rhino IgG and the anti-WRS reagents were highly effective in detecting the presence of RBC-bound rhino IgG.

Reactions of anti-WRS and anti-Hu C3 reagents against complement-coated Hu RBC and rhino RBC (Table 2) indicated that Hu RBC strongly coated with human C3c and C3d were weakly to moderately agglutinated by the anti-WRS. Stronger agglutination was observed against Hu RBC coated by the same method used to produce Hu RBC-Hu C3d and Hu RBC-Hu C3d, but substituting rhinoceros plasma for human plasma. Strongest reactions were observed against rhino RBC coated in rhinoceros plasma. Because purified rhinoceros C3c and C3d were unavailable, it was not possible to perform inhibition experiments needed to verify absolutely that the agglutination of RBC coated in rhinoceros plasma was due to bound rhinoceros C3. Purified human C3c and C3d did not cross-react sufficiently to inhibit the anti-WRS reactions. Neutralizing anti-WRS serum reagent with rhino IgG did not inhibit agglutination of the presumed rhinoceros C3-coated RBC, nor were such RBC agglutinated by the anti-rhino IgG reagent.

Reactivity of anti-rhinoceros globulin reagents with RBC from rhinoceroses with hemolytic anemia—The anti-WRS and anti-rhino IgG reagents became available on Apr 10,

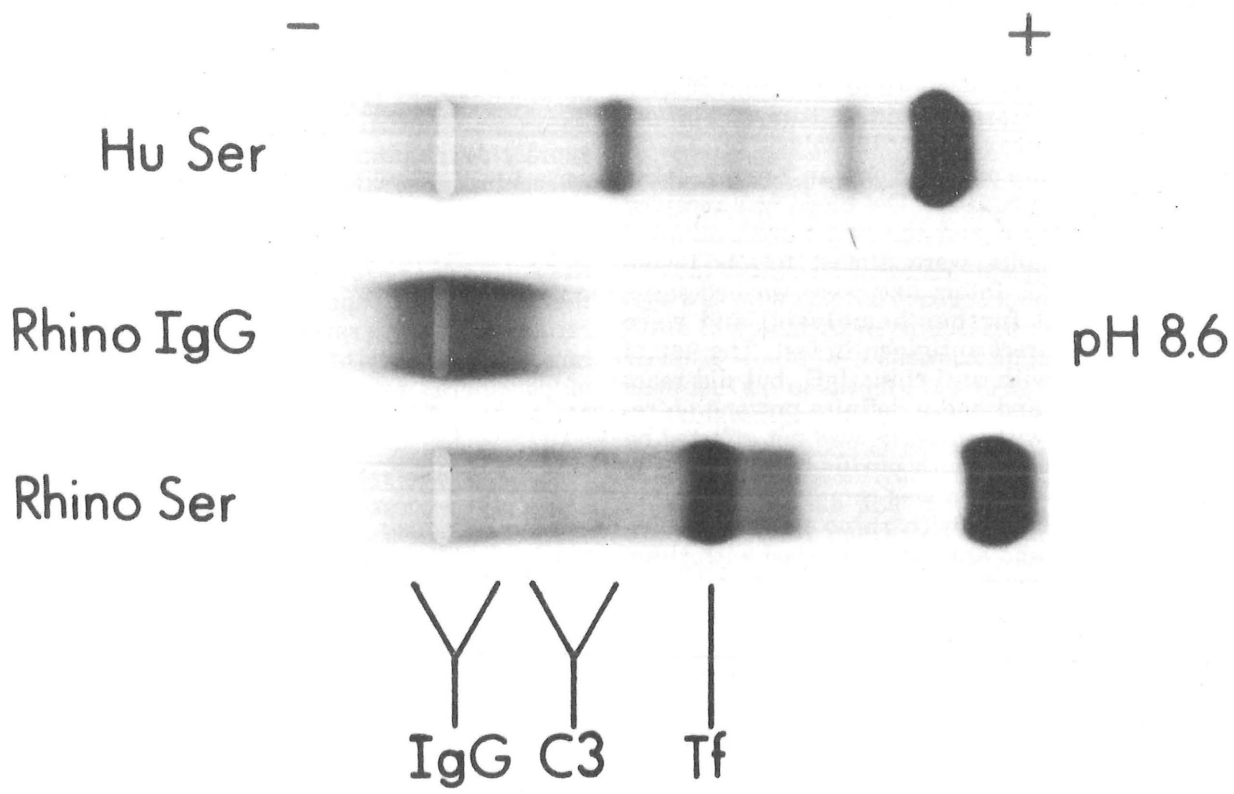

Fig 2—Agarose-gel electrophoresis of normal human serum (Hu Ser) and of serum from a healthy black rhinoceros (Rhino Ser = rhino 4) at pH 8.6. Rhinoceros IgG (Rhino IgG) was purified from rhino 4 serum, using DEAE-cellulose chromatography. Indicated below the Rhino Ser pattern are positions of rhinoceros IgG, C3, and transferrin as determined by use of immunofixation (not shown) and cross-reacting antisera specific for human IgG, C3, and transferrin. Results of immunofixation indicated that rhinoceros IgM migrated to the anodal edge of the application well.

TABLE 2—Comparison of anti-rhinoceros and anti-human antiglobulin reagents

Antiglobulin serum	RBC		Agglutination (neutralization not done)	Inhibition of agglutination after neutralization			
	Source	Protein coat		Hu IgG	rhino IgG	Hu C3c	Hu C3d
Anti-rhino whole serum	Human	None	Trace	–	–	–	–
		Hu IgG	Strong	Total	Marked	–	–
		Rhino IgG	Very strong	None	Total	–	–
		Hu C3b	Moderately strong	–	–	Moderate	–
		Hu C3d	Weak	–	–	–	None
		Rhino C3b	Moderately strong	–	–	None	–
		Rhino C3d	Moderately strong	–	–	–	None
	Rhino	Rhino C3b	Strong	–	–	None	–
		Rhino C3d	Moderately strong	–	–	–	None
Anti-rhino IgG*	Human	None	None	–	–	–	–
		Hu IgG	Moderately strong	Total	Moderate	–	–
		Rhino IgG	Very strong	None	Total	–	–
Anti-Hu IgG	Human	None	None	–	–	–	–
		Hu IgG	Very strong	Total	None	–	–
		Rhino IgG	Trace	–	–	–	–
Anti-Hu C3c	Human	Hu C3b	Very strong	–	–	Total	–
		Rhino 3Cb	None	–	–	–	–
	Rhino	Rhino 3Cb	None	–	–	Total	–
Anti-Hu C3d	Human	Hu C3d	Strong	–	–	–	Total
	Rhino	Rhino C3b	None	–	–	–	–

* Anti-rhino IgG did not react with any C3-coated RBC.

Rhino = rhinoceros; Hu = human; C3b = complement C3b; C3c = complement C3c; C3d = complement C3d; – = not applicable; Ig = immunoglobulin.

1984. After this date, blood samples were airmailed to the St Louis Zoo from 2 black rhinoceroses with acute intravascular hemolytic anemia (rhinos 1 and 2). Rhino 2 from the Denver Zoological Gardens had a moderate, self-limiting hemolytic episode (lowest hemoglobin concentration observed was 9.5 g/dl). The urine was dark red-brown for 11 days, and traces of hemoglobin were evident in the urine for 12 more days. The initial blood sample was collected and mailed to the Washington University School of Medicine in St Louis 4 days after the onset of hemoglobinuria was noticed. Using direct antiglobulin tests, rhino 2 RBC did not react with anti-WRS or anti-

rhino IgG. Similar results (data not shown) were obtained on a second sample obtained 1 day after hemoglobinuria had ceased and when rhino 2 was clinically improved (though the hematocrit had dropped to 28% from 35.7%).

Rhino 1 from Busch Gardens Zoo in Tampa, Fla, developed fulminating intravascular hemolysis on Jan 1, 1985. Blood samples were obtained on Jan 2 and 3, 1985; the rhino 1 died on Jan 6, 1985. The blood was received in St Louis on January 5 and the RBC were evaluated immediately. The samples were almost totally hemolyzed, with a PCV of 5%. Intact RBC were washed satisfactorily (ie, without further hemolysis) and were evaluated, using the direct antiglobulin test. The RBC of rhino 1 did not react with anti-rhino IgG, but did react weakly with anti-WRS and had a definite prozone on repeated tests. Reaction with anti-WRS was not affected by neutralization of antiserum with purified rhino IgG (20 mg/ml). However, the reaction was completely inhibited after absorption of anti-WRS with rhino RBC-rhino C3b. Results with anti-WRS and anti-WRS absorbed with rhino RBC-rhino C3b were consistent with weak C3-coating of the RBC of rhino 1.

Frozen stored RBC from rhino 3 were thawed, washed (saline solution), and incubated (1 hour at 37 C) with sera (which had been stored frozen at −20 C for 1 to 16 months) from the 3 healthy rhinoceroses (rhinos 3, 4, and 5), the 2 rhinoceroses with hemolytic anemia (rhinos 1 and 2), and the 1 rhinoceros with presumed blood loss anemia (rhino 6). After incubation, the RBC were washed again and tested against 10 serial doubling dilutions of anti-rhino IgG and anti-WRS. Agglutination was not observed.

Discussion

The present investigation was stimulated when a black rhinoceros in the St Louis Zoological Park died of fulminating hemolytic anemia in May 1981. To date, this devastating syndrome has been observed in 20 captive rhinoceroses with a mortality rate of 80%. Five of the 61 black rhinoceroses in North American zoos have been affected since 1980. The possible pathogenesis of the condition has been reviewed by Miller and Boever.[4]

In man, intrinsic RBC defects (eg, deficiencies of enzymes essential to erythrocyte metabolism and molecular abnormalities in hemoglobin structure) generally are heritable disorders. Although patients afflicted with these abnormalities may have chronic anemia, many remain clinically healthy, with normal hemoglobin values; however, exposure to unusual stress (ie, infections or contact with selected naturally occurring or synthetic chemicals), results in the underlying cellular defect causing a breakdown of normal erythrocyte homeostasis, and a hemolytic episode ensues. Although a familial incidence of these disorders is often observed, it is not invariable. For example, up to 30% of people with unstable hemoglobins do not have a family history of the disorder[28]; this has been attributed to variable expressivity of the heritable defect, insufficient family history, or spontaneous mutations at the stem cell level. Although 2 black rhinoceros families have had multiple siblings that have been affected, and an additional group had an affected mother, daughter, and granddaughter, the majority of affected rhinoceroses have not had such a familial pattern.[4] This epidemiologic data is insufficient to justify exclusive emphasis on an intrinsic RBC defect as an explanation of the hemolytic syndrome in these animals.

Results of the present study indicated that intravascular hemolysis was the hallmark of the illness in rhinoceroses. Marked hemoglobinemia was associated with rapidly developing anemia and was fatal in 1 of the 2 affected animals. Rare nucleated RBC were seen on the stained smear of the severely affected rhinoceros; nucleated RBC were somewhat more frequent (1/100 red) on the smear of the less severely affected rhino.[2] A similar finding has been reported in another rhinoceros.[4] Though reticulocytes were rarely seen in blood smears from these animals, this may be analogous to the reported absence of peripheral reticuloyctes in blood from the horse.[29] Marked erythropoietic marrow hyperplasia has been observed in affected rhinoceroses. Thus, the profound anemia appears to reflect the extraordinary severity of the hemolytic process, with insufficient marrow response to compensate for the rapid rate of RBC destruction. Results of the fresh and 24-hour-incubated osmotic fragility measurements on RBC from 2 healthy rhinoceroses (rhinos 3 and 4) and on the rhinoceros with only moderate hemolytic anemia (rhino 2) are not consistent with the possibility of an intrinsic rhinoceros RBC membrane abnormality similar to that in people with hereditary spherocytosis. The normal incubated fragility in rhino 2 was modest evidence against the possibility of rhinoceroses having one of several erythrocytic enzyme deficiencies seen in man (ie, wherein freshly collected RBC have normal osmotic fragility, but incubated RBC have increased fragility secondary to the defective RBC metabolism being unable to maintain the cell's resistance to hypo-osmotic lysis). Additional osmotic fragility studies need to be done, especially in severely affected rhinoceroses that recover to the point of having a normal PCV. A normal result on an incubated sample would cast further doubt on an intrinsic deficiency as the primary pathogenesis of this disorder.

Rhinoceros RBC membrane proteins were evaluated to determine whether the results would distinguish healthy rhinoceroses from rhinoceroses predisposed to hemolytic anemia. In man, such distinctions have been claimed for hereditary spherocytosis,[30,31] pyropoikilocytosis,[32] type II congenital dyserythropoietic anemia,[33] and elliptocytosis.[34] Application of the methods used for human RBC did not present apparent technical problems when applied to rhinoceros RBC. Although the membrane protein bands of rhino 2 (moderately affected) did not differ from those of 2 unrelated healthy rhinoceroses (rhino 3 and rhino 4), evaluations of severely affected rhinoceroses may rule out more conclusively an intrinsic structural membrane protein abnormality as a basis for the hemolytic process.

The limited hemoglobin electrophoresis comparisons between healthy rhinoceroses (rhinos 3 and 4) and rhino 2 do not indicate a hemoglobinopathy. However, the modest, self-limiting hemolytic anemia in rhino 2 may not represent the same hemolytic mechanism responsible for the rapidly fatal illness observed in the majority of affected rhinoceroses. Further electrophoretic studies are needed in more severely affected rhinoceroses. Unfortunately, the blood sample from rhino 1 in the present study contained barely enough intact RBC for the antiglobulin test studies. Black rhinoceros hemoglobin, even in apparently healthy animals, may be unstable, compared with

human hemoglobin. If such an hypothesis is true, minor infectious or chemical stress may result in life-threatening hemolysis, even in healthy rhinoceroses. Using isopropanol and heat tests, unstable hemoglobins should be evaluated in additional blood samples from healthy and affected rhinoceroses. These tests should be carried out on fresh blood samples (which was not done in the present study) because in vitro storage of human blood samples for more than a few days (even at 4 C) can result in alterations in otherwise stable hemoglobins, resulting in values consistent with intrinsically unstable hemoglobins.[28]

Interpretation of results, using anti-rhinoceros globulin reagents, assumes the reagents' reactivity with rhino IgG and with the third component of the rhinoceros complement system (C3). Within the human complement system, the third component (C3) is pivotal in modulating the classical and alternative pathyways of complement activation. In man, the concentration of C3 in normal serum is at least 5-fold greater than the concentration of any other complement components. High concentrations of IgG and C3 in rhinoceros serum were confirmed by strong precipitin cross-reactivity, using monospecific anti-human IgG and C3 reagents for immunofixation of rhinoceros serum proteins, after agarose-gel electrophoresis. Rabbits hyperimmunized with rhinoceros serum proteins produced antibodies against rhinoceros IgG and C3 (Table 2). The anti-rhino IgG reacted strongly with RBC coated by rhino IgG, and cross-reacted with RBC coated exclusively with Hu IgG. Similar anti-IgG reactivities were observed with the polyspecific anti-WRS. Anti-WRS also had anti-C3 reactivities, confirmed by cross-reactivity of anti-WRS with human RBC coated with human C3b and C3d. Anti-WRS strongly agglutinated rhinoceros RBC presumed to be coated strongly with rhino C3b. Strong agglutination of trypsinized rhino C3b-coated cells also indicated strong anti-rhino C3d reactivity in anti-WRS.

In immunologically mediated hemolytic human anemias, RBC may be coated with IgG alone, IgG and C3d, or C3d alone.[6] Although coating with IgM and IgA occasionally is observed,[6] these Ig almost always represent minor components in addition to IgG (ie, coating by IgM or IgA alone is rare). The same may be said for the 4th component (C4) of human complement (ie, C4d regularly occurs as a minor component accompanying RBC-bound C3d, but does not occur alone). Therefore, if immunologically mediated hemolysis in the rhinoceros resembles immunologically mediated hemolysis in man, the essential ingredients of antiglobulin reagents are reactivity against rhino IgG and against rhino C3d. Reagents used in the present investigation provided these ingredients.

Anti-rhino IgG and anti-WRS did not react with RBC from rhino 2 (obtained during the period of moderately active intravascular hemolysis). These data do not indicate an immunologically mediated pathogenesis of the hemolytic anemia of rhino 2. Whether the illness in rhino 2 represented a mild form of the disorder leading to death in the most affected rhinoceroses or whether rhino 2 had another disease with a different pathogenesis is not known. Results of tests on RBC from rhino 1 (fatal case) indicated weak coating with a serum protein other than IgG. By analogy with man, the most likely serum protein is C3d. Although this finding may constitute evidence for an immunologic basis for the hemolytic anemia of rhino 1, such a conclusion should be approached with caution for several reasons. The antiglobulin reaction was weak, even with the extremely severe hemolytic anemia. In man, the RBC of patient's dying of fulminating complement-mediated hemolysis generally are strongly coated with C3d.[6] Weaker coating may occur if complement becomes sufficiently depleted by the ongoing immunologic process and becomes rate limiting to the hemolytic mechanism. In the present study, evidence for this possibility in the rhinoceros was not explored because it was not possible to quantitate complement in rhinoceros serum. Another possible scenario would be a chemical immunologically mediated disorder (ie, reexposure to a drug, insect venom, or infection-related product to which the animal had been immunized by previous exposure), in which the chemical exposure results in lysis of strongly coated RBC, leaving in the circulation RBC less strongly coated with IgG or C3, along with noncoated RBC produced after chemical exposure had ceased. In the present study, evidence for or against such a hypothesis was not found. One finding consistent with a chemical immunologically mediated syndrome was the lack of indirect antiglobulin reaction of serum from rhino 1 against normal rhinoceros RBC. Using the indirect antiglobulin test, the sera of people with severe autoimmune hemolytic anemia generally react against all normal donor RBC because the autoantibody reacts against antigenic material common to human RBC, regardless of blood group system. Because chemical immunologically mediated hemolysis results from antibody directed against a chemical adherent to the RBC membrane, the indirect antiglobulin test will not detect antibody unless the chemical is added to the in vitro mixture of patient's serum and normal donor RBC. On the basis of observations in rhino 1, prior sensitization to a chemical, with subsequent chemical immunologically mediated hemolysis, appears more likely than the development of an autoimmune disorder.

Finding C3d-coated RBC in affected people does not necessarily indicate an immunologic basis for the complement coating. Chaplin et al[35] used a sensitive radioimmunoassay to quantitate RBC-bound C3d in 325 randomly selected patients in a large tertiary care hospital, and found supranormal values in almost a third of the blood samples evaluated. Although the amount of C3d bound was only slightly above normal in most patients, 10% of the samples evaluated had a sufficient amount of C3d to elicit a weak reaction, using the direct antiglobulin agglutination test. In most of these patients, the clinical problem was not a primary immunologic disorder. Severe trauma, septicemia, major surgical procedures, blood coagulation disorders, and metastatic cancer are examples of illnesses associated with weak anti-C3d antiglobulin reactions. Activation of the proteolytic coagulation cascade may have triggered the classical complement pathway, or bacterial or other infection-derived lipopolysaccharides may have triggered the alternative complement pathway, resulting in complement deposition on RBC in the proximity of activated complement components.

In the present study, a definitive cause of the fulminating hemolytic anemia in black rhinoceroses was not determined. There may be no single cause. Intravascular hemolysis may be the ultimate clinical manifestation of

RBC damage due to various causes. However, the clinical similarities among black rhinoceroses with acute fatal intravascular hemolytic anemia justify a major effort to seek a unitary explanation. The occasional familial clustering and the negative antiglobulin test results in rhino 2 may indicate a heritable intrinsic erythrocyte defect (ie, a hemoglobinopathy or enzyme deficiency). A background for more detailed studies of hemoglobin stability, using fresh hemolysates from healthy and affected rhinoceroses, has been provided by the preliminary studies reported here. Use of isopropanol and heat tests for unstable hemoglobins should have a high priority when rhinoceros blood samples become available. The weak antiglobulin test reaction consistent with complement coating of RBC from the single severely affected animal (rhino 1) indicates that the syndrome may have an immunologic basis. Therefore, direct and indirect antiglobulin testing should be an integral part of the evaluation of rhinoceroses with hemolytic anemia.

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