American Journal of Hematology 42:36–45 (1993)

Acute Episodic Hemolysis in the African Black Rhinoceros as an Analogue of Human Glucose-6-Phosphate Dehydrogenase Deficiency

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Sudden episodes of massive hemolysis have become the most common cause of death among captive black rhinoceroses, and there is evidence that they occur in the wild as well. We have observed radically unique enzyme and metabolite profiles in normal rhinoceros erythrocytes compared to humans and other mammals, including marked deficiencies of intracellular adenosine triphosphate (ATP), catalase, adenosine deaminase, and other enzymes involved in glycolysis, glutathione cycling, and nucleotide metabolism. Minimal concentrations of ATP appear to impair effective acceleration of hexosemonophosphate shunt activity in response to oxidants by restricting substrate generation at the hexokinase step. Antioxidant defenses are further compromised by catalase deficiency, which may be a general characteristic of rhinoceros erythrocytes, perhaps related to the common occurrence of severe mucocutaneous ulcerative disease. It is proposed that erythrocyte ATP deficiency in rhinoceroses may be an evolutionary adaptation conferring selective advantage against common hemic parasites, comparable to the role of human glucose-6-phosphate dehydrogenase (G-6-PD) deficiency in falciparum malaria. © 1993 Wiley-Liss, Inc.

Key words: erythrocyte enzyme, metabolic deficiency, hemolytic anemia, acatalasemia, catalase

PROLOGUE

Occasionally, a clinical problem provides more than the expected intellectual challenge or unanticipated insights. On special occasions, there may be an additional opportunity, (more accurately, a *need*), to return to basics, to reevaluate established facts and accepted dogma, and to review a given subject in broad historical context. One is then compelled to read again in detail classical papers that established both the major milestones along the course of discovery in that field as well as the enduring reputations of its pioneering explorers.

The following case has presented the author with such an opportunity, and it was especially rewarding to review the elegant early studies on erythrocyte antioxidant metabolism through the eyes of such creative researchers as Ernie Beutler, George Brewer, Harry Jacob, Jim Jandl, Frank Bunn, Dave Nathan, Alan Keitt, John Eaton, many others, and of course Ernst R. Jaffé, to whom this issue is warmly dedicated. In many instances, portions of the studies reported here and elsewhere have drawn from the thoughtful experimental approaches designed by those investigators. Additionally, Ernie Jaffé has lent his personal encouragement and support for these continuing studies on behalf of the endangered black rhinoceros for which they and I remain deeply ever grateful.

CASE REPORT

The index case was a 19-year-old immigrant East African male who had been in apparently good general physical health until just prior to referral, when he was noted to exhibit increasing signs of anorexia, lassitude, and weakness and the passage of dark red urine. He had been institutionalized since early adolescence and had remained essentially noncommunicative with visitors and attendants alike. One of the proband's brothers and a

Received for publication June 11, 1992; accepted September 30, 1992.

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Fig. 1. Subject T, the index case of hemolytic anemia referred and photographed by Dr. R. Eric Miller, Associate Veterinarian, Saint Louis Zoo.

maternal uncle and grandfather shared histories of poorly documented anemia, and his sibling had died during an apparent hemolytic crisis. A number of more distant relatives had suffered from acute and chronic recurrent ulcerative stomatitis and cutaneous ulcerations with and without anemia. Another female relative died in infancy with severe leukoencephalomalacia. The proband had been observed to be a strict vegetarian who shunned tobacco, alcohol, and safe sex.

On physical examination, the patient was a well-developed, well-nourished, corpulent male, who appeared lethargic, aphasic, and mildly irritable, with a widely divergent ocular gaze (Fig. 1). His heart and respiratory rates were moderately increased, but an accurate brachial artery pressure could not be obtained. His sclerae were grossly icteric, and mucous membranes were pallid as well as jaundiced. He had a greenish gray-coated tongue and fetid breath with gingival ulcers in varying stages of crusting and healing. His skin was sparsely hirsute with diffuse lichenification and dense focal hyperkeratotic excrescences, particularly prominent over the midline nasal bridge. Chest and abdomen were difficult to examine because of his corpulence and uncooperative attitude, but neither hepato- nor splenomegaly could be detected by palpation or percussion. He exhibited a flattened affect and complete disorientation with regard to time, place, and person. He was unable to interpret proverbs or to name any recent president. His deep tendon reflexes were intact, brisk, and provocative of aggressive behavior. He could not stand stationary on one foot, walk on his heels, or touch his nose with his eyes closed. Neither Babinski nor Romberg signs could be elicited.

Laboratory studies revealed a hemoglobin concentration of 5.0 g/dl, with rare reticulocytes and an indirect hyperbilirubinemia of 6.6 mg/dl. Moderate polychromasia and regenerative macrocytosis were evident in the peripheral blood film. Approximately 10% of the circulating erythrocytes contained a single prominent Heinz body, and scattered nucleated erythroblasts were present. Unstable hemoglobins could not be detected.

INTRODUCTION

Prior to referral of the index case to the UCLA Hematology Research Laboratory in late 1984, a number of similar occurrences had been observed sporadically in North America and Europe. In 1981, a massive and lethal hemolytic crisis in a female black rhinoceros at the St. Louis Zoological Gardens prompted its perspicacious Associate Veterinarian, Dr. R. Eric Miller, to initiate a survey that eventually revealed the startling occurrence of 24 similar episodes in 17 animals, 14 of which had been lethal [1]. These had been occurring at a time when zoological and wildlife institutions and individual conservationists were attempting to harvest threatened black rhinoceroses from the field to expand and protect a gene pool in zoos and private preserves, since poaching was rapidly decimating the few remaining native herds in Africa. At the rates of decline then extant, the entire species was projected for extinction in the wild as early as 1995, so recognition of the high incidence of sudden, massive, often lethal hemolysis presented a new and ominous threat to rhinoceros conservation, propagation, and potential repopulation efforts.

Investigative hematologists at several institutions were recruited to join the many zoological scientists and veterinarians participating in a Black Rhinoceros Species Survival Plan under the auspices of the American Association of Zoological Parks and Aquariums. Dr. Hugh Chaplin and his associates at Washington University in St. Louis developed an antirhinoceros globulin to investigate potential autoimmune phenomena associated with the hemolytic episodes [2]. Dr. Virgil Fairbanks at the Mayo Clinic subjected rhinoceros hemoglobin to extensive analyses to detect potential abnormalities or instabilities that might be responsible for premature hemolysis [3]. Simultaneously, intensive zoological research activities were occurring, both in laboratories and in the field, to evaluate possible contributory roles of dietary and nutritional factors, infections, parasitic diseases, natural and artificial toxins, idiosyncratic reactions, and epidemiologic factors [4]. Despite these efforts, the etiology of the hemolytic episodes remained unexplained.

Several findings were strongly suggestive of an intrinsic inherited metabolic defect in the erythrocytes of af-

fected rhinoceroses. Additionally, several clinical characteristics of this hemolytic syndrome were strongly reminiscent of human glucose-6-phosphate dehydrogenase (G-6-PD)-deficiency. Initial studies at the UCLA Hematology Research Laboratory, therefore, were undertaken to test the hypothesis that rhinoceros red cells in affected animals were deficient in G-6-PD or some other enzyme essential to antioxidant metabolism. As was reported previously [5], these studies indicated instead that both normal and affected rhinoceros erythrocytes had markedly *increased*, rather than decreased, activities of G-6-PD. This was found not to be a consequence of reticulocytosis (which rhinoceroses characteristically do not exhibit) nor of a young mean cell age.

Furthermore, several other enzymes of the oxidative hexose monophosphate (HMP) shunt were also elevated relative to erythrocyte activities in humans and other mammals, while many enzymes of anaerobic glycolysis and nucleotide metabolism were markedly diminished or undetectable. Intracellular concentrations of adenosine triphosphate (ATP) and total adenine nucleotides were markedly decreased (3–6% of human erythrocyte levels) in all the rhinoceroses studied to date. ATP deficiency has since proven to be a general characteristic of rhinoceros erythrocytes, and it may be responsible for producing a metabolic impairment and consequent hemolytic syndrome that is functionally equivalent to human G-6-PD deficiency [6,7]. The following summary of our ongoing studies presents data in support of that original hypothesis, but not yet in clear confirmation.

MATERIALS AND METHODS

Institutions participating in the Black Rhinoceros Species Survival Plan provided blood specimens whenever any clinical indication arose for tranquilizing or anesthetizing a rhinoceros, e.g., physical examination, surgery, sperm harvesting, or translocation. Informed consent was presumably provided by attending veterinarians who drew venous blood from ear or foreleg into heparin vials or directly into two volumes of 0.6 N perchloric acid for deproteinization and preservation of glycolytic intermediates, nucleotides, and other metabolites. Refrigerated specimens were shipped air express immediately to the UCLA Hematology Research Laboratory and to other participating laboratories to begin studies within 24–48 hr.

Enzyme assays were performed at the UCLA Hematology Research Laboratory on blood cell isolates prepared according to procedures standardized by the International Committee for Standardization in Hematology (ICSH) [8,9]. Concentrations of adenine ribo- and deoxyribonucleotides were determined by methods reported previously [10] or by those of Minakami et al. [11]. Glycolytic intermediates were also assayed according to Minakami et al. [11], except for 2,3-diphosphoglycerate (2,3-DPG), which was measured with Sigma Chemical Co. kit 665-PA. Reduced glutathione was measured according to Beutler et al. [12]. Ultraviolet absorption spectra were determined on perchloric acid extracts of washed erythrocytes at acid, neutral, and alkaline pH as described previously [13]. Isozymes of 5′-nucleotidase were assayed by phosphate evolution from a variety of substrates at acid and alkaline pH [14].

Leukocytes were separated into granulocytic and nongranulocytic fractions by flotation in Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ). Characteristics of cell incubation systems are described in Results.

RESULTS

Erythrocyte Enzymes

All rhinoceroses studied to date (including black [*Diceros bicornis*], a few white [*Ceratotherium simum*], and one Indian [*Rhinoceros unicornis*]) have uniformly exhibited extraordinarily divergent enzyme activity profiles compared to humans or other mammals. Figure 2 presents a diagrammatic comparison based solely on maximal in vitro activities as initially determined by ICSH techniques recommended for human blood. Disregarding species variations that might exist in enzyme kinetics, biochemical characteristics, or feedback control mechanisms, the marked differences observed in optimal activities alone suggest the evolution of a dominant glycolytic pathway in rhinoceros erythrocytes that favors antioxidant activity of the HMP shunt over adenosine triphosphate (ATP) generation.

In human erythrocytes, glucose is predominantly catabolized anaerobically via the Embden-Meyerhof pathway, with glycolytic rates controlled principally by hexokinase (HK) and phosphofructokinase (PFK). In rhinoceros erythrocytes, HK was markedly hyperactive (25-fold), whereas PFK showed only one-tenth the activity of human cells. All the subsequent enzymes in anaerobic glycolysis were also variably (and often considerably) less active than in humans, potentially restricting substrate flow through this pathway.

By contrast, rhinoceros erythrocytes had relatively hyperactive enzymes at key metabolic positions crucial to antioxidant activity of the HMP shunt: 1) The substrate for HMP shunt metabolism, glucose-6-phosphate (G-6-P), is generated by an HK 25 times more active than that in human red cells. 2) G-6-PD and glutathione peroxidase ($GSH\text{-}P_x$) were, respectively, three and 13.5 times more active in rhinoceros erythrocytes. 3) Markedly diminished PFK and twofold-hyperactive glucosephosphate isomerase (GPI) would likely combine to recycle HMP products preferentially back through the shunt rather than

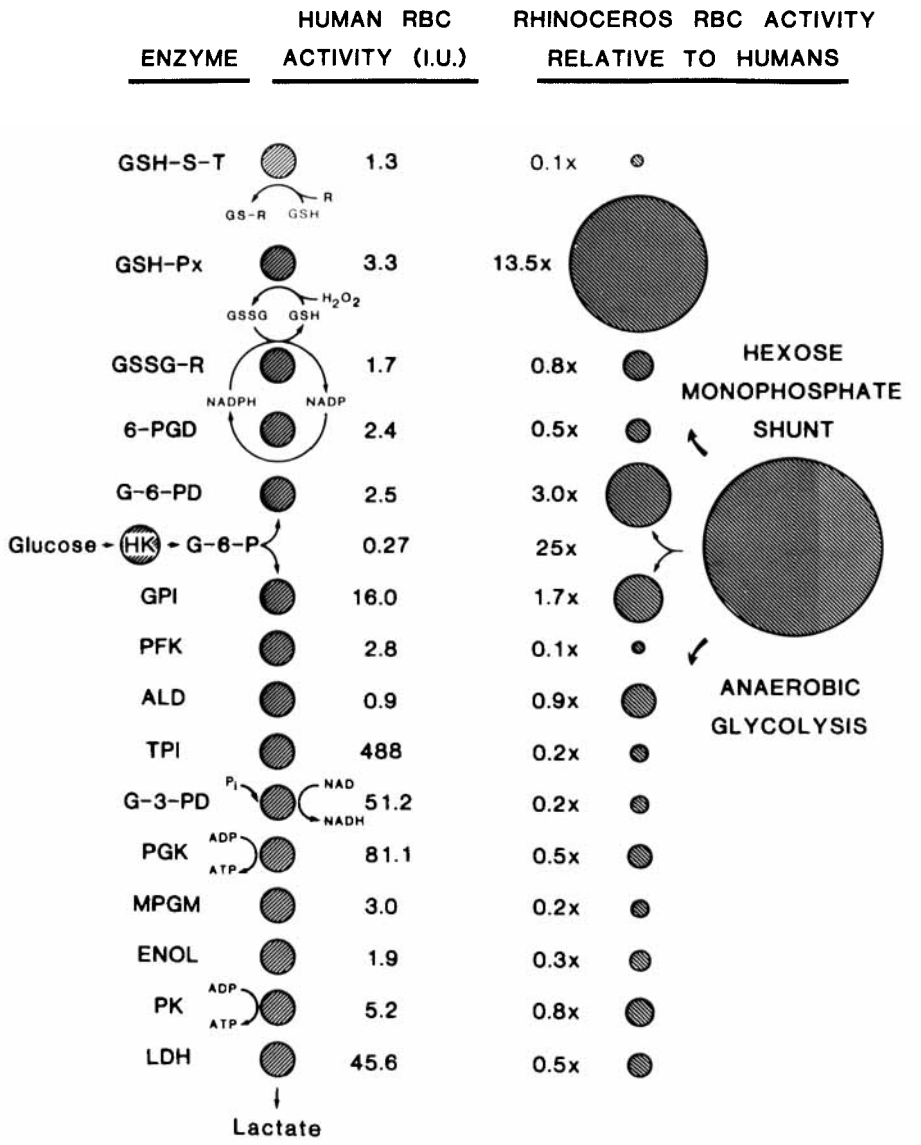

Fig. 2. Schematic comparison between human and rhinoceros red blood cell (RBC) enzyme activities as measured by ICSH-recommended assays (optimized for humans). Numerical values for normal human control means are presented in international units (I.U.) (micromoles of substrate converted per minute by 10^{10} RBC), and each is represented schematically on the left by a shaded circle of unit area. The activity of each enzyme in rhinoceros RBCs relative to human RBCs is indicated by a numerical factor and represented schematically by areas of the shaded circles at right. Glucose, phosphorylated to glucose-6-phosphate (G-6-P) by hexokinase (HK), can be degraded anaerobically via the Embden-Meyerof pathway to generate ATP and reducing equivalents in the form of NADH, the obligate cofactor for methemoglobin reductase activity. Alternatively, G-6-P can enter the HMP shunt to generate reducing equivalents as NADPH, the obligate cofactor for glutathione reductase (GSSG-R) activity. Oxidized glutathione (GSSG) is thereby converted back to its reduced state (GSH) to assist in peroxide neutralization catalyzed by glutathione peroxidase (GSH-P_x). Glutathione-S-transferase (GSH-S-T) catalyzes the conjugation of organic compounds (R) and glutathione, forming intermediates that are less toxic or more readily metabolized and excreted. Glucose-6-phosphate (G-6-P) catabolized via the HMP shunt loses its first carbon as CO_2, and the resulting pentose can rearrange through a series of reactions to reenter anaerobic glycolysis as triose or fructose-6-phosphate, which can recycle back through the HMP shunt. Additional abbreviations: ALD, fructose diphosphate aldolase; TPI, triosephosphate isomerase; G-3-PD, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; MPGM, monophosphoglyceromutase; ENOL, enolase; PK, pyruvate kinase; and LDH, lactic dehydrogenase. The latter enzyme is responsible for regenerating NAD as necessary (diagram not shown).

degrade them anaerobically, although this possibility has not yet been verified by monitoring catabolism of glucose with appropriate carbon labels. Possible rate-limiting roles of 6-phosphogluconate dehydrogenase (6-PGD) and glutathione reductase (GSSG-R), also remain to be investigated. The relative deficiency of glutathione S-transferase is of special interest since this enzyme has a key role in neutralizing the oxidant potential of a number of xenobiotics derived from plant nutrients [15–18].

The apparent dominance of antioxidant metabolism over high-energy phosphate generation prompted further investigation of related enzyme systems. Rhinoceros erythrocytes were found to have active NADH-dependent methemoglobin reductase and superoxide dismutase comparable to human erythrocytes, but they were markedly deficient in catalase. In the first three rhinoceroses studied [6], catalase activities were only 2–3% of those in human erythrocytes, and, interestingly, two had extensive mucocutaneous ulcerative disease, a problem commonly observed in captivity. While it is very intriguing to speculate that these two were afflicted with the rhinoceros equivalent of Takahara's disease, it remains to be established whether acatalasemia is truly pathologic or is simply a normal characteristic of the species in general.

Intracellular Nucleotides and Metabolites

Quantitative assays performed on erythrocytes from the index case and several other rhinoceroses indicated that most glycolytic intermediates were present in very low concentrations, comparable to those in human red cells [5]. The Eastern (*D. bicornis michaeli*) and Southern (*D. bicornis minor*) subspecies were found to be distinguishable on the basis of significant differences in red cell glutathione (GSH) and 2,3-DPG concentrations [5].

Erythrocyte ATP concentrations (mean 0.20 μmol/g Hgb) were only 3–6% of those found in humans and other mammals, and the partitioning of adenosine mono-, di-, and triphosphates favored ADP by as much as a factor of two compared to the six- to eight-fold dominance of ATP in human red cells. Ultraviolet absorption spectra of rhinoceros red cell extracts revealed the presence of a significant component of nonadenine nucleotides. We have observed a similar ultraviolet absorption spectrum in blood from a tapir, but not from horses, both of which are phylogenetically related to rhinoceroses in the family of odd-toed ungulates.

These findings (relative ATP deficiency, unusual adenine nucleotide partitioning, and increased nonadenine nucleotides) appear to be characteristic of black rhinoceroses in general, and similar patterns have been observed in the few white rhinoceroses studied as well. Despite the absence of adenosine deaminase (ADA) activity, rhinoceros erythrocytes did not contain detectable amounts of deoxyribonucleotides, as do children with ADA deficiency causing severe combined immunodeficiency disease. In contrast to human ADA deficiency, we found abundant ADA activity in lymphocyte and granulocyte fractions of rhinoceros blood.

The precise nature of the nonadenine nucleotides (approximately 20% of total nucleotides) present in rhinoceros erythrocytes remains to be determined. Limited chromatographic analyses of acid extracts by procedures described previously [13] so far indicate that two major uridine compounds are present that are not simple nucleotides but are apparently composed of uridine complexed with still unidentified ligands.

Erythrocyte Glycolysis

Preliminary studies indicated that the catabolism of glucose to pyruvate and lactate occurred at very slow rates in intact rhinoceros red cells compared to human erythrocytes. Furthermore, the unusual equilibrium and extremely low concentrations of erythrocyte adenine nucleotides were not significantly altered by in vitro exposure of washed cells to glucose or to a number of other six-, five-, and three-carbon sugars under a variety of incubation conditions. When a carbon source was supplemented with adenine in the form of base or nucleoside, however, rhinoceros erythrocytes proved capable of expanding their adenine nucleotide pools to levels equal to or greater than those in human red cells.

Figure 3 shows an ATP-generation study that was typical of the responses of several different rhinoceroses, which differed only in their rates of purine substrate utilization and conversion to adenine nucleotides. Incubation of washed erythrocytes in isotonic saline-phosphate buffer with 1 mM adenosine and 5 mM glucose consistently resulted in linear increases of intracellular ATP and total adenine nucleotides, reaching ten to 20 times initial concentrations after 4 hr (levels equivalent to those found normally in human erythrocytes).

Quantitative assays of those enzymes principally responsible for adenine nucleotide generation and conversion [adenosine kinase (AdoK), adenine phosphoribosyl transferase (APRT), ribosephosphate pyrophosphokinase (RPK), and adenylate kinase (AK)] indicated that direct phosphorylation of adenosine to AMP by AdoK and its subsequent conversion by AK was most likely the principal salvage pathway for maintenance of the precariously low adenine nucleotide pool, although direct incorporation of adenine also occurred to a lesser extent. Rhinoceros AK was only 15% as active as in human red cells, but AdoK was two to three times more active than the human erythrocyte enzyme.

Among the first, apparently normal, animals assayed for AdoK activities, two groups have emerged, one with approximately one-half or less of the activity exhibited by the other. This suggests the possible existence of a heterozygous deficiency state in some rhinoceroses that

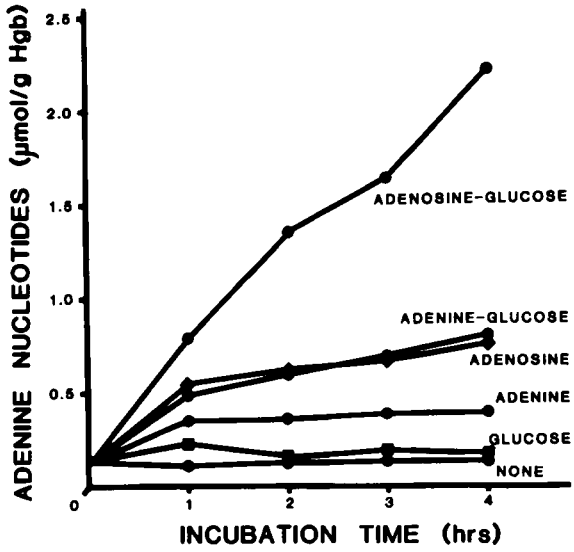

Fig. 3. Effect of various substrate additives (1 mM adenosine, 1 mM adenine, 5 mM glucose) on the generation of intracellular adenine nucleotides in intact rhinoceros erythrocytes incubated in isotonic phosphate buffer, pH 7.4, at 37°C. Each point is the mean of duplicate experiments in two separate black rhinoceroses of the same subspecies. Approximately 90% of the total adenine nucleotide complement was equally divided between ATP and ADP.

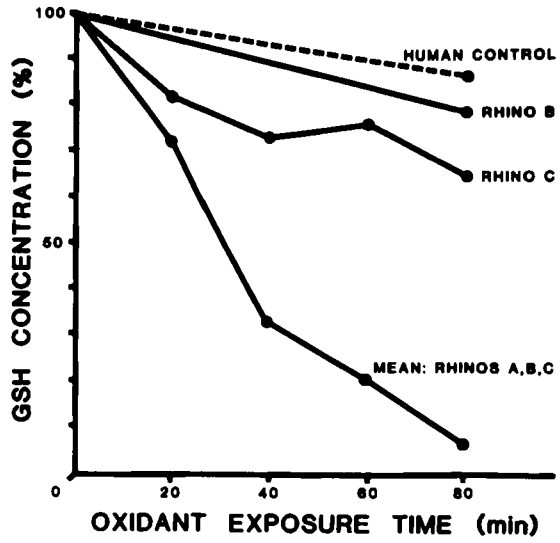

Fig. 4. Glutathione instability of rhinoceros erythrocytes compared with two samples preincubated with adenosine and glucose to increase intracellular ATP and to a typical normal human control. Points on the lower curve are means of separate determinations in three animals. Individual curves for two of those animals thus studied are shown after preincubation to increase ATP concentrations.

might impair this crucial ATP-generation pathway sufficiently to induce hemolysis in and of itself or to do so in homozygous offspring. Moderate or marked deficiency in AdoK activity would then be expected to compound the inherent ATP deficiency, further predisposing the cells to premature hemolysis when confronted by a sudden surge of oxidants. Data continue to be collected to test this hypothesis, but as yet there has been no opportunity to study a rhinoceros with a hemolytic history with assays of AdoK activity.

Glutathione Stability and HMP Shunt Metabolism

Despite the hyperactivity of several enzymes of the HMP shunt, this pathway's capacity to neutralize oxidants remained suspect [7]. We observed that 10–15% of circulating erythrocytes in apparently normal rhinoceroses contained single large Heinz bodies, and others have made similar observations [19–21]. In two animals thus studied, exposure to acetylphenylhydrazine increased the percentage of cells containing multiple Heinz bodies to ~90% within 30 min, but only to 25% if the cells had been preincubated with adenosine and glucose to increase their intracellular ATP (see below). The ascorbate-cyanide test [22] in these animals was similarly positive.

Applying Beutler's classical glutathione stability test [23,24], we observed very rapid deterioration of GSH concentrations in rhinoceros erythrocytes under oxidant (acetylphenylhydrazine) challenge. Gluthathione was also markedly unstable when cells were incubated with low concentrations of ascorbic acid, which generates intracellular hydrogen peroxide by a coupled oxidation reaction with oxyhemoglobin [25]. GSH instability was found to be a dependent variable of ascorbate concentration between 1 and 20 mM and was additionally accompanied by florid Heinz body formation.

To study the relation between relative ATP deficiency and the apparent impairment of HMP shunt antioxidant activity, rhinoceros erythrocytes were preincubated for 4–18 hr with 1 mM adenosine and 5 mM glucose in isotonic saline-phosphate buffer to increase their intracellular ATP concentrations. Figure 4 presents results of a typical experiment with cells from three rhinoceroses compared to a human control. Glutathione instability with acetylphenylhydrazine was prevented when cells from two of these three were preincubated to elevate ATP concentrations. Similar results have been obtained with ascorbic acid as the oxidant generator in some (but not all) other animals so far studied, and preliminary data suggest a response pattern that again may be dependent on ATP concentration.

At this stage, these preliminary data are consistent with the hypothesis that low ATP concentration in rhinoceros erythrocytes can be an important rate-limiting factor in acceleration of HMP shunt activity in response to oxidant stress, but other as yet unstudied factors may be involved as well, e.g., NADP/NADPH concentrations and ratios

and the activities and characteristics of 6-PGD, GSSG-R, and GSH-P_x.

DISCUSSION

Clearly, much remains to be learned about normal metabolic processes in rhinoceros erythrocytes, and the precise pathogenesis of the hemolytic and mucocutaneous ulcerative diseases remains far from certain. In many ways, the results of investigations in this area have continued to raise more questions than they have answered. Such uniformly low levels of endogenous ATP, for example, challenge traditional concepts regarding the roles of diminishing ATP in limiting normal mammalian erythrocyte life spans and in the induction of premature hemolysis in cells with various metabolic deficiencies [26]. Are the relatively high concentrations of ATP in human and other mammalian erythrocytes truly necessary or are they simply buffers against metabolic depletion, which the rhinoceros has sacrificed in deference to more demanding evolutionary pressures?

The precise reasons for such low intracellular concentrations of ATP and total adenine nucleotides remain a mystery. The echidna, an oviparous animal of the lowest mammalian order, *Monotremata*, is the only other mammal known to possess such low erythrocyte ATP levels, though others may exist beyond our knowledge. Rhinoceros 5′-nucleotidase appears to differ in many respects from the isozymes in human erythrocytes, but preliminary experiments did not show that it was likely to be responsible for draining the adenine nucleotide pool by direct dephosphorylation of AMP. The presence of uridine-containing complexes may be a reflection of rhinoceros nucleotidase properties or of other enzymes, perhaps in phospholipid metabolism, that remain unstudied. The unusual partitioning of ATP, ADP, and AMP suggests that a complex interplay among various enzymes affecting adenine nucleotide salvage and degradation may ultimately account for the equilibrium existent at such very low concentrations. Additionally, hypophosphatemia has been a source of concern in some rhinoceroses, particularly in association with hemolytic episodes, and this condition is also known to result in diminished glycolysis, decreased red cell ATP and glutathione, and hemolytic anemia in cattle [27] and humans [28–30]. Further investigations of both biochemical and genetic control mechanisms are clearly needed.

There are several intriguing parallels between human G-6-PD deficiency and hemolytic disease of the black rhinoceros: 1) the clinical presentation of sudden episodes of acute, moderate to massive hemolysis in otherwise apparently healthy individuals, 2) evidence of chronic hemolytic anemia in subsets, 3) an hereditary tendency, and 4) multiple diverse factors associated with initiation of hemolysis, including infections; parasitemias secondary to leptospirosis, babesiosis, trypanosomiasis, and theileriasis; tocopherol deficiency; drug administration, including Isoniazid; exposure to toxins and chemicals such as phenolics and organic phosphorous compounds; and trauma and stress related to capture, immobilization, and confinement during translocation.

These clinical similarities between G-6-PD deficiency and hemolytic anemia in rhinoceroses are compounded by biochemical similarities. Rhinoceros erythrocytes appear highly susceptible to oxidant stresses in vitro as well as in vivo and, like G-6-PD-deficient cells, exhibit positive ascorbate-cyanide and Heinz-body tests and marked glutathione instability with increased Heinz bodies when challenged by oxidants such as acetylphenylhydrazine or ascorbate-generated hydrogen peroxide. The partial or complete correction of some of these test results, by preincubating cells under conditions that elevate their intracellular adenine nucleotides, suggests that ATP may play a rate-limiting role in the acceleration of HMP shunt catabolism in response to oxidant stimulation, perhaps by restricting substrate generation from glucose to glucose-6-phosphate at the hexokinase step, a hypothesis that will be amenable to experimental testing as specimens become available.

Significantly diminished red cell ATP concentrations have been documented in a number of conditions and have been postulated to contribute to the relative resistance of certain erythrocytes to malarial parasitization [31], although other mechanisms may contribute as well. It is tempting to speculate that restricted availability of intracellular ATP would similarly provide a less hospitable host for intraerythrocytic and other hemic parasites that commonly infect rhinoceroses in the wild, a hypothesis also proposed independently by du Toit and Paul [20]. Though some of these currently prevalent parasites do not have an intraerythrocytic phase, it is not known what parasites might have existed hundred or thousands of millennia ago to provide the evolutionary pressure necessary for this prominent biochemical divergence from virtually all other mammals.

The data so far accumulated indicate that severely restricted ability to handle oxidant stresses may be a characteristic of rhinoceroses in general, a rather ominous possibility in an endangered species facing ever more frequent encounters with human intrusion and artificial environments. Natural habitats may present dangers as well. Fava beans are a well-known initiator of hemolysis in certain G-6-PD-deficient individuals, and similar hemolytic episodes have been documented in domestic animals consuming *Brassicae* plants [1] and in horses following ingestion of mulched red maple leaves [32–35], wild onions [36], and oak leaf browse [37]. Smith et al. [38] have shown that equine erythrocytes also have very low HMP shunt response to oxidants and low GSH regeneration rates, despite relatively high G-6-PD activity.

Since horses and rhinoceroses are related within the family of odd-toed ungulates, more credence should be given to accumulating evidence that acute hemolytic episodes might be induced in the black rhinoceroses by consumption of certain natural plants, particularly of the *Urginea* and *Pavetta* variety (du Toit, personal communication). As emphasized by du Toit and Paul [20], some historically massive series of black rhinoceros deaths (approximately 300 in 1960-1961), previously ascribed to "nutritional anemia," may well have been hemolytic episodes, and these could have been initiated by nutritional factors such as postdrought blooms of toxic plants or unusual variations in vegetation available for browse. The metabolic basis for such events might well reside in the extremely low activity of glutathione-S-transferase, which could severely impair the rhinoceros erythrocyte's ability to neutralize toxic metabolites such as polycyclic hydrocarbons, aflatoxins, and certain alkaloids [15–18], further increasing their vulnerability to specific plant nutrients.

Despite the general species sensitivity of rhinoceroses to oxidant stresses, the possibility (and the hope) remain that hemolytic episodes have been isolated to a subset of black rhinoceroses that has been rendered especially vulnerable by some potentially identifiable, inherited metabolic alteration. If, for example, the observed deficiency of erythrocyte ATP is indeed a crucial factor in maintaining reducing equivalents, further ATP losses might be incompatible with continued red cell viability. Adenosine kinase is particularly suspect in this regard, since it appears to mediate the principal nucleotide salvage pathway in rhinoceros erythrocytes. The partial or severe deficiency of this enzyme, therefore, might further restrict ATP reserves or generation rates sufficiently to affect resistance to oxidant-induced lysis. In ten animals so far studied, adenosine kinase activity has segregated into two groups, one with approximately one-half the activity of the other. The lower activity level may be sufficient in itself to cause problems, or it may represent a heterozygous carrier state that might contribute to hemolysis in homozygotes. As yet, no animal with a history of hemolysis has been available for assay of this enzyme.

As indicated in Figure 2, other enzymes are also positioned to play crucial rate-limiting roles in HMP shunt response to peroxides and other oxidants. Both 6-phosphogluconate dehydrogenase and glutathione reductase have activity levels well below those in human red cells and far below other rhinoceros enzymes of aerobic glycolysis, particularly if hexokinase is viewed as the first step of the HMP pathway. Glutathione reductase activity was not appreciably affected in several animals by addition of flavin adenine dinucleotide, but little else is known about the characteristics of this enzyme or others in rhinoceros red cells. Additionally, glutathione peroxidase has also been observed to be significantly lower in a small subset of apparently normal rhinoceroses, justifying similar speculation about possible severe deficiency states contributing to hemolytic disease or leukoencephalomalacia.

The role of red cell catalase deserves special attention. Of the three animals so far assayed, catalase activity was only 2–3% of that found in human erythrocytes [6]. One of these animals was afflicted with chronic anemia, severe ulcerative stomatitis, and extensive mucocutaneous ulcerations, a very common and sometimes lethal disorder of black rhinoceroses [39–42]. The other two animals were siblings. One, assayed in the newborn period, had no apparent clinical abnormalities. The other, studied at the time of death before 2 years of age, had severe leukoencephalomalacia coupled with mucocutaneous ulcerative disease similar to the other acatalasemic rhinoceros. Again, the clinical resemblance to a human disorder, hereditary acatalasemia (specifically, Takahara's disease) [43], is too compelling to ignore. It remains to be determined whether these findings reflect isolated cases of hereditary acatalasemia (or acatalasia) or whether this is another general species characteristic comparable to ATP deficiency. Catalase deficiency would almost certainly compound the restricted antioxidant capacity already inherent in rhinoceros erythrocytes. Whether this deficiency contributes to the pathogenesis of acute hemolytic anemia or mucocutaneous ulcerations, or perhaps to both, must await future studies.

CONCLUSIONS

Studies of rhinoceros erythrocytes have revealed biochemical and metabolic characteristics radically unlike those of other mammalian species. These include significant deficiencies (compared to humans) of intracellular ATP and total adenine nucleotide concentrations (~5%), nucleoside phosphorylase (~7%), AMP deaminase (~7%), glutathione-S-transferase (~10%), phosphofructokinase (~10%), adenylate kinase (~15%), and adenosine deaminase (undetectable). Despite the latter, rhinoceros erythrocytes do not accumulate deoxyribonucleotides, although ~20% of their intracellular nucleotide complement is composed of uridine complexes.

Other enzymes are more or less active in a manner suggestive of evolutionary development favoring antioxidant metabolism of the HMP shunt over ATP generation via anaerobic glycolysis. Nonetheless, in vitro studies indicate a marked susceptibility of rhinoceros red cells to oxidants, which may be ameliorated by artificially increasing intracellular adenine nucleotides to levels comparable to humans. ATP concentration appears to be rate limiting for acceleration of HMP shunt activity, perhaps contributing to the sudden episodes of massive hemolytic anemia that are the commonest cause of death among black rhinoceroses in captivity, and perhaps in the wild as well.

Adenosine kinase appears to be the principal pathway for maintenance of the precariously low adenine nucleotide concentrations in normal erythrocytes, and a partial deficiency consistent with heterozygosity has been observed in a subset of rhinoceroses, possibly increasing their susceptibility to hemolysis. Catalase deficiency (2–3%) has been detected in another subset of three black rhinoceroses (the only ones so far assayed), two of which had associated severe mucocutaneous ulcerations strongly reminiscent of Takahara's disease in humans. If acatalasemia proves to be another general species characteristic, it would further compromise the ability of rhinoceros erythrocytes to neutralize oxidant stresses and therefore might contribute both to mucocutaneous ulcerative disease and to a predisposition to acute episodic hemolysis.

Evidence is presented to support the hypothesis that these multiple metabolic deviations from other mammals produce a product-deficiency disorder in rhinoceros erythrocytes that is functionally analogous to human G-6-PD deficiency in its biochemical, metabolic, and clinical consequences. It is further proposed that marked relative deficiency of intracellular ATP may be an evolutionary adaptation conferring selective advantage against common hemic parasites, comparable to the role of human G-6-PD or ATP deficiency in falciparum malaria postulated by others [31,44–46,47].

ACKNOWLEDGMENTS

Portions of these studies were supported by USPHS grant HLB-12944 and by private donors. The author is grateful for the technological contributions of Misae Nakatani and Richard Brockway, and to Zohra Patail, Fernando Casimiro, Linda Pride, and Carol Appleton for assistance with manuscript preparation. Dr. Mark Scott, Children's Hospital Oakland Research Institute, kindly measured erythrocyte superoxide dismutase and catalase activities in a specimen from rhino C (Fig. 4), confirming the degree of relative catalase deficiency (2–3%) that we had observed in rhinos A, B, and C. Concerned veterinarians from a number of public and private zoological institutions have contributed blood specimens for study and analysis. Especially rewarding discussions have been held with Raoul du Toit of the Rhino Conservancy Project in Harare, Zimbabwe; Richard C. Cambre, DVM, of the Denver Zoological Gardens; Ellen S. Dierenfeld, PhD, nutrition advisor for the Rhino Taxon Advisory Group; and Eric H. Harley, MD, PhD, of the University of Capetown. The author owes a special note of gratitude to Dr. R. Eric Miller, Veterinary Advisor for the Black Rhinoceros Species Survival Program, for the opportunity to participate with him and his national and international cadre of dedicated associates in this most compelling project.

NOTE ADDED IN PROOF

Subsequent studies now indicate that acatalasemia is a general species trait not shared by white rhinoceroses. Three animals with active hemolytic processes all had adenosine kinase activities in the intermediate-low range. Intensive phosphate therapy in one of these was accompanied by correction of hypophosphatemia, increased red cell ATP to near human concentrations, cessation of active hemolysis, and a return to a hematocrit of 38% from a nadir of 16%.

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