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Erythrocytes of the Black rhinoceros *Diceros bicornis:* susceptibility to oxidant-induced haemolysis

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Growth of the captive Black rhinoceros *Diceros bicornis* population has been limited by four diseases whose aetiologies are poorly understood (Miller, 1993). These syndromes, haemolytic anaemia, oral and skin ulcers, encephalomalacia and fungal pneumonia, play a critical role in the maintenance of a species that is undergoing precipitous decline in the wild and numbers fewer than 90 animals in the North American population.

Haemolytic anaemia now ranks as the leading cause of death among captive Black rhinoceroses, accounting for *c.* 40% of adult mortalities (Miller & Boever, 1982). At time of writing, 44 episodes of haemolysis have been documented in 36 captive Black rhinoceroses of which 75% died during either their initial or a subsequent episode of haemolysis. Iron deposition in tissues (haemosiderosis) suggests that in some animals chronic haemolysis may also occur (Kock *et al.*, 1992). There is justifiable but undocumented suspicion that lethal or transient haemolytic episodes also occur in the wild, where they are unlikely to be detected and documented.

The occurrence of chronic oral and/or skin ulcers is second in incidence and clinical importance. This syndrome has been observed in over 30 captive Black rhinoceroses in North America (Munson, 1993). Lesions vary from shallow self-limited ulcers over pressure points, to more severe necrosis affecting vast expanses of skin. Oral and colonic ulcers may occur either separately or in association with skin ulcers (Ott *et al.*, 1982). Histopathologically, initial lesions appear to be non-inflammatory and are characterized by marked intra- and intercellular oedema, epithelial hyperplasia and parakeratosis. In more chronic cases, secondary infection and inflammatory changes are often noted (Munson, 1993). Attempts to identify an underlying viral or auto-immune aetiology have been unsuccessful (Munson, 1993).

Massive and usually fatal encephalomalacia, primarily affecting white matter, has been noted in four Black rhinoceroses (Miller *et al.*, 1990). Despite extensive diagnostic evaluation, a cause for this disorder has not been found. Lastly, fungal pneumonia (usually *Aspergillus* sp)

has been identified in six Black rhinoceroses (Gillespie *et al.*, 1990; M. T. Barrie, pers. comm.; S. Citino, pers. comm.; R. Faust, pers. comm.; R. McManamon, pers. comm.), suggesting the possibility of immunodeficiency.

CHARACTERISTICS OF THE HAEMOLYTIC DISORDER

Haemolytic crises have occurred in otherwise healthy Black rhinoceroses and as terminal events complicating diverse systemic diseases. Initial investigations into the aetiology of haemolysis were based on known causes of similar syndromes in domestic animals and man (Jubb *et al.*, 1985). Haemoglobinopathies, autoimmune disease and copper toxicity were evaluated and eliminated as likely causes (Miller & Boever, 1982; Chaplin *et al.*, 1986; Fairbanks & Miller, 1990). In some cases drug exposure (for example, isoniazid) and hypophosphataemia have been suggested as contributing causes (Miller & Boever, 1982; Gillespie *et al.*, 1990). Others have speculated that low levels of serum vitamin E (α-tocopherol) may contribute to red blood cell instability (Dierenfeld *et al.*, 1988; Ghebremeskel *et al.*, 1988). Infection with serovars of the spirochaete bacterium *Leptospirosis interrogans*, has been suggested by titre or fluorescent antibody techniques in nearly 50% of the Black rhinoceroses undergoing 'primary' haemolysis, that is, without signs of other underlying disease (Asakura *et al.*, 1960; Douglass *et al.*, 1980; Mikulica, 1986; Jessup *et al.*, 1992). However, not all cases of haemolysis have been associated with this organism (Jessup *et al.*, 1992) and in the majority of haemolytic Black rhinoceroses, a definitive cause has not been identified.

Clinical similarities among Black rhinoceroses undergoing haemolysis prompted efforts to identify a single common denominator for this syndrome, that is, a basic biochemical or metabolic defect that would account for a number of factors or agents initiating haemolysis. To that end, studies of the metabolism of erythrocytes in Black rhinoceroses were initiated, based on the established association of red cell enzyme deficiencies with virtually identical haemolytic syndromes in man (most notably glucose-6-phosphate dehydrogenase deficiency) (Paglia *et al.*, 1986; Paglia, 1993).

RHINOCEROS ERYTHROCYTE METABOLISM

Background Initial studies conducted at the UCLA Hematology Research Laboratory established a general pattern of energy metabolism in Black rhinoceros erythrocytes that departed radically from all other known species (Paglia *et al.*, 1986). A number of cellular enzymes exhibited quantitatively increased (two- to 25-fold) activities relative to most mammalian erythrocytes, whereas many others were markedly decreased ($\leqslant 10\%$); and some, like adenosine deaminase, were essentially undetectable (Paglia *et al.*, 1986; Paglia, 1993). Perhaps the most extraordinary finding was the dearth of intracellular high-energy phosphate in the form of adenosine triphosphate (ATP), which was reduced to only a few per cent of the concentrations found in other mammalian red cells.

These unique metabolic features now appear to be general species characteristics and, to date, no specific enzyme deficiency has been identified to distinguish those animals experiencing haemolysis from any others. The investigative focus therefore shifted from detection of differences between affected and unaffected animals, to testing the hypothesis that ATP restriction, or some other unusual but 'normal' metabolic characteristic, predisposed *all* Black rhinoceros erythrocytes to the lytic effects of diverse agents.

Human G-6-PD deficiency served as the paradigm because of its strong clinical resemblance to the rhinoceros haemolytic syndrome. Several standard qualitative screening tests for G-6-PD deficiency were found to be strongly positive when healthy Black rhinoceroses were tested,

despite quantitative activities of G-6-PD that were three to five times greater in rhinoceros red cells than in humans (Paglia, 1993). These included ascorbate-cyanide (Jacob & Jandl, 1966a), glutathione stability (Beutler, 1957) and Heinz-body (Beutler *et al.*, 1955) tests. Approximately 10–15% of the circulating red cells in clinically normal rhinoceroses contained prominent Heinz bodies and this number rose to 90% or more if the cells were exposed to an oxidant such as acetylphenylhydrazine (Paglia, 1993).

Intracellular concentrations of reduced gluathione (GSH) were equal to *(Diceros bicornis minor)* or double *(D. b. michaeli)* the levels found in humans, but GSH was rapidly oxidized by either acetylphenylhydrazine or ascorbic acid, where the latter generates hydrogen peroxide by a coupled reaction with oxyhaemoglobin (Jacob & Jandl, 1966b). An example of the ascorbate effect is shown in Fig. 1, which also demonstrates the dependence of GSH maintenance on oxidant concentration. GSH decay rates characteristic of human G-6-PD deficiency coincide with those shown for 10–20 mM ascorbate, whereas normal human erythrocytes sustain *c.* 90% of their GSH complement under standard oxidant challenge over the same period.

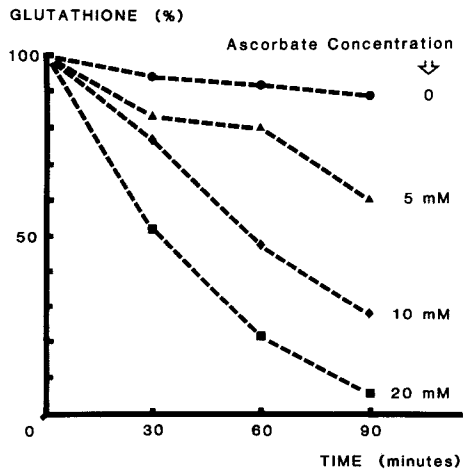

Fig. 1. Effect of ascorbic acid on reduced glutathione (GSH) concentration in Black rhinoceros *Diceros bicornis* erythrocytes incubated in isotonic phosphate buffer containing 200 mg/dl glucose. Each point is the mean of single or duplicate determinations on two to five animals. GSH stability of normal human red cells, under oxidant challenge with acetylphenylhydrazine or ascorbate, is the same as shown for the control curve devoid of ascorbate. G-6-PD deficient erythrocytes generally display rapid GSH-decay rates such as those shown by rhinoceros erythrocytes exposed to 10–20 mM ascorbate.

Metabolic basis for oxidant-induced haemolysis Explanations for such pronounced sensitivity to oxidants may reside in certain metabolic restrictions within the pathways of glucose catabolism (Fig. 2). Most mammalian erythrocytes rely on glucose or other simple sugars as substrate for two primary metabolic pathways, both of which are essential to normal cell function, viability and life span. Anaerobic glycolysis via the Embden-Meyerhof pathway generates ATP to fuel the cation pump and other important biochemical reactions. If any of a dozen enzymes that catalyse reactions in this pathway are defective, ATP concentrations may decrease sufficiently to result in premature cell death, producing *chronic* haemolytic anaemia.

The second metabolic pathway, known as the hexose monophosphate (HMP) shunt, usually operates at about 5–10% of the capacity of the first but can be stimulated 20- to 30-fold when needed to neutralize oxidants, such as hydrogen peroxide, that are generated by a number of diverse conditions, including, for example, infection, stress, inflammation, and certain drugs, chemicals and foodstuffs. Humans or animals with disorders of this metabolic pathway often have no ill effects until they encounter a substance or condition that results in a sudden oxidative challenge. Under these conditions, if the enzymatic or biochemical impairment prevents a defensive response sufficient to neutralize the oxidants, susceptible proteins in the affected red cells may be damaged or irreversibly denatured and an apparently healthy subject can suddenly

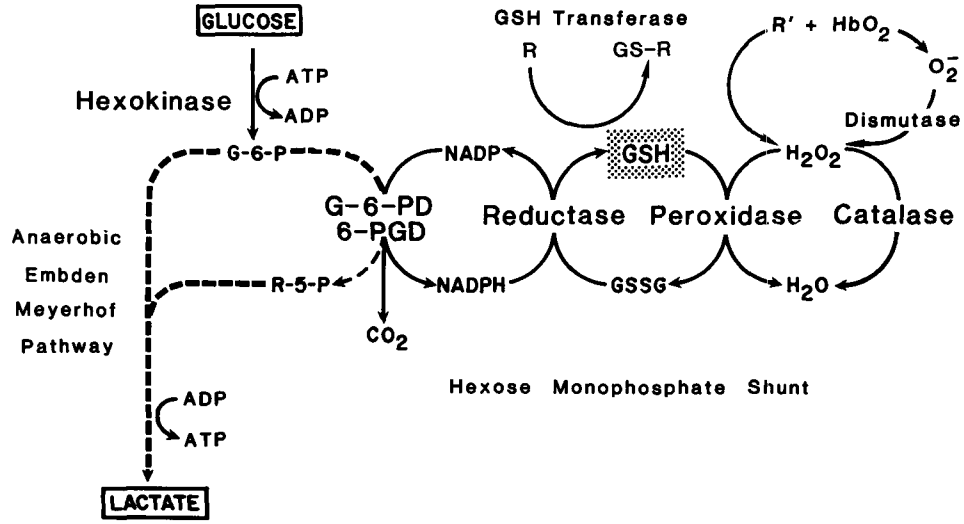

Fig. 2. Pathways of glucose catabolism in mammalian erythrocytes. Individual reactions of anaerobic glycolysis are not shown. HMP shunt metabolism is responsible for maintaining reduced glutathione (GSH), which serves as a sacrificial reductant to neutralize ambient oxidents. Compared to humans and other mammals, the Black rhinoceros is markedly deficient in ATP, catalase and GSH transferase, with intermediate activities of glutathione reductase and 6-PGD, and hyperactive hexokinase (25 ×), G-6-PD (5 ×) and GSH peroxidase (13·5 ×).

experience an episode of *acute* haemolytic anaemia.

Rhinoceros erythrocytes were found to be capable of glucose degradation, but they do not respond with increased glycolytic rates when challenged *in vitro* with oxidant stimuli, such as acetylphenylhydrazine or ascorbic acid, or with a redox dye (methylene blue) that normally activates HMP shunt activity in other mammals (Paglia, 1993; D. E. Paglia & M. Nakatani, unpubl. obs.). Low endogenous concentrations of ATP may be responsible for this impaired response, since GSH instability has been at least partially corrected in some rhinoceroses by preincubating erythrocytes under *in vitro* conditions that increase ATP concentrations to human levels or above (Paglia, 1993). Since ATP is required by hexokinase to generate substrate (glucose-6-phosphate) for the HMP shunt, restricted availability of ATP could well be rate-limiting for oxidant-induced acceleration of the HMP pathway, even though hexokinase and G-6-PD in rhinoceros erythrocyte are respectively 25 and five-fold more active than in human cells.

Other metabolic restrictions may exist at the reactions mediated by 6-phosphogluconate dehydrogenase and glutathione reductase, since those enzymes possess only 50–75% of the *in vitro* activities of their human red cell counterparts. The most significant erythrocyte deficiencies, which we have observed in all Black rhinoceroses so far studied, involve catalase and glutathione S-transferase, which measured 2–3% and $<1\%$, respectively of human red cell activities. Catalase and GSH S-transferase deficiencies are potentially of great clinical importance since they would likely impair neutralization of hydrogen peroxide and potentially toxic intermediate metabolites or xenobiotics generated by infections or from catabolism of plant nutrients. Fettman (1991) has comprehensively reviewed the interactions and importance of some of these reactions in contributing to oxidant susceptibility in animals.

Clinical significance of metabolic restrictions Either alone or in combina-

tion, these unusual metabolic characteristics of rhinoceros erythrocytes may create an impairment in the cell's ability to defend against oxidants that is *functionally* equivalent to human G-6-PD deficiency (Paglia, 1993), even though G-6-PD is itself several times more active than in human red cells. On this basis, we previously issued preliminary warnings (Paglia & Miller, 1992a, unpubl.) that *all* rhinoceroses should be regarded as clinically equivalent to G-6-PD deficient humans and stringently protected from those agents and conditions known to initiate haemolytic episodes in the latter. These include several classes of pharmaceuticals, for example, anti-malarials, sulphonamides, sulphones, nitrofurans, acetanilid, chloramphenicol, vitamin K analogues and miscellaneous others (Table 1), and a number of chemical compounds, particularly those containing cyclic hydrocarbons such as naphthalene and phenols. The latter are of special concern, since creosote has been used as a wood preservative on protective corrals in Africa for animals awaiting relocation. Recent episodes of hepatic failure and subsequent haemolysis in such animals are being studied with a specific focus on known hepatotoxins and haemolytic agents (R. E. Miller, R. Orth, J. Stover & E. S Blumer, unpubl. obs.).

Some individuals with G-6-PD deficiency are also sensitive to haemolytic factors in broad (fava) beans *Vicia faba*, raising the possibility of analogous effects induced by dietary metabolites derived from rhinoceros browse. Haemolysis has been observed in domestic animals, particularly horses, following consumption of wild onions *Allium canadense* (Pierce *et al.*, 1972), and oak *Quercus* spp (Duncan, 1961) and red maple *Acer rubrum* leaves (Tennant *et al.*, 1981; Divers *et al.*, 1982; George *et al.*, 1982; Plumlee, 1991). Equine erythrocytes also respond poorly to oxidants, exhibiting low GSH regeneration rates and low HMP shunt stimulation (Smith *et al.*, 1972).

We believe that some rhinoceros deaths in the wild may be explained by similar processes, particularly massive simultaneous die-offs such as the 1960–1961 episode mentioned by du Toit & Paul (1987). These incidents should be re-investigated to search for plant species that might have become predominant under prevailing conditions like droughts. Neutralization of toxic metabolites, including aflatoxins, polycyclic hydrocarbons and alkaloids, generally depends on GSH S-transferase activity, which is virtually undetectable in erythrocytes from either *D. bicornis* or White rhinoceroses *Ceratotherium simum*.

In addition to drug, chemical and dietary factors, several other clinical conditions are likely to be associated with haemolysis in the Black rhinoceros: infec-

CLASS	COMPOUNDS
Analgesics	acetanilid, acetominophen, aminopyrine, aspirin, phenacetin
Anti-malarials	chloroquine, pamaquine, pentaquine, primaquine, quinacrine, quinine
Nitrofurans	nitrofurantoin
Sulphonamides	sulphacetamide, sulphadiazine, sulphadimidine, sulphamerazine, sulphanilamide, sulphamethoxazole, sulphamethoxypyridazine, sulphapyridine, sulphisoxazole, sulphoxone
Sulphones	dapsone (diaminodiaphenylsulphone), thiazolsulphone
Miscellany	chloramphenicol, dimercaprol, doxorubicin, Levo-dopa, methylene blue, nalidixic acid, naphthalone, niridazole, phenazopyridine, phenylhydrazine, probenecid, toluidine blue, trinitrotoluene, vitamin C, vitamin K analogues

Table 1. Pharmaceutical and chemical agents reported in association with haemolysis in humans with G-6-PD deficiency. Many of these compounds have been associated with haemolytic episodes but have not been proven to be causative. (Adapted from Luzzatto & Mehta (1989).)

tions, hypophosphataemia and acidosis. Bacterial, viral or rickettsial infections are the most common haemolytic initiators in human G-6-PD deficiency, justifying preventive immunizations and aggressive therapy of active infections in rhinoceroses with judiciously selected antibiotics. *Leptospirosis* has been the most conspicuously associated agent in the Black rhinoceros (Miller & Bolin, 1988; Jessup *et al.*, 1992).

Conditions that result in acidosis are also likely to induce haemolysis by direct suppression of red cell glycolytic activity, which is crucial for maintenance of the precariously low ATP concentrations characteristic of rhinoceros erythrocytes. Hypophosphataemia has a similar inhibitory effect on anaerobic glycolysis, and is known to cause decreased erythrocyte ATP and GSH concentrations and haemolysis, both in humans and in cattle (Lichtman *et al.*, 1969, 1971; Lichtman & Miller, 1970; Jacob & Amsden, 1971; Ogawa *et al.*, 1989). Since the converse is also true, namely, that *hyper*phosphataemia directly increases red cell ATP, we have begun to investigate the therapeutic potential of phosphate supplementation. Both *in vitro* and *in vivo*, the increased availability of inorganic phosphate has been observed to increase erythrocyte ATP concentrations ten- to 20-fold or more. In one adult Black rhinoceros experiencing a *de novo* episode of primary haemolysis, reversal of hypophosphataemia to a hyperphosphataemic state was associated with a rapid marked but transient elevation of red cell ATP, cessation of haemolysis and return of haematrocrit from a nadir of 16% back to normal levels (Paglia, 1993; D. E. Paglia, M. T. Barrie & M. Nakatani, unpub. obs.). Optimal forms and modalities of phosphate administration remain to be determined, but this appears to be a very promising approach to possible prevention as well as treatment of haemolytic anaemia in the Black rhinoceros.

In addition to impaired anti-oxidant defence, we believe catalase deficiency is likely to play an important pathogenetic role in mucocutaneous ulcerative disease, which now ranks as the second most common cause of Black rhinoceros deaths in captivity. In the Japanese form of human acatalasaemia (Takahara's disease), as many as 50% of affected subjects present with necrotizing, even gangrenous ulcerations, usually of the oropharynx. The precise underlying mechanism remains uncertain but stringent oral hygiene has markedly reduced the incidence of this complication in humans, suggesting that periodontal infections with peroxide-producing pyogenic bacteria may be the initiating source that then progresses to extensive necrotizing ulceration.

We have now confirmed that catalase deficiency is a species-wide characteristic of Black rhinoceroses that is apparently not shared by White rhinoceroses (Paglia *et al.*, 1992). *Ceratotherium simum* possesses most of the other unusual erythrocyte metabolic features exhibited by *D. bicornis*, including ATP and GSH S-transferase deficiencies, but White rhinoceroses are not known to be susceptible to either primary or secondary haemolytic anaemia or to mucocutaneous ulcerative disease. This species distinction strongly supports a likely pathogenetic role for erythrocyte catalase deficiency in both of these major diseases so commonly affecting the Black rhinoceros population.

The evolutionary pressures that produced these marked metabolic divergences from other mammalian erythrocytes may well reside in their potential protective effects against various haemic parasites. Again, parallels exist among human diseases: G-6-PD deficiency and decreased erythrocyte ATP concentrations are both associated with increased resistance to parasitization by the most malignant form of falciparum malaria. Indeed, this protective effect is often cited as responsible for the evolutionary persistence of G-6-PD deficiency, which is the commonest human enzymopathy known and coincides precisely in geographic

distribution with the world's malaria belt. Since rhinoceroses are also commonly affected by haemic parasites, it seems likely that similar protective mechanisms may be operative. This hypothesis is potentially amenable to experimental testing, since ATP content of rhinoceros erythrocytes can now be artificially manipulated *in vitro* (Paglia, 1993).

SUMMARY

Studies on the cellular metabolism of rhinoceros erythrocytes have revealed characteristics that contrast radically with those evolved by all other known species. Either alone or in combination, deficiencies of intracellular ATP and certain enzymes render erythrocytes of the Black rhinoceros extremely susceptible to multifactorial oxidant-induced lysis. Among these, catalase deficiency may play an important pathogenetic role in both the haemolytic and ulcerative syndromes that constitute the two most common causes of death in the captive population of these species. Strategies for the prevention and treatment of the haemolytic disorder can be derived from an understanding of these metabolic restrictions and characteristics and from consideration of analogous human syndromes. The evolutionary pressures responsible for such unique biochemical features seem likely related to protective effects against certain haemic parasites.

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