Polymorphism of glucose-6-phosphate dehydrogenase in black rhinoceroses: a possible link with haemolytic anaemia

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*The high incidence of haemolytic anaemia in black rhinoceroses (*Diceros bicornis*) is a matter of concern to conservationists because of the mortality attributed to this syndrome during capture or handling operations: intravascular haemolytic episodes may be induced in animals by oxidative stress and often those episodes are fatal. Blood samples of 105 black rhinoceroses from different natural habitats were investigated and a genetic polymorphism of the erythrocyte enzyme glucose-6-phosphate dehydrogenase (G-6PD) is described. This is the first time a polymorphism for this enzyme has been described for a wild animal: a deficiency of the enzyme in humans is associated with intravascular haemolysis. Two common alleles resulting in three phenotypes were detected by means of starch gel electrophoresis and specific enzyme staining. The gene product of the variant allele, an anodally faster migrating enzyme, showed a markedly reduced activity (~ 50%) compared to the 'normal', slow isoenzyme. The allele frequencies of the deficient allele appear to fit with the incidence of haemolytic anaemia observed in the geographic areas sampled. Further genetic and haematological studies need to be performed to prove the causative role of this enzyme variant for haemolytic anaemia in black rhino.*

Since 1982, it has been well documented that black rhinoceroses (*Diceros bicornis*) suffer from acute intravascular haemolytic anaemia, especially when the animals are exposed to stress. This is a matter of particular concern about this rapidly diminishing species as the syndrome is characterized by intravascular haemolysis, which often gives rise to kidney failure, resulting in sudden death due to spontaneous haemolysis soon after capture or immobilization.[1] Paul *et al.*[2] obtained baseline haematological data by analysing blood samples of 31 wild black rhinoceroses from Zimbabwe and suggested an unstable haemoglobin was responsible. However, Fairbanks and Miller[3] described a polymorphism of the rhinoceros' haemoglobins and concluded that this polymorphism appears to be unrelated to acute haemolytic anaemia. In addition, the red-cell enzymes of rhinoceroses have been studied[4] but no evidence for red-cell enzyme abnormalities was found. Van Vliet[5] examined blood samples of 22 white and 31 black rhinoceroses. Of these animals, 30% of the black and 50% of the white rhinoceroses suffered from intravascular haemolytic anaemia as described for captive rhinoceroses.[4] It was found that when the animals were exposed to several types of stress, intravascular haemolytic anaemia occurred in some cases resulting in kidney failure and death.[5] Kock *et al.*[6] investigated the effects of capture and translocation on black rhinoceroses and, although no animal died at capture, there was a mortality rate of 14% between 1 week and 2 months after capture.

These reported incidences of haemolytic anaemia in black rhinoceroses prompted us to look into the possible genetic variation of the enzyme glucose-6-phosphate dehydrogenase (G-6PD). Glucose-6-phosphate dehydrogenase deficiency in humans is well described and has been studied extensively. In short, G-6PD catalyses the first step in the pentose-phosphate shunt. The enzyme catalyses the oxidation of glucose-6-phosphate to 6-phosphogluconate and NADPH is generated from NADP. The NADPH is needed to reduce oxidized glutathione to reduced glutathione, which is essential to maintain a functional red blood cell.[7] In the case of G-6PD deficiency, the red blood cells are unable to withstand oxidative damage. Red-cell destruction is intravascular and may result in haemoglobinuria and kidney failure. G-6PD deficiency is inherited and can lead to haemolytic anaemia when triggered by some drugs and infections.[8] Inheritance of human G-6PD further shows a characteristic X-linked pattern. A selective advantage of G-6PD deficiency in humans is that it can confer relative resistance against malaria and clinical data indicate that this is confined to heterozygous females.[8]

Materials and methods

Blood samples of 105 black rhinoceroses of different natural habitats were collected by personnel of the Zambezi Rhino Project and Natal Parks Board. The samples came from Zambezi Valley, Zimbabwe (n = 37), Mkuzi Game Reserve, Natal (n = 31), Hluhluwe/Umfolozi (n = 25) and Etosha, Namibia (n = 6). Blood samples were obtained from ear veins and acid-citrate–dextrose or heparin were used as anti-coagulants in the case of the Zambezi Valley rhinoceroses. Acid-citrate–dextrose was used for the Natal and Etosha animals. Blood samples were kept on ice in the field and centrifuged as soon as possible to separate the plasma, buffy coat and compacted red blood cells. The plasma and red blood cells were frozen in liquid nitrogen and transferred to the laboratory where the blood was kept at –20°C. Prior to electrophoresis, 200 µl of erythrocyte lysate was pipetted into a reaction tube, 20 µl of an aqueous solution containing 5% vol^{-1}. mercaptoethanol and 10% vol^{-1}. Triton X-100 was added. Another 200 µl of a water-based gel consisting of 20% sucrose and 4.5% Sephadex G-200 was added and mixed. The samples were applied to preformed slots in the starch gel about 8 cm from the cathodal end.

Starch gel electrophoresis: This was performed using a water-cooled electrophoretic apparatus. The bridge buffer consisted of 0.2 M Tris and 0.23 M histidine – HCl, pH = 7.4. The gel buffer was a 1:7.7 dilution of the bridge buffer. The conditions for electrophoresis were as follows: 18 h at 4°C at 300 V 300 mA^{-1} at 5 V cm^{-1}. After electrophoresis the first 10 cm of the anodal part of the gel was sliced into slabs of 2-mm thickness and stained. The staining solution consisted of 10 mg disodium NADP (Boehringer), 15 mg monosodium glucose-6-phosphate (Boehringer), 20 mg MTT, 1 mg PMS dissolved in buffer (pH = 7.4): 0.1 M Tris-HCl + 0.2 M $MgCl_2$. Filter paper (Schleicher and Schüll, no. 2316) 10 × 19 cm was soaked with the staining solution and laid over the cut starch gel and covered with a transparent household plastic wrap. The gel was incubated in the dark for about 30 min at 37°C until the banding pattern could easily be detected.

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In order to quantify the G-6PD activity for the three phenotypes, we used two techniques on blood samples which were available in sufficient volume and were fresh enough for reliable analysis:

Scanning: Lanes of the electrophoretic separation (Fig. 1) were scanned densitometrically to measure the intensity of enzyme bands at 540 nm, using a Cliniscan 2 (Helena Laboratories, Beaumont, Texas). The areas under the curves produced by the densitometer were used as an index of enzyme activity. Seven lanes (three of each homozygote and one heterozygote) were scanned.

Spectrophotometric measurements: Because of several problems involved in the quantitative assessment of electrophoresed samples, glucose-6-phosphate dehydrogenase activity was determined spectrophotometrically using a Lambda II spectrophotometer (Perkin-Elmer). Monosodium glucose-6-phosphate and disodium NADP were used as substrates. The rate of increase of NADPH was measured at 340 nm over a period of 9 min. A blank sample, without glucose-6-phosphate, was used as a standard. The reaction solution consisted of the following: 2 ml Tris buffer (pH = 7.2: 0.1 M Tris-HCl + $MgCl_2$), 100 µl disodium NADP (78.7 mM), 100 µl monosodium glucose-6-phosphate (70.9 mM), erythrocyte lysate (500 µl buffer + 100 µl lysate from compacted erythrocyte sediment). Since the assay of G-6PD is influenced by the number of erythrocytes used, haemoglobin concentration (assayed using the ferricyanide method[9]) was used to estimate the number of erythrocytes lysed for analysis and which contributed to G-6PD activity. A longer period elapsed between collection of samples from Etosha and laboratory analysis compared with the corresponding period for the Zimbabwe samples: this complicated direct comparison of the figures for Etosha and Zimbabwe because of a higher expected degree of denaturation of G-6PD in the samples from Etosha. We therefore performed separate analyses on the two sets of samples.

Data interpretation: Allele frequencies were calculated in the standard way for sex-linked loci. Sampling variances of the allele frequency estimates as well as the significance of differences in allele frequency between populations were calculated following Nei.[10]

Results

A polymorphism of the red-cell enzyme glucose-6-phosphate dehydrogenase was found in the black rhinoceros material we examined. Three different banding patterns (Fig. 1) allowed the formal genetic interpretation of two alleles (fast and slow; F/S) at one locus. The homo- and possible hemizygous animals showed a single band with a minor pre-band, whereas the heterozygous animals showed a double-banding pattern with a minor pre-band. The F isoenzyme, besides having a faster electrophoretic mobility, also stained a lighter intensity, which suggested a reduced activity.

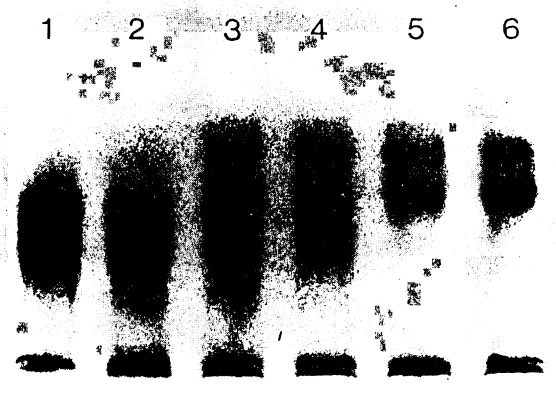

Fig. 1. An electrophoretic isoenzyme separation pattern indicating the different G-6PD phenotypes (F, FS and S) among black rhinoceroses from the Zambezi Valley. Each isoenzyme is represented by two bands on the gel due to post-translational modification of the enzymes. 1 = SS female, 2 = S male, 3 = FS female, 4 = FS female, 5 = F male, 6 = F Female. F stands for fast allele, S for slow.

Table 1. Phenotypes and allele frequencies of the four black rhinoceros populations for the glucose-6-phosphate dehydrogenase locus. S = slow allele, F = fast allele. Since standard ANOVA procedures for the analysis of sex-linked allele frequencies are not available for application to our data, the bottom part of the table presents pairwise statistical comparison of the allele frequencies.[10]

	Population			
	Mkuzi	H/U/C	Zambezi	Etosha
Sample size	31	25	37	6
Phenotype				
F	0	0	3	4
FS	1	0	4	1
S	30	25	30	1
Allele frequencies				
S	0.98±.019	1.0±0.0	0.88±.04	0.25±.11
F	0.02±.019	0.0±0.0	0.12±.04	0.75±.11
Statistical differences in allele frequencies (*P*)				
MKuzi	–	<0.33	<0.034	<0.0001
H/U/C	–	–	<0.005	<0.0001
Zambezi	–	–	–	<0.0001

Table 1 shows the distribution of the three phenotypes and the gene frequencies of the two alleles within the sampled black rhinoceros populations. The frequency of the F allele was much higher in the Etosha population than in the other populations ($\chi^2 = 40.69$, d.f. = 6, $P < 0.001$) and the fast allele was more common in the Zambezi population than in those from Mkuzi and Hluhluwe/Umfolozi/Corridor (Table 1). All six assayed heterozygous females yielded electrophoretic patterns of two bands with the more cathodal band noticeably fainter than the other.

Scanning of electrophoretic gel lanes revealed that F phenotypes stained 17% lighter than did S phenotypes (Mann-Whitney $U = 278$, $n1 = n2 = n3$, $P < 0.01$) (Table 2). These results

Table 2. Densitometric measurements on the staining intensities of electrophoretic isoenzyme bands of the different erythrocytic G-6PD phenotypes. Figures in the 'Density' column represent the proportion of areas under densitometric peaks associated with the relevant isoenzyme bands. The haemoglobin (Hb) variant observed for each individual is indicated in the last column.

Rhino subject	Sex	G-6PD phenotype	Density	Hb genotype
Etosha 1	M	S	97.25	A
Mkuzi 20	F	S	100.00	B
Mkuzi 23	F	S	93.95	B
Etosha 2	F	FS	95.32	B
Etosha 3	F	F	76.11	A
Etosha 4	F	F	80.67	A
Etosha 5	F	F	83.06	A

Table 3. Spectrophotometrically determined activity of G-6PD in three black rhinoceros phenotypes. Five individuals were assayed and separate analyses were conducted for samples from Etosha and from Zimbabwe (see methods). Specific activity is expressed as μM substrate converted per minute per gram of haemoglobin.

Rhino subject	Sex	Phenotype	Specific activity ($\mu M\ min^{-1} g^{-1}$)	Relative activity (%)
A				
Etosha 1	M	S	1.47	100.0
Etosha 2	F	FS	1.13	76.2
Etosha 3	F	F	0.83	56.6
B				
Zambezi 6	F	S	2.23	100.0
Zambezi 10	M	F	0.71	34.8

strongly suggest that the F isoenzyme has a lower enzymatic activity than the S isoenzyme.

Direct spectrophotometric evaluation of reaction mixtures to make accurate measurements of the enzymatic activity of erythrocytic G-6PD corresponding to the different G-6PD genotypes of five black rhinoceroses confirmed the results obtained from gel scanning. Table 3 shows that the F phenotype had a lower activity than the S phenotype and that the single heterozygote (FS) had an intermediate activity. The erythrocytic G-6PD activity of the F phenotypes ranged between 35% and 57% of that of the S phenotypes.

Two variants of haemoglobin[3] were observed during our analyses, with one variant being restricted to the samples from Etosha.

Discussion

This is the first time that a polymorphism at the G-6PD locus, similar to that found in humans, has been described in a wild animal. The existence of a wild animal analogue of this polymorphism has obvious implications for medical research. Humans with G-6PD deficiency can be asymptomatic but acute haemolytic anaemia can be triggered by a number of drugs, infections or the ingestion of fava beans.[8] We suggest that the stress associated with capture procedures and the administration of some drugs to black rhinoceroses induces haemolytic anaemia if the animals are G-6PD deficient, resulting in the high reported incidence of haemolytic disease.[5] Heterozygous females are protected against malaria in human populations with a G-6PD polymorphism. We speculate that a similar protection mechanism against some parasites helped to establish the G-6PD polymorphism in the black rhinoceros. However, for those animals with the G-6PD deficient variant, this polymorphism can be detrimental and stress-related events may induce the negative effects noted above.

Rhinoceroses with the F allele come almost exclusively from Etosha and Zambezi and only one F allele was observed in the Natal population. If our hypothesis resulting from the enzymatic activity measurements holds true, animals with the fast variant are more likely to develop haemolytic anaemia. The Etosha and Zambezi populations would then be particularly vulnerable because of the high frequency of the deficient, faster-migrating allele. Almost no figures have been published about the incidence of mortality after the capture of black rhinoceroses in different areas, but available data suggest that this is the case. Kock *et al.*[6] reported mortality greater than 10% after capture and immobilization of black rhinoceroses in the Zambezi Valley. Losses were also encountered during capture operations in Namibia (B. Fox, Namibia Directorate of Wildlife Conservation and Research, pers. commun.). On the other hand, haemolytic anaemia was never observed while 163 black rhinoceroses were immobilized during planned capture operations and an estimated 150 injured or snared black rhinoceroses were immobilized in Natal over the same period (J. Flamand, Natal Parks Board, pers. commun.). These differences in mortality may be due to genetic differences, at the G-6PD locus, between the rhinoceros populations.

We observed a haemoglobin polymorphism in the Etosha population. However, this seems unrelated to haemolytic disease in black rhinoceroses[3] and was, in addition, not linked to G-6PD deficiency in black rhinoceroses (Table 2). Our results therefore suggest that glucose-6-phosphate dehydrogenase deficiency is a more likely the cause of haemolytic anaemia in black rhinoceroses. However, our data are inferential and do not demonstrate a direct cause and effect relationship between erythrocytic G-6PD deficiency and haemolytic anaemia in black rhinoceroses. Direct haematological and genetic studies on live animals should be conducted to establish such a relationship. Our results suggest also that drugs affecting the pentose phosphate shunt should not be used for immobilizing black rhinoceroses.

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