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Haematological studies on wild black rhinoceros (*Diceros bicornis*)—evidence of an unstable haemoglobin

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(With 1 figure in the text)

Baseline haematological data were obtained through routine analyses of blood samples from 31 wild black rhinoceroses captured in the Mana Pools National Park, Zimbabwe. Additional tests showed that the haemoglobin of this population is unstable; this observation helps explain the attacks of acute intravascular haemolysis documented in captive animals.

Contents

Introduction

Acute intravascular haemolytic anaemia is a recurrent veterinary problem in captive populations of black rhinoceroses, being associated with about 40% of mortalities of adults in zoos (Miller & Boever, 1982; Chaplin *et al.*, 1986). Blood samples from a few of these animals have been studied in detail for an underlying cause; no evidence has been found for red cell enzyme abnormalities (Paglia *et al.*, 1986), and investigations of osmotic fragility, erythrocyte membrane protein composition and immune mediated lysis have not yielded an explanation for the haemolysis (Chaplin *et al.*, 1986). The latter works did, however, note a degree of instability in

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haemoglobin subjected to isopropanol testing and saw two haemoglobin bands on electrophoresis; but the low number and poor quality of their samples prevented them from drawing firm conclusions on the stability of rhinoceros haemoglobin.

Investigation of the haemolytic anaemia problem has been hampered not only by the difficulty of obtaining quantities of fresh blood from healthy and afflicted animals in zoos, but also by a lack of data on the haematology of animals in the wild. In mid-1986, the translocation of a large number of black rhinoceroses from the Zambezi Valley of Zimbabwe to other parts of the country (to build up populations in less poached areas) provided an opportunity for the collection of blood samples in order to acquire baseline haematology data as well as to investigate the haemolytic tendency further.

Methods

All the black rhinoceroses were captured in the Mana Pools National Park of Zimbabwe (bordering the Zambezi River) during mid-1986 (the cool dry season) while the availability of browse and water was good and the animals could be expected to be in prime natural condition. Of those from which blood samples were taken, 11 were adult males, 16 were adult females and 4 were juveniles.

The method of capture involved darting each animal with an immobilizing drug, securing it in lateral recumbency on a sled as quickly as possible after collapse, winching it on to a truck and conveying it to holding pens where the blood was collected just prior to administrating an antidote to the analgesic drug. Etorphine hydrochloride was used to immobilize most of the rhinoceroses, at a dose of 1·75 mg (sometimes in conjunction with 50 mg hyoscine hydrobromide); the successfully darted animals mostly collapsed in 7–20 min. The remaining captured animals were drugged with 2–3 mg carfentnyl citrate, which generally showed more rapid effect.

Blood was obtained from an ear vein and collected into EDTA, heparin and CPD anticoagulant. Initial processing and testing of the blood commenced in a field laboratory 15–50 min after bleeding. Blood films were made and fixed in methanol for later Romanowsky staining and differential white cell counts (all the differential counting being done by one person (BP), and for parasite screening following Giemsa staining. Reticulocyte preparations were made with new methylene blue. Osmotic fragility was investigated by standard methods (Dacie & Lewis, 1984), and erythrocyte sedimentation rate (ESR) was measured in a Wintrobe tube, using heparinized blood.

Blood samples were transferred to a haematology laboratory in Harare on wet ice as soon as possible—usually within 48 hours. Blood counts were then carried out with EDTA specimens using a Coulter S Plus machine with quality assurance provided by both internal and external reagents (Coulter 4C, and National External Quality Assurance, UK). Manual red blood cell (RBC) and white blood cell (WBC) counts and microhaematocrit were also performed in the field laboratory but only Coulter data are presented here since the results were similar.

In Harare, the following tests were carried out on EDTA blood using standard methods (Dacie & Lewis, 1984): acidified glycerol lysis time ($AGLT_{50}$), haemoglobin stability by both heat and isopropanol precipitation (17% isopropanol v/v with tris/HCl buffer, pH 7·4, at 37 °C), staining for Heinz bodies by methyl violet and examining wet preparations. Human specimens kept under similar conditions were used as controls. Haemoglobin electrophoresis was performed on cellulose acetate at pH 8·9.

Glucose-6-phosphate dehydrogenase (G6PD) in CPD blood was assayed with a commercial test kit (Sigma, St. Louis, USA) which includes maleimide as an inhibitor of 6-phosphogluconate dehydrogenase. All results were corrected to a temperature of 30 °C using data provided by the manufacturer.

The significance of differences in haematology values was assessed by means of Student's *t*-test (probability level, $P = 0{\cdot}05$).

TABLE I

Means, standard deviations and ranges of haemoglobin, red cell count and indices

Characteristic (Sample size)	Unit	Mean	S.D.	Range
Haemoglobin (29)	g/l	158·1	18·2	108–192
Red cell count (29)	$\times 10^{12}$/l	5·08	0·59	3·89–6·38
Haematocrit (29)	l/l	0·445	0·049	0·308–0·513
Red cell distribution width (20)		14·4	1·88	10·3–17·0
Mean cell volume (29)	fl	87·2	5·8	75·3–97·1
Mean cell haemoglobin (29)	pg	31·2	2·35	27·9–36·1

Results

Red blood cells

The values obtained for haemoglobin (Hb), RBC counts, haematocrit, red cell distribution width (RDW), mean cell volume (MCV), and mean cell haemoglobin (MCH) are shown in Table I.

The animals have been grouped as a whole as there was not a significant difference between adult males and females (mean Hb levels were $165{\cdot}4 \pm 2{\cdot}15$ and $157 \pm 1{\cdot}15$ g/l, respectively); there were too few calves to evaluate separately. No gross morphological abnormalities of the red cells were noted in the peripheral blood smears.

We found the MCV to be higher in adult females than males (means $90{\cdot}2 \pm 4{\cdot}39$ and $85{\cdot}9 \pm 4{\cdot}44$ fl, $t = 2{\cdot}41$, $P < 0{\cdot}05$). There were no significant sex differences for MCH. The lowest haemoglobin value measured was 108 g/l in a male calf caught together with its mother (Hb 154 g/l, with normal RBC indices), and this calf also had a low MCV and MCH (75·3 fl and 28·0 pg, respectively), suggestive of iron deficiency. Another calf also had RBC indices at the lower limit of our range (MCV 77·7 fl; MCH 27·9 pg) but a normal Hb of 152 g/l.

Reticulocytes could not be accurately counted due to very low numbers; none was detected in some animals and the highest count was 0·5%. These cells showed a coarsely clumped pattern. No cells showed a finely stippled pattern typical of HbH in man (tetramers of the β globin chain).

White cells and platelets

The values for total leucocyte counts and differential counts, platelet counts and ESR are shown in Table II. There was a wide range of leucocyte counts with all the higher counts being due to higher percentages of neutrophils. Females were readily identified by a small proportion of neutrophils having typical drumstick nuclear appendages. Eosinophil counts were also variable, the wide distribution being due to five animals having counts over $1{\cdot}1 \times 10^9$/l. Basophils were identified in most films (at levels of under 0·5%). Morphology of lymphocytes was similar to that in

TABLE II

Means, standard deviations and ranges for total and differential leucocyte counts, platelet counts and erythrocyte sedimentation rate

Characteristic (Sample size)	Unit	Mean	S.D.	Range
White cell count (30)	$\times 10^9$/l	13·98	4·77	5·6–26·2
Neutrophils (30)	$\times 10^9$/l	8·39	3·30	1·8–18·5
Lymphocytes (30)	$\times 10^9$/l	3·86	1·88	1·01–9·49
Monocytes (30)	$\times 10^9$/l	1·05	0·76	0·37–3·86
Eosinophils (30)	$\times 10^9$/l	0·69	0·56	0·09–2·36
Platelets (18)	$\times 10^9$/l	223·5	67·6	126–410
ESR (29)	mm/h	23	17·5	2–78

humans, apart from occasional cells being larger (approximately 14 μm diameter) with a cleft nucleus. It was not difficult to differentiate between monocytes and lymphocytes.

Lymphocyte counts were higher in the four calves than in the group as a whole $6{\cdot}65 \pm 1{\cdot}91$ and $3{\cdot}86 \pm 1{\cdot}88 \times 10^9$/l, respectively, $P < 0{\cdot}02$).

Platelet counts were low in some specimens due to activation and aggregation during collection, and these results are not included.

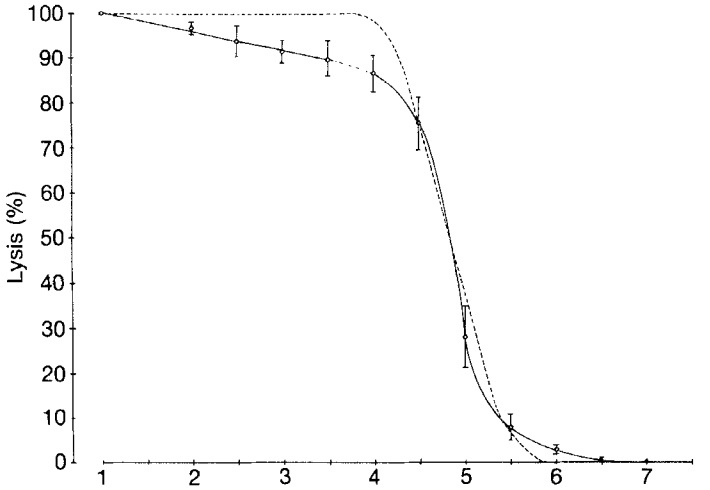
ESR showed marked variation which in part can be attributed to ambient temperature variation (range of shade temperature was 11–36 °C, although most tests were done at 22–28 °C).

Blood parasites

Screening for blood parasites (by Dr C. Foggin of the Government Veterinary Research Laboratory, Harare, and Dr V. Clarke of the Department of Biological Sciences, University of Zimbabwe) revealed intracellular *Theileria*-type parasites in 9/32 animals, *Babesia*-type parasites in 1/32 and *Trypanosoma brucei* in 1/32. The counts of the *Theileria*-type organisms ranged from one per 120 high-power fields to one per 15 (each high-power field containing about 200 red cells). The *Theileria*- and *Babesia*-type parasites have not been identified but appear similar to those described in white rhinoceroses by Bigalke, Keep, Keep & Shoeman (1970).

Haemolytic tendency

The tendency towards spherocyte formation was assessed by osmotic fragility and $AGLT_{50}$; the former results are shown in Fig. 1. The mean corpuscular fragility was 4·85 g/l NaCl. All



Sample size	28	28	20	24	24	23	19	22	26	28	28	28
Mean lysis (%)	100	97	94	91	90	86	75	28	8	3	1	0
S.D.	-	3·7	7·6	6·1	9·8	9·9	12·9	16·2	7·6	2·8	1·4	-

FIG. 1. Osmotic fragility data from the sample of wild black rhinoceroses (—⊕—, mean ± 2 S.E.), with the curve of the horse (- - - -) indicated for comparison. Horse data obtained by researchers at College of Veterinary Medicine, Ohio State University (C. W. Kohn, pers. comm.).

rhinoceroses showed an $AGLT_{50}$ response time of more than 30 min, and longer tests were not performed.

When testing haemoglobin stability, it was found that freshly prepared haemolysates ($n = 30$) were all stable at 50 °C for 60 min, but all showed dense precipitation at 5 min in isopropanol buffer. Methyl violet staining was positive in all animals with up to 10% of red cells showing a single Heinz body. This test was positive even after storage of EDTA specimens at 4 °C for two weeks. Haemoglobin electrophoresis showed a similar pattern in all animals tested ($n = 30$) with two fast-moving (anodally) bands of seemingly equal density (densitometry was not performed).

G6PD was assayed on 19 animals, and gave the following activities:

Mean 755·7 ± 140·3 (range 532·5–1032·0) units/10^{12} red cells or 24·6 ± 4·35 (range 18·7–34·1) units/g Hb.

Discussion

The results for red cell indices conform closely with data obtained from seven captive black rhinoceroses by researchers of the Zoological Society of London (R. Kock, pers. comm.), with data from a further 18 analyses of blood from captive animals (Maruska, Dresser & Barden, 1986), and with 'normal' data from six animals referred to by Chaplin *et al.* (1986). The mean values for Hb, RBC count and haematocrit are similar to those of adult men (Dacie & Lewis, 1984). The

RDW is wider than the normal range for man (8·5–11·5), indicating a greater degree of anisocytosis (only specimens received within 48 h are included).

The total white cell, neutrophil and eosinophil counts tended to be higher in the Zambezi animals than in the zoo animals. No obvious relationship between haematological parameters and parasitaemia was noted. The calves had higher lymphocyte counts than adults; children have higher counts than adult humans (Dacie & Lewis, 1984). Subtyping into B and T cells was not done.

The osmotic fragility results show a typically sigmoid curve, but with a flattish gradient at the lower salt concentrations, which may be partly due to inaccuracies arising under field conditions. The mean corpuscular fragility (MCF) is the same as that of the horse, with the red cells being more fragile than those of humans, in which the MCF is 4·25 g/l NaCl (Dacie & Lewis, 1984). There was no indication of spherocyte formation as judged by a shortened acidified glycerol lysis time, which is a more sensitive test for the human hereditary spherocytosis defect than osmotic fragility (Zanella *et al.*, 1980).

The rapid precipitation of haemoglobin in isopropanol and the high proportions of Heinz bodies shown in the methyl violet staining of fresh red cells are good evidence of the instability of rhinoceros haemoglobin. Hereditary Heinz body haemolytic anaemias are well recognized in humans with over 30 haemoglobin variants described (White, 1976). The clinical features in humans are very variable but continuing haemolysis is usually demonstrable and can be rapidly worsened by infection, pregnancy and oxidant compounds (White, 1974). Low-grade haemolysis was not apparent in these wild rhinoceroses. In animals affected by intravascular haemolysis, it would be useful to test for dipyrroluria due to abnormal catabolism of Heinz bodies or free haem groups.

G6PD activities were two to three times the equivalent in humans, and similar results were seen in eight healthy rhinoceroses in captivity (Paglia *et al.*, 1986). The latter workers have also examined other enzyme activities in the species, and found high activity of hexokinase and glutathione peroxidase that would also appear to be consistent with an unstable Hb; glutathione levels did not appear particularly low. In humans, unstable haemoglobinopathies are frequently associated with increased activity of the Embden-Meyerhof and pentose phosphate pathways (White, 1974). A relative deficiency of adenine nucleotides (Paglia *et al.*, 1986) may exacerbate the tendency of haemoglobin instability.

Understanding of the pathophysiology of intravascular haemolysis in the black rhinoceros could be enhanced through analysis of the amino acid sequence of the haemoglobin together with crystallography to reveal tertiary structure. It is unlikely that there will be a single agent responsible for triggering haemolytic episodes; these are probably the end result of a variety of oxidant stresses on the red cells.

Permission for blood collection was granted by the Director, Department of National Parks and Wildlife Management, Zimbabwe, and the research was facilitated by Mr C. Coetsee and his staff of the Umtshibi Capture Unit (of that department). Assistance in the provision of equipment, materials and advice was given by Mr G. West of the Blood Transfusion Service, Harare, Dr D. Smith of the Veterinary Faculty, University of Zimbabwe, Dr S. Grace of the Department of Physiology, University of Zimbabwe, Mr R. Jooste and other members of the Government Veterinary Research Laboratory, Harare. Dr E. Miller of St. Louis Zoological Park, USA, provided the G6PD assay kit. Dr C. Foggin of the Government Veterinary Research Laboratory and Dr V. Clarke of the Department of Biological Sciences, University of Zimbabwe, undertook the parasite screening. R. du Toit is funded by the World Wildlife Fund.

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