INVESTIGATION OF THE HAEMOLYTIC EFFECTS OF *ANCYLOSTOMA CEYLANICUM* : OBSERVATIONS ON INFECTED DOGS *IN VIVO* AND HUMAN AND DOG BLOOD *IN VITRO*

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INTRODUCTION

Hookworm anaemia is a major cause of ill-health and mortality. In the early parts of this century, a number of investigators found in *in vitro* studies, haemolysins in various species of hookworm (Alessandrini, 1904; Noc, 1908; Preti, 1908; Whipple, 1909; Schwartz, 1920; Fulleborn and Kikuth, 1929; Cruz, 1933). Nevertheless, it became clear from *in vivo* studies using radiolabelled erythrocytes that the loss of blood from the gastrointestinal tract was the major cause of anaemia and the possible role of haemolysins produced by these worms lost favour (Lane, 1937; Roche and Layrisse, 1966).

In the studies of dogs infected with varying numbers of *Ancylostoma ceylanicum*, however, we noted during the preparation of lymphocytes on Ficoll-paque columns, that considerable haemolysis was present in those columns layered with blood from the more heavily infected animals. We have, therefore, re-examined the possible role of haemolysis in the genesis of hookworm anaemia. We sought evidence of haemolysin in the blood of dogs with varying intensities of infection and studied the effects of adult worm extract, adult worm excretory/secretory (E/S) products and living adult worms on normal dog and normal human erythrocytes.

MATERIALS AND METHODS

A. ceylanicum: Worms were obtained from an infected dog in Malaysia. The acquisition of the worm, maintenance of the life cycle and methods of infection have been described in detail elsewhere (Carroll *et al.*, 1983). Recent studies have shown that patent infections with this strain develop in humans (unpublished observations). Living adult worms were removed from the intestine of a dog which had been infected percutaneously six weeks prior to autopsy. Soluble protein extracts of adult worms were prepared as described earlier (Carroll *et al.*, 1981) and then sterilised by passage through a 0.22 μm Millex-GV filter (Millipore Corporation, Bedford, MA). Excretory/secretory (E/S) products of adult worms were also obtained. The preparation, concentration and sterilisation of these products have been described elsewhere (Carroll *et al.*, 1984). Protein concentrations of adult worm extract and E/S products were estimated by the method of Hartree (1972).

Dogs: Male mongrel dogs, three to five months old were obtained from the general public. Six dogs were prepared, housed and infected percutaneously as described previously (Carroll *et al.*, 1983). Two dogs in each group were infected with either 150, 1,350 or 12,150 filariform larvae of *A. ceylanicum*. Six weeks after infection, blood was taken by venepuncture and collected in tubes containing EDTA or sodium citrate (Venoject, Terumo, Tokyo, Japan) or mucous sodium heparin (Commonwealth Serum Laboratories, Melbourne, Australia).

Normal blood: Pooled uninfected human group O blood and pooled uninfected dog

blood were collected in tubes containing either sodium citrate or citrate-phosphate-dextrose solution.

Techniques: Blood films were prepared and complete blood counts were analysed on a Coulter Counter S-Plus (Coulter Electronics Inc., Hileah, FL). Plasma haptoglobin (semi-quantitative electrophoretic technique), haemoglobin and methaemalbumin concentrations were measured using the methods of Dacie and Lewis (1975). Autohaemolysis and osmotic and mechanical fragilities of red cells were measured by the methods of Dacie and Lewis (1968). Haemolysis, with and without added glucose, and erythrocyte mechanical fragilities were measured in the presence of worm extract. Serum total bilirubin levels were measured in a SMA-6 Plus autoanalyser (Technicon Instrument Corporation, Tarrytown, NY) and urine porphobilinogens were sought using the method of Wallace and Diamond (1925).

Red cells were radiolabelled with ^{51}Cr (Amersham Laboratories, England) by the method of Dacie and Lewis (1975). Following several washes with sterile 0.9% sodium chloride, the labelled red cells were resuspended as a 5% suspension in RPMI 1640 (300 mOsm) (Grand Island Biological Company, Grand Island, NY) containing 5 µg/ml tobramycin sulphate and 8 µg/ml cefoxitin, henceforth referred to as medium. Living adult worms were removed from the mucosa of the small intestine of a dog at autopsy, placed directly into medium at 37°C and washed gently several times. One male or one female worm was added individually to separate tubes containing 200 µl of the red cell suspensions. These tubes and tubes without worms were incubated at 37°C in an atmosphere of 5% CO_2 for 24 hours. Following incubation, 0.5 ml 0.1M phosphate buffered saline (pH 7.4) was added to each tube. The contents of the tubes were agitated gently and centrifuged at 75 g for 10 minutes. The supernatant of each tube was removed and placed in a separate container before the radioactivity of both cells and supernatant were counted for 100 seconds using an LKB 1280 Ultragamma Counter (LKB, Sweden). The percentage of ^{51}Cr released from the red blood cells after incubation with adult worms was compared with that of the control tubes. Similarly, 100 µl of either E/S products (1,520 µg/ml) or medium were added to 10 tubes each containing 200 µl of ^{51}Cr-labelled red blood cell suspensions prepared from either pooled human or pooled dog blood. Tubes were incubated and the released ^{51}Cr measured as described above.

The morphology of red cells from each of the infected dogs was examined by scanning electron microscopy. One ml of whole blood was added to 10 ml of 0.04 M phosphate buffer pH 7.4 (PB) containing 0.9% sodium chloride and washed gently several times in the same buffer. After the final wash, cells were placed in 2.5% glutaraldehyde in PB. Cells were then attached to poly-L lysine-coatea coverslips and dehydrated in graded ethanol solutions to superdry alcohol. These were then critical-point dried (Polaron Apparatus, Polaron, Watford, England), mounted on aluminium studs and coated with 20 nm of platinum using the E 5100 SEM Coating Unit (Polaron). The preparations were examined on a Philips P-SEM 500 electron microscope at an accelerating voltage of 25 kV.

RESULTS

Studies of infected dogs: The most heavily infected dogs developed a severe microcytic anaemia six weeks after infection (Table 1). The blood films showed that dogs with the heaviest infection had a mean reticulocyte count to 4.9% with occasional normoblasts. This contrasts with the mean reticulocyte

Table 1

Relationship between infective dose of larvae and haemoglobin, and MCV before and six weeks after infection.

Infective dose	Pre-infection		Post-infection	
	Hb* gm/dl	MCV* fl	Hb gm/dl	MCV fl
150	12.6	70.5	15.8	70.1
1,350	15.1	69.4	15.5	70.6
12,150	16.7	70.2	9.5	59.5

Hb = haemoglobin concentration
MCV = mean corpuscular volume
* mean value for two dogs

count of 1.9% for the dogs with the lightest infections.

Serum haptoglobin concentrations were reduced in the least heavily infected dogs and increased in the most heavily infected animals (Table 2). Plasma haemoglobin and serum methaemalbumin were not detected in any animal. Autohaemolysis was increased in the most heavily infected dogs, both in the presence and absence of glucose (Table 2). When the data from dogs infected with 150 or 1,350 worms were combined and compared with animals infected with 12,150 larvae, this difference was statistically significant ($p < 0.001$, "t" test) in the case of erythrocytes incubated in the presence of glucose. There was no significant difference between dogs with respect to osmotic fragility (Table 2). Serum total bilirubin levels were not elevated in any dog (Table 2) and urobilinogen was not found in the urine of any animal. Scanning electron microscopical examination disclosed no apparent differences in the surfaces of red cells obtained from the various animals.

Effects of worm extracts on erythrocytes *in vitro*: The effects of varying quantities of adult worm extract on haemolysis of pooled human or dog erythrocytes in the presence or absence of glucose are shown in Table 3; no significant differences were observed.

Mechanical fragility of red cells was measured both immediately on addition of worm extract (final concentration 400 µg/ml) and after two hours incubation with extract at 37°C. No significant haemolysis of either dog or human erythrocytes was seen.

Table 2

Relationship between infective dose of larvae and haptoglobin, autohaemolysis in the presence (+) and absence (–) of glucose, osmotic fragility and bilirubin.

Infective dose	Haptoglobin concentration gm/l	Autohaemolysis glucose+	glucose–	Osmotic fragility*	Serum bilirubin concentration µmol/l
150	trace	0.5	1.7	0.43	< 2
150	<0.5	4.8	1.4	0.47	< 2
1,350	0.5-1.0	0.6	1.0	0.45	< 2
1,350	0.5-1.0	6.3	1.3	0.46	< 2
12,150	>1.0	49	18.0	0.39	< 2
12,150	>1.0	64	4.0	0.43	< 2

* Concentration of NaCl producing 50% erythrocyte lysis.

Table 3

Effects of varying concentrations of adult worm extract on haemolysis of both human and dog erythrocytes in the presence (+) and absence (–) of glucose.

Final conc. adult worm extract µg/ml	Haemolysis* Human erythrocytes glucose+	glucose–	Dog erythrocytes glucose+	glucose–
400	0.5	0.8	0.9	2.9
100	0.5	1.0	0.6	1.8
25	0.5	0.8	2.1	2.9
6	0.2	0.7	0.8	1.8
Buffer only	0.5	0.9	0.4	3.8

* mean value of three tests.

Effects of worm E/S products on erythrocytes *in vitro:* ^{51}Cr release from labelled dog and human red cells was measured in the presence and absence of E/S products (final concentration 510 µg/ml). No significant differences were observed, there being 3.5±0.3% and 3.4±0.4% release from control and test dog red cells, respectively, and 4.9 ± 0.5% and 4.5 ± 0.3% release from control and test human red cells, respectively.

Effects of living worms on erythrocytes *in vitro:* ^{51}Cr release from labelled dog and human red cells was measured in the presence and absence of living male and female adult worms (Table 4). No significant increase in release of ^{51}Cr was observed from dog red cells for either male or female worms. With human cells, however, the release of ^{51}Cr was increased significantly with both male and female worms ($p < 0.005$, "t" test).

Table 4

Release of ^{51}Cr from erythrocytes in the presence or absence of living adult worms. Ten samples were in each group.

	Per cent release of ^{51}Cr from erythrocytes		
	Controls	Male worms	Female worms
	mean±S.D.	mean±S.D.	mean±S.D.
Dog erythrocytes	7.7 ± 1.8	7.1 ± 2.2	7.9 ± 1.9
Human erythrocytes	3.7 ± 0.3	5.3 ± 0.5	5.0 ± 1.1

DISCUSSION

In1904, Alessandini first demonstrated a haemolysin in an extract of *A. duodenale*. This was confirmed by Preti (1908), Whipple (1909) and Schwartz (1920) in this species, in *Necator americanus* by Noc (1908) and Whipple (1909) and in the dog hookworm, *A. caninum* by Whipple (1909), Fulleborn and Kikuth (1929) and Cruz (1933). On the other hand, Loeb and Fleisher (1910) failed to demonstrate haemolysins in extracts of *A. caninum*.

Firstly, we examined dogs with varying hookworm burdens. We observed a severe microcytic anaemia in the most heavily infected animals. There was little evidence of haemolysis in these animals, however. Bilirubin levels were not elevated, plasma haemoglobin, serum methaemalbumin and urinary urobilinogens were not detected, nor was there increased osmotic fragility in the red cells of any of these animals. Similarly, scanning electron microscopical examination revealed that the surfaces of erythrocytes were normal. Furthermore, serum haptoglobulin concentrations were actually increased, whereas reduced levels should have

been found if there were significant haemolysis. It is well recognised that increased serum haptoglobin concentrations are found in inflammatory disorders (Nyman, 1959). It is likely that the present observations reflect the marked necrosis and inflammation of intestinal mucosa found in the heavily infected dogs (Carroll *et al.*, 1984). The only laboratory test indicative of defective red cells was the increased autohaemolysis of erythrocytes obtained from the more heavily infected dogs.

Secondly, we examined the effects of various worm preparations on normal dog and human erythrocytes *in vitro*. Again, little evidence of haemolysis could be found. Soluble extracts of *A. ceylanicum* did not enhance haemolysis nor did they alter the mechanical fragilities of red cells. Similarly, adult worm E/S products did not cause significant release of ^{51}Cr from labelled red cells. When such cells were incubated with living worms, however, there was increased release from human but not dog radiolabelled erythrocytes.

In conclusion, the weight of evidence from both *in vivo* and *in vitro* studies indicates that worm haemolysins are at most only a minimal factor in the aetiology of hookworm anaemia.

SUMMARY

Investigations into the probable role of haemolysins in the causation of hookworm anaemia have been undertaken in living infected dogs. Secondly, the effects of living hookworms and various worm products on erythrocytes *in vitro* have been assessed. In dogs infected with varying numbers of *A. ceylanicum*, severe microcytic anaemia developed in the most heavily infected animals six weeks after infection. Erythrocytes from the latter animals showed significantly greater autohaemolysis in the presence of added glucose. When serum bilirubin and methaemalbumin, plasma haemoglobin, urinary urobilinogin and osmotic fragility of their red cells were measured, however, no evidence of haemolysis was detected. Erythrocytes from these animals appeared normal under scanning electron microscopy. In *in vitro* studies varying concentrations of adult worm extract had no effect on the haemolysis of either dog or human erythrocytes in the presence or absence of glucose nor on their mechanical fragilities. There was no increase in ^{51}Cr release from dog or human labelled red cells when incubated with either adult worm extract or excretory/secretory products of worms. Living adult worms caused an increase in ^{51}Cr release from human but not dog labelled erythrocytes. Thus, the role of haemolysins in the genesis of hookworm anaemia is minimal.

ACKNOWLEDGEMENTS

This study was supported by a grant from the National Health and Medical Research Council of Australia. The authors are grateful to Dr. J.M. Papadimitriou for the electron microscopical examination.

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