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## Anaemia in trypanosomiasis: Mechanisms of erythrocyte destruction in mice infected with *Trypanosoma congolense* or *T. brucei*

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### Summary

Studies in mice infected with *T. brucei* or *T. congolense* showed that increased red cell destruction in the spleen occurred as from the third day of patent parasitaemia and this resulted in a marked reduction of the half-life of transfused syngeneic  $^{51}\text{Cr}$  labelled cells. There was a progressive increase in the osmotic fragility of the red cells, especially in *T. congolense* infected mice which also showed a more severe anaemia. The antiglobulin test was only rarely positive in the late stages of *T. brucei* infection. Incubation of normal red cells with plasma from infected mice in vitro did not result in haemolysis, but in the case of plasma from *T. brucei* infected mice, it caused an appreciable reduction in the half-life of the cells when transfused into normal mice. It is suggested that mechanisms of red cell destruction in trypanosome infections are complex and may vary with the species of trypanosomes, the host and the stage of infection.

### Introduction

Anaemia occurs in human and animal trypanosomiasis and is regarded as the main pathological feature in livestock infected with *Trypanosoma congolense* and *T. vivax* which constitute the haematic group (Fiennes, 1970; Losos and Ikede, 1972). Opinion is divided on the pathogenesis of the anaemia but it is generally agreed that three broad factors: increased red cell destruction, inadequate bone marrow response and haemodilution (or hydraemia), contribute to the anaemia (Boycott and Price-Jones, 1913; Fiennes, 1954, 1970).

Fiennes (1954) reported the occasional presence of a haemolysin in the plasma of cattle infected with *T. congolense*. He noted that its occurrence was transitory at the rare intervals of active trypanolysis, and could therefore be

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easily missed. There is no report of a follow-up of this study, but a haemolytic factor was recently isolated from an in vitro suspension of living trypanosomes (*T. brucei*) (Huan et al., 1975).

Mackenzie and Cruickshank (1973) observed marked erythrophagocytosis in sheep infected with *T. congolense*. They suggested that the phagocytic activity was probably due to a) the coating of cells with parasitic antigen, b) the production of defective cells, and c) an autoimmune reaction. Woodruff et al. (1973) observed a shortened life span of erythrocytes in human trypanosomiasis and demonstrated complement on the red cell membrane. Similarly Jennings et al. (1974) observed a marked reduction in the half life of erythrocytes in rats and mice infected with *T. brucei* and they suggested that the anaemia observed was due mainly to extravascular haemolysis. However, since red cell destruction was evident within a few days of infection, they were of the opinion that immune mechanisms were unlikely, but that the reticulo-endothelial system was probably acting non-specifically following stimulation by trypanosomes. Fiennes (1954) had earlier made a similar suggestion in cattle infected with *T. congolense*. Recently, Woo and Kobayashi (1975) observed adsorption of *T. brucei* antigen onto normal rabbit red cells after incubating both in vitro at 37° C. They then postulated that in infected rabbits, trypanosome antigen-antibody complexes were coated on the red cell surface and that these cells under certain conditions were lysed by complement or agglutinated and removed by the spleen.

The present studies were undertaken to determine the possible individual role played by plasma and red blood cells in the anaemia of mice infected with *T. congolense* or *T. brucei*.

### Materials and methods

CD1 strain of mice weighing about 25 g at the start of each experiment were used. They were infected with trypanosomes by intraperitoneal injection of  $1 \times 10^3$  parasites in 0.25 ml of phosphate-glucose-saline pH 7.4. The strains of parasites used were *T. congolense* derived from a stablate of TREU 938 and *T. brucei* derived from a stablate of TREU 667. Both strains cause a subacute to chronic infection of up to 2½ months duration, but anaemia is usually marked within 2–3 days (*T. brucei*) and 6 days (*T. congolense*) of patent parasitaemia.

*Evidence of erythrophagocytosis.* Five mice (2 infected with *T. congolense*, 2 with *T. brucei* and 1 control) were killed daily during the first cycle of parasitaemia (5–15 days) and onset of anaemia as judged by the packed cell volume (PCV). Impression smears of spleen were made from each mouse, stained with May-Grunwald-Giemsa stain, and examined under the microscope using an oil immersion ( $\times 100$ ) objective.

*Test for a haemolysin.* Heparinised cardiac blood was obtained from the above mice killed daily. The erythrocytes were separated from plasma by centrifugation at room temperature, and the plasma stored in aliquots at  $-20^\circ\text{C}$  until all the mice had been sacrificed (about two weeks). Thereafter the effect of plasma from infected mice on normal mouse red blood cells (MRBC) was tested basically as described by Fiennes (1954). A 5% suspension of washed MRBC was prepared in 0.01 M phosphate buffered, 0.015 M saline (PBS) pH 7.4. To a tube containing 0.1 ml of thawed plasma or distilled water, 0.2 ml of the MRBC suspension was added and then incubated at 37° C for 30 min. After centrifugation, the optical density (O. D.) of the 0.2 ml of the supernate diluted to 1

ml with PBS was determined in a portable EEL colorimeter using a blue filter<sup>1</sup>. The O. D. of the diluted plasma alone was taken as 0% and that of the MRBC suspension in distilled water as 100% haemolysis.

*Osmotic fragility test.* Fifteen mice were used for this study, 5 infected with *T. congolense*, 5 with *T. brucei* and 5 controls. Heparinised blood was collected by bleeding from the orbital sinus at weekly intervals for 5 weeks. The fragility of the red cells was determined as described by Mackenzie and Boreham (1974) using serial dilutions of sodium chloride in 0.01 M phosphate buffer pH 7.4 and ranging from 0.85% to 0.1%. Results were expressed as concentration of saline giving 50% haemolysis  $\pm$  standard error.

*Coombs' direct antiglobulin test.* Commercial preparations<sup>2</sup> of swine anti-mouse Ig, rabbit anti-mouse IgG<sub>1</sub>, rabbit anti-mouse C<sub>3</sub> ( $\beta$ 1C) as well as locally produced rabbit anti-mouse IgM were used. Serial doubling dilutions of the antisera were made in normal saline, starting from 1/4. Two groups of 5 mice infected with either *T. congolense* or *T. brucei* as well as 5 normal mice were tested for 6 weeks. Adult female New Zealand Black (NZB) mice served as positive controls for anti-Ig and anti-IgG<sub>1</sub> antisera. The procedure was a slight modification of that outlined by East et al. (1965). The mice were warmed in an incubator and bled from the tail into warm heparinised saline. After three gentle washes, a 50% suspension of the red cells was prepared in warm saline. A drop of each dilution of antiserum and saline control was placed in an appropriate division on a microscope slide using a disposable pasteur pipette. An equal drop of the rbc suspension was placed alongside the antiserum and mixed gently for 10 min. The slides were then examined with the naked eye and under a low power ( $\times 5$ ) objective of the microscope for agglutination which was graded from 0 to 4+.

*Red cell half life measurements.* The half life of MRBC transfused into normal and *T. congolense* infected mice was first determined as described by Jennings et al. (1974) for *T. brucei* except that infection and transfusion were done simultaneously. Subsequently, at 4 weeks of infection with *T. brucei* or *T. congolense*, groups of infected and control mice were sacrificed, cardiac blood was obtained in heparin and incubated with Berenil ( $10^{-4}$  molar soln) for 4 h at 4° C to render any trypanosomes present non-infective (Williamson, personal communication). The red cells were separated by centrifugation and washed twice with PBS pH 7.4. They were then labelled by incubating with <sup>51</sup>Cr as sodium chromate for 30 min at 37° C. After washing, equal volume of pooled normal mouse plasma was added to red cells from infected mice.

Similarly, an equal volume of plasma from each infected mouse was added to pooled MRBC. Each suspension was incubated at 37° C for 30 min washed twice, and injected into normal mice, using the tail vein. Each mouse received about 10  $\mu$ Ci of <sup>51</sup>Cr. The half-life of the injected red cells was then determined as described by Jennings et al. (1974) except that blood was obtained from the orbital sinus and the counting vial contained 1 ml of 0.5% NaOH. Vials were stored at room temperature until sampling was completed in two weeks. All samples were counted on the same day on a gamma scintillation counter<sup>3</sup> and expressed as percentage of the initial starting count (Jennings et al., 1974). The results were analysed by the method of Kreier (1969).

## Results

*Erythrophagocytosis in spleen.* In both groups of infected mice, erythrophagocytosis was evident 2 days after patent parasitaemia and was marked up to the 15th day post-inoculation. Macrophages contained single, or more often, multiple erythrocytes (up to 5) and in later stages, haemosiderin pigments were

<sup>1</sup> EEL OB 10 filter

<sup>2</sup> Nordic Immunological Laboratories, Maidenhead, Berks, U.K.

<sup>3</sup> Philips Scintillation Counter Assembly comprising Type PW 4032 (Counter) Type PW 4062 (Timer) and Type PW 4022 (High voltage supply and amplifier).

Table 1. Percentage haemolysis following incubation of normal red cells with plasma from mice infected with *T. congolense* or *T. brucei*

Days post infection	5	6	7	8	9	10	14	15
Mean* % haemolysis ( <i>T. congolense</i> )	—	4.9	7.5	6.6	4.3	8.6	—	8.6
Mean* % haemolysis ( <i>T. brucei</i> )	5.0	6.3	7.3	6.3	6.5	—	5.3	—

\* Mean of 2 mice per day. Range for 8 control mice 2.9–6.8%.

present as well. This was associated with progressive enlargement of the spleen and a marked reduction in the PCV (from an average of 50% to about 30% in *T. congolense* infected mice). Non-infected mice showed no appreciable erythrophagocytosis and the PCV did not change during the period of observation.

*Test for a haemolysin in plasma.* When plasma from the above mice showing erythrophagocytosis and anaemia was incubated with normal mouse erythrocytes in vitro at 37° C for 30 min, there was no significant haemolysis of the red cells as compared with incubation with normal plasma (Table 1).

*Osmotic fragility test.* The mean saline dilutions giving 50% haemolysis at weekly intervals in infected and control mice are shown in Table 2, while Fig. 1 shows the mean and range of percentage haemolysis at different saline dilutions 4 weeks after infection with *T. congolense* or *T. brucei*. As compared with the controls, red cells from *T. congolense* infected mice were significantly more fragile as from week 2 ( $P < 0.05$ ) increasing up to week 5 ( $P < 0.001$ ). *T. brucei* infected mice had more fragile red cells than control mice only during the third and 5th weeks corresponding with the time of maximum drop in PCV. Fig. 1 also shows that haemolysis started at higher saline concentrations (above 0.6%) in *T. congolense* infected mice than in *T. brucei* infected (0.55%) and normal (0.5%) mice.

*Coomb's direct antiglobulin test.* Up to the 5th week of infection, all mice infected with *T. brucei* or *T. congolense* remained negative when tested with anti-mouse Ig and IgG<sub>1</sub>. On the 6th week two out of the 5 mice infected with *T. brucei* were positive at 1/16 dilutions of anti-Ig and anti-IgG<sub>1</sub>. Control NZB mice were positive at up to 1/128 for anti-Ig and 1/64 for anti-IgG<sub>1</sub>. All mice, including the NZB group, were negative when tested with the anti-IgM and anti-C<sub>3</sub> antisera.

*Red cell half-life measurements.* The half-life of MRBC injected into 4 *T. congolense* infected mice was  $5.5 \pm 1.1$  days (S. E.) whereas that of MRBC injected into 4 normal mice was  $11.4 \pm 0.8$  days. The difference is highly significant ( $p < 0.01$ ). When MRBC were incubated with plasma from normal mice and from mice infected for 4 weeks with *T. brucei* or with *T. congolense* and then transfused into normal mice, the mean half-lives in days were  $12.6 \pm 0.2$ ,  $10.4 \pm 0.2$  and  $11.7 \pm 0.8$  respectively (Fig. 2). Hence incubation of MRBC with plasma from *T. brucei* infected but not from *T. congolense* infected mice caused

Table 2. Mean saline dilutions causing 50% haemolysis of RBC from normal mice and mice infected with *T. congolense* or *T. brucei* for 1–5 week

Group of mice	Saline dilutions <sup>Δ</sup> causing 50% haemolysis				
	Week 1	Week 2	Week 3	Week 4	Week 5
Group A – normal	0.469 ± 0.005	0.465 ± 0.007	0.479 ± 0.007	0.437 ± 0.011	0.478 ± 0.020
Group B – <i>T. congolense</i>	0.474 ± 0.012	0.491 ± 0.018*	0.552 ± 0.013**	0.486 ± 0.013**	0.521 ± 0.037**
Group C – <i>T. brucei</i>	0.466 ± 0.011	0.476 ± 0.012	0.051 ± 0.016**	0.444 ± 0.135	0.510 ± 0.032*

<sup>Δ</sup> Mean of 5 mice plus standard error.

\* Significantly different from that of group A at 5% level ( $p < 0.05$ ).

\*\* Significantly different from that of group A at 0.1% level ( $p < 0.001$ ).



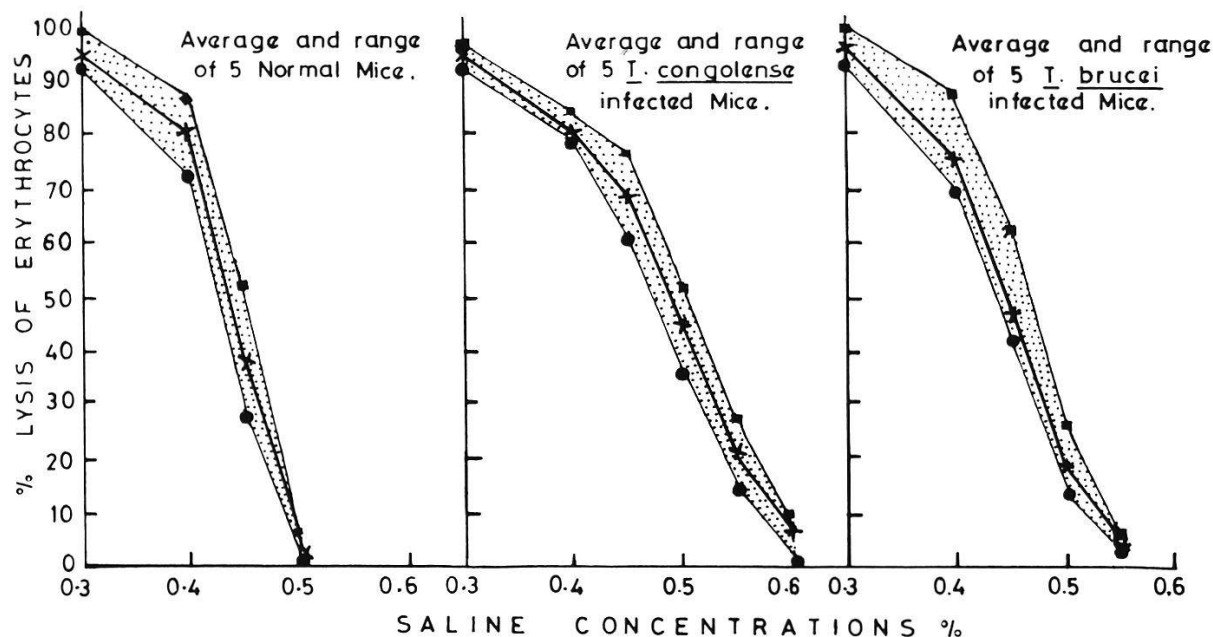


Fig. 1. Osmotic fragility curves of erythrocytes from normal mice and mice infected with *T. congolense* or *T. brucei* for 4 weeks. Infection with trypanosomes resulted in increased fragility more marked in *T. congolense* infection.

a more rapid erythrocyte destruction than incubation in plasma from non-infected mice before transfusion. When red cells from infected mice were incubated with normal plasma and transfused into non-infected mice, their half-life was markedly increased (over 20 days). This was attributed to increased number of reticulocytes and immature red cells in the circulation of infected mice.

## Discussion

The results indicate that factors responsible for red cell destruction in mice infected with trypanosomes may lie within the red cells as well as in the plasma of infected animals. Red cell destruction in the spleen started early during infection with either *T. congolense* or *T. brucei*, but with the strains of trypanosomes used, anaemia was more marked in *T. congolense* infections. On the other hand, parasitaemia was heaviest in *T. brucei* infected mice.

The marked drop in PCV in *T. congolense* infected mice was accompanied by a marked reduction in the half-life of transfused red cells. No haemolysin was demonstrable in the plasma of mice infected for up to 15 days, although there was progressive anaemia and parasitaemia. However, incubation of normal MRBC with plasma from *T. brucei* infected mice caused a more rapid elimination of the cells when injected into normal mice. This would suggest the presence of some mildly toxic substance in the plasma of *T. brucei* infected mice. Recently, Huan et al. (1975) demonstrated and purified a haemolytic factor in supernatant of trypanosome (*T. brucei*) suspensions in vitro. In livestock where

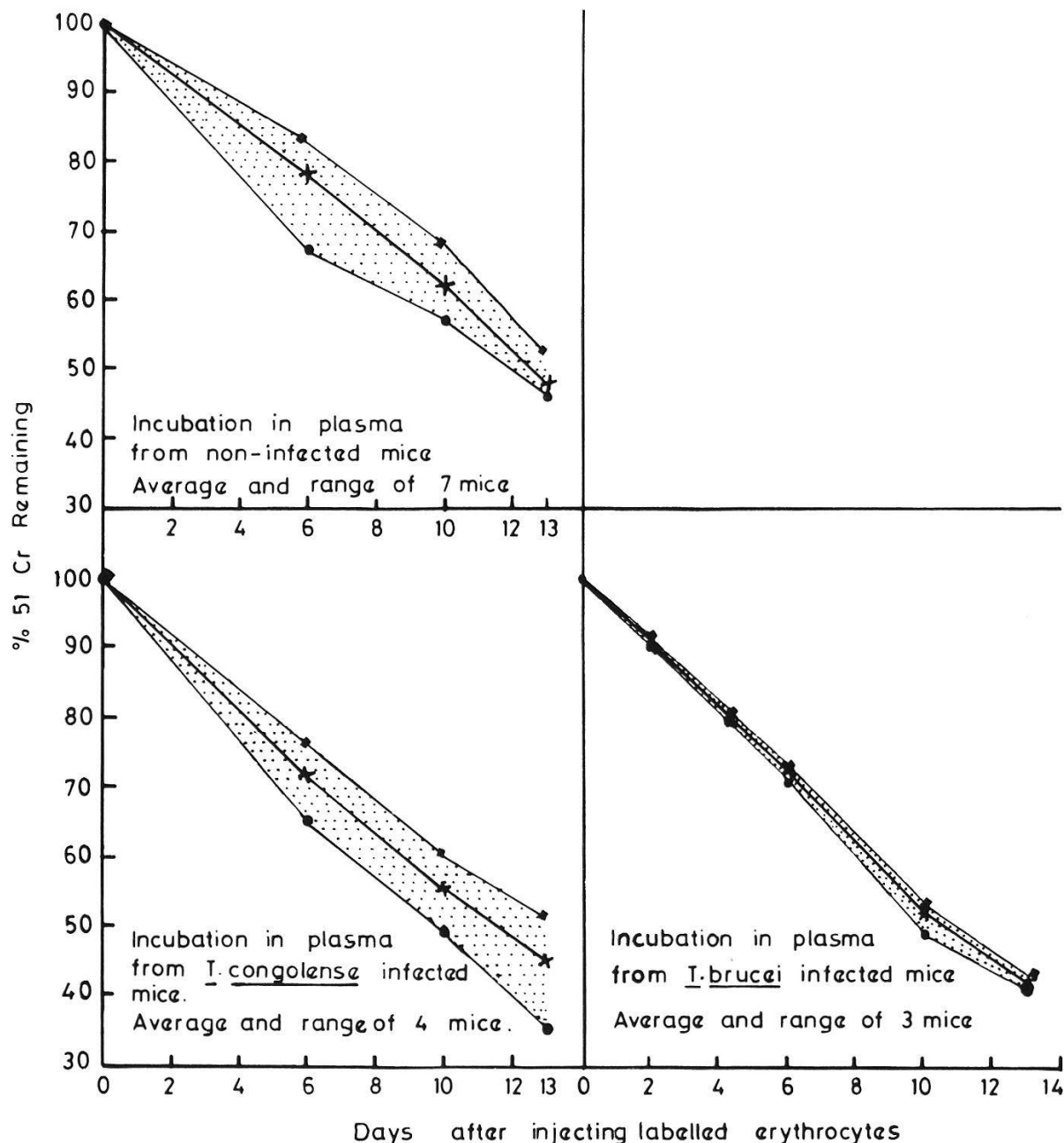


Fig. 2. Effect of incubation in plasma on survival of erythrocytes. Incubation of erythrocytes from uninfected mice in plasma from *T. brucei* but not from *T. congolense*, infected mice caused a more rapid erythrocyte destruction than incubation in plasma from non-infected mice before transfusion ( $p < 0.02$ ).

anaemia may occur without corresponding heavy parasitaemia, some other factors, in addition to a possible haemolysin, must be sought.

The osmotic fragility of red cells obtained from *T. congolense* infected mice was significantly increased as from the 2nd week of infection. Similar but less marked changes were observed in *T. brucei* infected mice. However antiglobulin tests were always negative in *T. congolense* infected mice and rarely positive in *T. brucei* infected mice suggesting that the increased fragility of the red cells



may not be related to surface coating with immunoglobulins. In rabbits infected with *T. brucei*, MacKenzie and Boreham (1974) had equivocal results with the direct antiglobulin test and no alteration in red cell fragility. On the other hand, Woo and Kobayashi (1975) using the more sensitive immunofluorescent techniques have provided some evidence for the presence of immune-complexes on the membranes of red cells incubated in vitro with *T. brucei*. It seems likely therefore, that the mechanisms of red cells destruction in infected animals are complex and may vary not only with the species of trypanosomes, but also with the host and the stage of infection.

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