

Zeitschrift:	Acta Tropica
Herausgeber:	Schweizerisches Tropeninstitut (Basel)
Band:	31 (1974)
Heft:	4
Artikel:	Miscellanea : Autoimmunity in trypanosome infections. Part III, The antiglobulin (coombs) test
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DOI:	https://doi.org/10.5169/seals-311965

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Miscellanea

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Autoimmunity in Trypanosome Infections III. The Antiglobulin (Coombs) Test

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Abstract

Direct antiglobulin tests have been carried out in rabbit, cattle and human trypanosomiasis. Positive results were obtained in human patients up to 36 months after treatment. Under the conditions of these tests cattle were negative and the rabbit result equivocal. Indirect tests on the sera of lions infected with trypanosomes suggest the presence of an autoantibody but iso-antibodies cannot be excluded. No change was seen in the fragility of erythrocytes of rabbits infected with *Trypanosoma brucei*. The results provide an example of an abnormal immunological response in human trypanosomiasis.

Introduction

The antiglobulin (Coombs tests), originally developed by Moreschi in 1908 (see COOMBS & GELL 1968, p. 19) and later redescribed by COOMBS et al. (1945), is designed to detect non-agglutinating erythrocyte antibodies such as rhesus factors. These antibodies were regarded as 'incomplete' and although this name is still used its meaning has changed. The term must not be taken to imply an abnormality of the antibody but simply that the antibodies will not cause agglutination of red cells although combination with red cell antigens occurs. The major agglutinins found in human serum comprising the ABO system are macroglobulins (IgM) while the rhesus antibodies in the main are IgG. It is probable that the 'incompleteness' of the antibody results from a spacial arrangement of the antigenic determinants on the red cell surface which will not allow direct agglutination.

The antiglobulin test detects amongst others, the rhesus antibodies which will not agglutinate by themselves. It should be remembered that non-agglutinating levels of 'complete' IgG and IgM could also be detected by this test. The test relies on the ability of anti-species γ -globulin serum to agglutinate red cells coated with specific red cell antibody. An indirect method is also available in which indicator cells are first exposed to the test serum and then agglutinated with an antiglobulin serum. The indirect test, however, requires careful interpretation because of the existence of iso-antigens. A positive direct test provides evidence of autoimmunity.

Positive antiglobulin tests have been found in a wide range of disease states. Autoimmune haemolytic anaemia is probably the best known example and has been reviewed by DACIE (1962). Haemolytic anaemias associated with the use of

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certain drugs result in sensitisation of autologous red cells with immunoglobulin (SWANSON 1973). ZUCKERMAN (1964) has reviewed the occurrence of red cell auto-antibodies in rodent malaria infections, and also in blackwater fever of cattle. ZUCKERMAN & SPIRA (1961) doubt the significance of positive antiglobulin tests in the rat infections since bleeding alone produced a similar effect. The cells involved in this reaction appeared to be mainly reticulocytes.

ZOUTENDYK & GEAR (1951) reported that human cases of malaria showed positive antiglobulin tests. More recent work by TOPLEY et al. (1973) confirms this finding. Positive antiglobulin tests have also been found in kala azar (CHATTERJEE & SEN GUPTA 1970; WOODRUFF et al. 1972). Two reports of single cases of positive antiglobulin tests in human trypanosomiasis exist (ZOUTENDYK & GEAR 1951; BARRETT-CONNOR et al. 1973). The latter authors record that after treatment of their patient with pentamidine isethionate and suramin sodium the antiglobulin test returned to negative within one week. More recently WOODRUFF et al. (1973) found γ -globulin on the surface of the erythrocytes of three out of six patients with trypanosomiasis. In this case titres ranged from 1/4 to 1/8.

A number of features of trypanosomiasis suggest that autoimmune phenomena may be expressed at the red cell surface, for example the severe anaemia often seen in cattle trypanosomiasis (LOSOS & IKEDE 1972), the elevated IgM levels MATTERN et al. (1961) and the immunosuppression effects reported by FREEMAN et al. (1973) and MURRAY et al. (1973). The work reported here examines the incidence of positive antiglobulin tests in trypanosome infections of four species, rabbit, cattle, man and lion.

Materials and Methods

Trypanosome Infections

(a) Rabbits. New Zealand White rabbits of either sex weighing 2.5–3.0 kg were obtained from A. E. Moss, White Cloud Farm, Tring, Herts., England. The rabbits were infected by subcutaneous injection of between 5×10^7 and 2×10^8 washed trypanosomes which had been separated on a DEAE cellulose column (LANHAM 1968). Two strains of *Trypanosoma brucei* were used. *T. brucei* 427 produces a chronic infection lasting 4–6 weeks with a low grade parasitaemia while *T. brucei* S42 produces a more acute infection lasting 2–4 weeks, with greater parasitaemias. Full details of these strains are given by BOREHAM & FACER (1974 a). Blood samples were collected for the antiglobulin test from the marginal ear veins of rabbits at different periods throughout the infection, using heparin as an anticoagulant.

(b) Cattle. Zebu cattle bred at the East African Trypanosomiasis Research Organization (EATRO) farm at Tororo, Uganda, were used. Two cattle were infected with *T. brucei* EATRO 1386 and two more with *T. congolense* EATRO 505. Blood samples were taken from the jugular veins of these cattle for a period of five weeks after infection. In addition, blood was collected at post-mortem examination from five cattle with old *T. congolense* infections and three control cattle. All the animals examined at post-mortem had *Haemonchus contortus* infections and three were also infected with *Fasciola gigantica*. It is possible that the cattle experimentally infected also had concurrent helminth infections but this was not investigated. In addition, previous work has shown that a high proportion of the cattle at EATRO are infected with *T. theileri* (REID et al. 1967).

(c) Man. Blood was collected at the EATRO hospital from patients infected with *T. rhodesiense*. Some samples were from active cases and others from patients undergoing follow-up examinations.

(d) Lion. Serum was collected from eight wild lions naturally infected with

trypanosomes in the Serengeti National Park, Tanzania. The lions were captured as part of the 1971 survey lead by Professor Geigy. For details of the method of capture of the lions, see GEIGY & KAUFFMANN (1973). All of the lions tested had concurrent infections of *Hepatozoon* and *Babesia*. Lion erythrocytes were kindly supplied by Dr. Christine Hawkey from a young circus lion brought for examination to the Veterinary Hospital, Zoological Society of London.

The antiglobulin test

(a) Preparation of reagents. Anti-rabbit serum was prepared by the intramuscular injection of alum precipitated whole rabbit serum into a goat, following the method of WEITZ (1952). The antiserum was absorbed with an equal volume of packed normal rabbit erythrocytes for 1 h at 37 °C followed by 1 h at 4 °C. Pooled cells from a number of rabbits were used. Absorption was repeated until all red cell agglutinins had been removed. The reagent used in the cattle tests was an anti-bovid serum prepared as above, except that the antiserum was raised in a rabbit. Absorptions were carried out with normal zebu cattle erythrocytes.

An anti-cat serum was prepared by the injection of an emulsion of cat serum in Freunds complete adjuvant into lymph nodes of rabbits (BOREHAM & GILL 1973). This antiserum was used for the indirect antiglobulin test on lion serum.

Insufficient lion erythrocytes were available to absorb this serum, but since only very slight agglutinating activity was observed, it was possible to work at dilutions greater than the agglutinating titres. Wellcome Coombs reagent was used for the tests on human erythrocytes.

(b) Direct antiglobulin test (rabbit, cattle and man erythrocytes). Serial doubling dilutions of the appropriate reagent were made in 'Microtitre' plates commencing at a $\frac{1}{2}$ dilution. This ensured that the optimum dilution was used for the test. When testing rabbit erythrocytes it was found necessary to use 5% normal goat serum, previously heat inactivated and absorbed with normal rabbit erythrocytes as the diluent, to prevent panagglutination. An equal volume of 2% test erythrocytes washed three times was added to these dilutions and the plate incubated at 37 °C for 1 h. Normal rabbit red cells were used as a negative control. As a positive control rabbit red cells were coated with rabbit γ -globulin using bis-diazotized benzidine as the coupling agent. Settling patterns were graded from negative (no agglutination) to 4+ (complete agglutination).

When using cattle cells in the direct antiglobulin test blood from a non-trypanosome infected ox was used as a negative control, but it was not possible to include a positive control. Group O rhesus positive cells were used as a negative control for the test on human erythrocytes and the same cells passively sensitised with anti-D serum as the positive control.

(c) Indirect antiglobulin test (lion erythrocytes). Two drops of the test serum were mixed with two drops of 2% lion cells and either two drops of saline or two drops of a 20% solution of bovine serum albumin (BSA) (Ortho Diagnostics). After incubation at 37 °C for 30 min the cells were washed three times in saline and 2 drops of a 1/20 or 1/200 antiglobulin reagent added to the pellet. The cells were mixed, allowed to settle for 5 min at room temperature and then centrifuged lightly at 500 g for 1 min. The results were read macroscopically by gently agitating the pellet and grading the degree of agglutination from negative to 4+.

Fragility tests

Serial dilutions of sodium chloride were prepared in phosphate buffer pH 7.4 ranging from 0.85% to 0.1%. Blood from rabbits was collected from the marginal ear vein in EDTA before infection and 10 and 30 days after infection. The blood

samples were aerated and 0.02 mls added to 4 mls of each saline dilution. After mixing, the cells were allowed to stand at room temperature for 30 min and then centrifuged at 1000 g for 5 min. Haemolysis was measured in a colorimeter using the 0.85% sample as the blank showing no haemolysis and the 0.1% sample as 100% haemolysis. The results are expressed as the concentration of saline giving 50% haemolysis \pm standard error of the mean.

Results

Rabbit

The results of antiglobulin tests on five rabbits infected with *T. brucei* and three control rabbits are shown in Table 1. Positive antiglobulin tests were only

Table 1. Results of antiglobulin tests in rabbits. The first number indicates the greatest dilution showing maximum agglutination and the second in brackets the degree of agglutination on a negative - 4+ scale at that titre

Rabbit	Strain	Days after infection									
		0	7	14	17	19	21	25	31	40	
S184	427	-	-	4 (2+)	16 (3+)	8 (2+)	NT	NT	8 (3+)	2 (3+)	-
S185	427	-	-	8 (3+)	16 (3+)	16 (3+)	NT	NT	8 (3+)	2 (2+)	-
S203	427	-	-	-	NT	NT	-	-	NT	-	NT
S192	S42	-	-	-	-	killed					
S193	S42	-	-	-	-	killed					
S165	Control	-	-	-	2 (2+)	1 (2+)	NT	NT	4 (2+)	-	-
S189	Control	-	-	-	-	-	NT	NT	NT	NT	NT
S202	Control	-	-	128 (1+)	NT	NT	128 (\pm)	-	NT	-	NT
NT = Not tested											
- = Negative											

consistently seen in two rabbits, S184 and S185, both infected with *T. brucei* 427. Neither of the rabbits with strain S42 showed any positivity up to 17 days when they had to be killed because of the severity of the infection. A third rabbit S203 infected with strain 427 did not show any positive results. Two of the three control rabbits gave transient positive responses. Control rabbit S165 gave low titres of less than 4 on three occasions while rabbit S202, also an uninfected control, gave much higher titres of 128 on two occasions. It should be noted that on no occasion was 4+ complete agglutination recorded and in the control rabbits the degree of agglutination was less than in the infected rabbits. In control experiments the reagent did however give 4+ agglutination of rabbit red cells coated with rabbit γ -globulin.

Results of the fragility tests carried out on control sera and sera taken 10 and 30 days after infection with *T. brucei* 427 are shown in Table 2. They suggest

Table 2. Erythrocyte fragility tests on rabbits infected with *T. brucei* 427. The results are expressed as the percentage of saline \pm standard error causing 50% haemolysis of the red cells

	Days after infection		
	0	10	30
Fragility	0.486 \pm 0.009	0.491 \pm 0.013	0.491 \pm 0.009
No. of rabbits	11	9	6

that there is no significant change in the fragility of rabbit erythrocytes during the infection ($P < 0.01$).

Cattle

No positive results were obtained for antiglobulin in either the experimentally infected or the post-mortem samples of cattle infected with trypanosomiasis.

Man

The incidence of positive antiglobulin tests in human cases of trypanosomiasis and in follow-up patients are shown in Table 3. Six control samples from other patients at the hospital are included.

Table 3. Results of antiglobulin tests in human cases of trypanosomiasis and follow-up cases. For comparison the results of six other patients are included

Active cases

SS. No.	Duration	Titre	Result
1011	2 days *	32	+
1013	5 days	64	+
1019	5 weeks	< 2	–
1020	8 months	64	+
1021	4 months	64	+
1023	1 month	16	+
1024	1 month	64	+
1025	4 months	128	+
1026	3 months	64	+

Follow-up cases

SS. No.	Time after infection	Titre	Result
859	36 months	32	+
866	32 months	64	+
944	18 months	64	+
956	15 months	64	+
975	9 months	< 2	–
992	7 months	32	+
1002	3 months	32	+
1004	3 months	64	+
1005	3 months	64	+

Controls

Initials	Diagnosis	Titre	Result
SM	Malaria	32	+
AA	Mumps	< 2	–
EF	Adenitis	< 2	–
MW	Conjunctivitis	32	+
MO	Bruising	< 2	–
SE	Tropical ulcer	< 2	–

* Reinfection

Lion

Table 4 shows the results of the indirect antiglobulin tests on the sera of 8 lions. Two results are shown for each sample, one in the presence of 20%

Table 4. Indirect antiglobulin test on lion sera. Results are expressed as the degree of agglutination on a negative - 4+ scale

Coombs reagent	Saline control	Lion sera							
		245	246	247	272	276	277	278	280
1/20 Saline	2+	4+	4+	4+	4+	4+	4+	4+	4+
1/20 Albumin	-	-	-	-	2+	-	-	-	±
1/200 Saline	±	-	-	-	-	-	±	-	-
1/200 Albumin	-	-	-	-	-	-	-	-	±

albumin and the other in saline. Two dilutions of antiglobulin reagent were used in each case.

Discussion

The antiglobulin reagents used in this study were prepared in different animal species and by different methods. The exact specificity of these reagents is not known although they can probably be assumed to have a broad spectrum of activity since they were raised against the whole sera. The extent to which the reagents react with IgM for instance, is uncertain. The goat anti-rabbit serum did not develop a β_2 M precipitin line on immunoelectrophoresis and, therefore, was unlikely to agglutinate cells sensitised with IgM. The anti-human reagent however was known to react with IgM coated cells at low dilutions.

A complication in the rabbit test was the poor settling of normal erythrocytes in saline. 5% serum was necessary to ensure reproducible settling patterns and for this purpose heat inactivated (56 °C) red cell absorbed normal goat serum seemed the most appropriate.

Since some 'incomplete' antibodies fail to cause agglutination in saline but will do so in 15–30% BSA it was decided to carry out indirect tests in both 20% BSA and saline. The results however, show that less agglutination occurred with BSA present than in its absence. This was presumably due to a masking of any immunoglobulin on the red cell surface by a coating of albumin.

Positive antiglobulin tests were found in man and lion and negative results in cattle. The results for the rabbits are inconclusive. Two rabbits showed transient positivity with maximum titres of 1/16, and 3 rabbits showed no response at all. These results must be treated with caution since one control (S165) showed weak (2+) activity at a maximum titre of 1/4 and a second control rabbit (S202) showed transient very weak (1+) agglutination but at titres up to 1/128. This might represent a non-immunological coating of cells with plasma components other than immunoglobulins which are recognised by the reagent. A repeat of these experiments with monospecific antisera would probably resolve this point.

The antiglobulin tests in man seem to indicate the presence of an autoantibody since the direct test will detect antibody fixed *in vivo* or shortly after the blood is taken. In either case autologous antibody would be involved. The class of antibody is not clear. The optimum titre of the reagent for IgG-anti-D sensitised rhesus positive red cells was 1/320. In the present work agglutination was never seen at this dilution. The highest dilution showing maximal agglutination was 1/128 but 1/32 or 1/64 was more usual. This implies that the antibody being measured is not strictly speaking an incomplete (IgG) antibody but is in fact IgM presumably at sub-agglutinating doses. If this is so then the antibody is

probably the cold agglutinin shown to occur in trypanosomiasis by YORKE (1911). This could be checked by carefully collecting the blood at 37 °C and maintaining it at this temperature throughout the washing procedure.

From these experiments no evidence of globulin on the surface of infected cattle erythrocytes was found. The lack of a positive control makes interpretation difficult. The positive indirect tests seen in lions suggest that an autoantibody may occur. However, since no investigation was made of blood groups of the animals in this study, the possibility of natural blood group antibodies to the test lion cells cannot be ruled out. It is not known whether the high concentration required to give a positive result indicates IgM sensitisation or simply a poor reagent.

The anaemia in trypanosomiasis has not been satisfactorily explained. The erythrocytes in the circulation are predominantly young, many containing nuclear material while the older cells often show crenation and are preferentially removed by the spleen and liver. Haemodilution is a contributory factor to the observed anaemia (BOREHAM 1967).

Immunological phenomena may be causative factors of the abnormal shape of the red cells or contribute towards haemolysis of the cells. WOODRUFF et al. (1973) found the C₃ factor of complement on the surface of erythrocytes in 4 out of 6 patients with *T. rhodesiense*. They suggest that immune cytolysis is an important factor in the anaemia. The presence of C₃ may indicate dissociation of previously fixed immunoglobulin from the cell as suggested by SCHUR & AUSTIN (1968) but equally the fixed-C₃ receptor of primate erythrocytes may be involved (NELSON 1963).

The results obtained indicate a high incidence of Coombs positivity in human trypanosome infections up to 36 months after treatment. In contrast WOODRUFF et al. (1973) obtained positive results in only 3 out of 6 cases. This probably relates to the specificity of the reagent and the method of testing. In the present work the temperature of the blood samples was allowed to fall to room temperature, which would allow the fixation of cold agglutinins to erythrocytes (YORKE 1911) which would be detected by the antiglobulin reagent. If, in Woodruff's experiments, the samples were maintained near 37 °C during the washing a lower incidence of Coombs positivity might be expected. The controls used by WOODRUFF et al. (1973) were "7 persons free from demonstrable haematological abnormality". In this work patients undergoing treatment for conditions other than trypanosomiasis were used. The results suggest that the proportion of positive antiglobulin tests may be relatively high in the population at large.

The probable negative result in rabbit is unexpected since anaemia occurs in this species, and an alteration of the erythrocyte is suggested by the frequent adherence of erythrocytes to trypanosomes as seen in wet film preparations of infected blood. Nevertheless, if the cells are abnormal in any way it does not appear to alter their fragility as judged by the results reported here. A careful look for IgM on the surface of rabbit erythrocytes might provide further information on this subject. Similarly further work on cattle blood with more carefully defined antiglobulin reagents would be useful.

In those cases where positive tests were found, it is probable that the anaemia is at least partly autoimmune. The deeper implications of such autoimmunity is that a breakdown in tolerance is somehow induced by the trypanosomes. A number of autoimmune features are known to occur in trypanosome infections. These include the appearance of immunoconglutinins (INGRAM & SOLTYS 1960), rheumatoid-like factor (HOUBA et al. 1969), anti-tissue and anti-Wassermann auto-antibodies (MACKENZIE & BOREHAM 1974) and an autoantibody directed against a component of the fibrin/fibrinogen system (BOREHAM & FACER 1974 b). It is

therefore apparent that an immunological imbalance exists. Work by FREEMAN et al. (1973) and MURRAY et al. (1973) clearly indicates that this imbalance is not restricted to autoimmunity but a deficiency of the immune system as a whole which may be expressed as either enhancement or suppression.

Acknowledgements

This work was undertaken while one of us (ARM) held a Medical Research Council Scholarship for training in research methods. We wish to thank the Overseas Development Administration of the Foreign and Commonwealth Office and the Medical Research Council for financial support. Our thanks are due to the Director of the East African Trypanosomiasis Research Organisation for laboratory facilities for part of this study and to Professor Geigy for his hospitality in allowing one of us (ARM) to join his survey in the Serengeti.

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