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Mechanisms of natural resistance to trypanosomal infection

ROLE OF COMPLEMENT IN AVIAN RESISTANCE TO *TRYPANOSOMA CRUZI* INFECTION

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Summary. The natural resistance of chickens to *Trypanosoma cruzi* infection and the capacity of their sera to lyse blood (trypomastigote) forms of the parasite *in vitro* were found to be complement-dependent phenomena. Parasites given intravenously to decomplemented chickens were detectable in their bloodstream for at least 24 h post-infection, whereas in untreated animals they became undetectable after 1 min (and destroyed flagellates were observed). One millilitre of serum had the capacity to lyse as many as $10–30 \times 10^6$ organisms. The lytic activity of serum *in vitro* was not impaired in chickens that had been immunosuppressed by four different procedures and was present in the absence of antibodies. *In vitro* lysis of *T. cruzi* by either normal or antibody-free chicken sera occurred in the absence of calcium ions but required magnesium ions, indicating that complement was activated via the alternative pathway. Administration of normal chicken serum to mice infected with *T. cruzi* provoked a marked decrease in their parasitaemias.

INTRODUCTION

Birds (Dias, 1944; Nery-Guimares, 1972) and some poikilothermic animals (Bruni, 1926; Dios, Werngren and Perez, 1929; Dias, 1933) are known to be naturally resistant to *T. cruzi* (Goble, 1970), a protozoan flagellate which causes Chagas' disease in humans. Blood (trypomastigote) forms of *T. cruzi* obtained from infected mice were reported readily lysed by fresh sera from hens, frogs and toads but not by normal mouse, human or guinea-pig sera (Rubio, 1956; Budzko, Pizzimenti and Kierszenbaum, 1975). Heat inactivation of the effective sera abolished the effect and suggested the involvement of complement in the process. Attempts to reproduce these results by utilizing parasites obtained from infected rats under different experimental conditions were unsuccessful (Warren, 1958) and created controversy about the subject. Although *T. cruzi* infection may be established during embryonal life (Ganapati, 1948; Nery-Guimaraes, 1972) newly hatched chickens are already resistant (Nery-Guimaraes and Lage, 1972). However, no signs of infection were demonstrable in chickens which hatched from infected embryos. Hormonal bursectomy or treatment with corticosteroids did not affect the refractory state (Nery-Guimaraes and Lage, 1972).

We have devoted the present study to exploring the mechanism of resistance to *T. cruzi* in chickens. It will be shown that this resistance is complement-

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dependent and that the *in vitro* lysis of the parasites is the result of an antibody-independent reaction in which chicken complement is probably activated through the alternative pathway.

MATERIALS AND METHODS

Parasites

Both Y and Tulahuén strains of *T. cruzi* were maintained by serial passages in 4-week-old CD1 mice. The trypomastigotes were obtained by decanting the supernatant plasma of heparinized infected blood at 4° for 2–18 h. Parasite counts were performed by a standardized microscopic method described previously (Kierszenbaum and Saavedra, 1972). The suspensions utilized in the *in vitro* tests contained $3–20 \times 10^6$ *T. cruzi* per millilitre.

Chickens

The birds were hatched from fertile eggs of specific pathogen free flocks of White Leghorn chickens received from Wickham Laboratories (SPF Farms) Ltd.

Sera

Blood samples were obtained from normal White Leghorn chickens at different ages and from chickens 3 weeks after one of the following treatments: (a) neonatal bursectomy; (b) administration of three daily intraperitoneal doses of 0·2 mg of cyclophosphamide monohydrate (Koch-Light Laboratories, Colnbrook, Bucks.), starting on the day of hatching; (c) combined bursectomy and cyclophosphamide treatment as above. Other samples were drawn from chickens immunosuppressed by neonatal infection with infectious bursal disease virus (IBDV) (Ivanyi, 1975; Ivanyi and Morris, 1975) and from complement-depleted chickens. In all cases the clot was allowed to form at 37° for 60 min. Sera were either fresh or kept at − 70° until used.

Chicken IgG

IgG was purified from serum as described by Korinek and Paluska (1961).

Cobra venom factor (CVF)

CVF was prepared from the venom of the cobra snake *Naja naja* (Ross Allen Reptile Institute, Silver Springs, Florida) as described by Müller-Eberhard and Fjellström (1971).

Complement depletion

In vivo. Depletion of complement in chickens was accomplished by intraperitoneal administration of 200 u of CVF per kilogram of body weight. The total dose was distributed in three equal injections given over 24 h. *In vitro.* Complement was inactivated by adding 2·5 ml of CVF to the reaction mixture 15 min before incorporating the parasites. The effectiveness of the *in vitro* or *in vivo* CVF treatments was evidenced by an either marked or complete reduction in the complement haemolytic activity of the sera.

Determination of the extent of lysis of T. cruzi

To give optimal conditions for complement activation, calcium and magnesium ions were incorporated into the reaction mixtures except where the effect of inhibition by chelating agents was to be tested. The concentration of magnesium ions was adjusted in some cases to reach a given value. The temperature and time of incubation were fixed at 37° and 30 min respectively, although lysis of *T. cruzi* may take place at room temperature and in less time. The rationale underlying quantitative lytic tests for *T. cruzi* trypomastigotes has been described previously (Budzko *et al.*, 1975).

To perform the test, 0·1 ml of the material to be tested was mixed with 0·05 ml of a solution containing 0·005 M magnesium chloride and 0·0015 M calcium chloride. Complement inhibitors, when added, were contained in 0·05 ml except CVF which was in 0·005 ml. Volume adjustments, if required, were made with a phosphate-buffered saline solution, pH 7·0, containing 4 per cent bovine serum albumin. After incubation, the numbers of live trypanosomes were counted in all tubes and the percentage of lysis established by the following formula: percentage lysis $= [(N-n)/N] \times 100$, where n and N are the numbers of *T. cruzi* per millilitre counted in the mixture containing the material tested and the buffer, respectively.

Active haemagglutination

The antibody response to sheep red blood cells (SRBC) was measured by active haemagglutination as described by Ivanyi, Valentova and Cerny (1966).

RESULTS

The lytic effect

T. cruzi trypomastigotes were readily lysed by chicken

sera. The effect was reduced and eventually lost by dilution of the sera (Table 1). Although variable, the lytic capacity of chicken sera was found to be relatively large. Thus, the capacities of two samples were found to be 3×10^7 and 1×10^7 parasites per millilitre of serum.

Table 1. Effect of dilution of normal chicken serum on lysis of *T. cruzi* trypomastigotes*

Reciprocal of dilution	Percentage lysis	
	Serum 1†	Serum 2‡
Undiluted	100	100
2	100	100
4	100	91·7
8	74·2	41·6
16	51·8	0
32	2·8	0
64	0	0
128	0	0

* Each tube contained: 0·2 ml of the dilution tested, 0·1 ml of PBS, 0·05 ml of the solution containing calcium and magnesium ions and 0·05 ml of the suspension of the parasites.

† The number of *T. cruzi* (Y strain) added to each tube was $8{\cdot}4 \times 10^5$.

‡ The number of *T. cruzi* (Y strain) added to each tube was $5{\cdot}7 \times 10^5$.

Lysis of *T. cruzi* by chicken sera is complement-dependent

Both the preferentially reticulotropic Talahuén strain and the myotropic Y strain of *T. cruzi* were lysed by chicken sera. The reaction was found to be complement-dependent as it was inhibited either by heating the sera at 56° for 30 min or by different types of complement-inhibiting agents (Table 2). Selective chelation of calcium ions with sodium ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*-tetra-acetate (EGTA) (final concentration 0·01 M) in the presence of 0·001 M magnesium ions did not alter significantly the lytic capacity of chicken sera. Consistent with this, chelation with disodium ethylenediaminotetra-acetate (EDTA) in the presence of excess magnesium ions (final concentrations of EDTA and magnesium ions were 0·01 M and 0·08 M respectively) did not interfere with the lytic reaction. These are conditions under which magnesium but not calcium ions are available, i.e. which inhibit the classic but not the alternative pathway of complement activation.

Lack of resistance to *T. cruzi* infection following complement depletion

The role of complement in the destruction of *T. cruzi in vivo* was studied in chickens whose complement levels had been depleted by administration of CVF. Blood samples were obtained both from normal and

Table 2. Complement-mediated lysis of *T. cruzi** blood forms by chicken sera

Material tested	Percentage lysis*					
	Expt 1	Expts 2 and 3	Expt 4	Expt 5	Expts 6 and 7	Expts 8–10
NCS†	100	100	100	100	100	100
Heat-inactivated NCS	—	0	0	0	0	—
NCS+CVF	0	—	—	0	—	—
NCS+EDTA	0	0	0	6·6	—	—
NCS+EGTA	90·1	100	—	86·6	—	—
NCS+EDTA+Mg^{2+} excess	73·9	—	—	80·0	—	—
EDTA	0	0	—	0	—	—
EGTA	0	0	—	0	—	—
Mg^{2+}	0	—	—	0	—	—
Serum from CVF-treated chicken	—	—	—	—	—	0

— = Not determined.

* Y strain (expts 1–4 and 8–10) and Tulahuén strain (expt 5–7) of *T. cruzi* were used.

† NCS = normal chicken serum.

Table 3. Lysis of *T. cruzi** trypomastigote forms by the sera from immunosuppressed chickens

Treatment of serum donors	Serum treatment	Percentage of lysis		
		Expt 1	Expt 2	Expt 3
None	None	100	100	100
None	Heat inactivation	0	2·8	—
Surgical bursectomy	None	100	—	—
Cyclophosphamide	None	100	—	—
Bursectomy and treatment with cyclophosphamide	None	100	—	—
Infection with IBDV	None	—	100	100
Infection with IBDV	Heat inactivation	—	0	0
Infection with IBDV	EDTA	—	0	0
Infection with IBDV	EGTA	—	95·7	—

— = Not determined.
* Y strain (expts 1 and 2) and Tulahuén strain (expt 3) of *T. cruzi* were used.

decomplemented chickens before infection and subjected to lytic tests. No lysis was observed with the sera of chickens that were depleted of complement by the administration of CVF (Table 3). The animals were given 6×10^6 parasites (Y strain) intravenously, after which their blood was examined microscopically to establish the presence of the organisms at various time intervals. The flagellates were demonstrable in the blood of decomplemented chickens for at least 24 h after infection. In marked contrast, samples obtained from untreated animals 1 min after receiving the parasites showed that very few of them were motile and, after 4 min, living organisms were no longer detectable despite intensive searching. One minute post-infection, parasites found in the blood of normal chickens showed distinct signs of destruction such as, for example, highly granular cytoplasm, loss of the flagellum and membrane damage. Parasitaemia was measured quantitatively in one decomplemented animal starting 60 min after infection. The values obtained were $2{\cdot}7 \times 10^5$, $1{\cdot}5 \times 10^5$, 9×10^4, 6×10^4 and $7{\cdot}5 \times 10^3$ *T. cruzi* per millilitre at 60, 100, 130, 180 and 1400 min respectively.

Does lysis of *T. cruzi* trypomastigote forms require antibody?

Tests were performed with the sera of animals which had been immunosuppressed in four different ways. First, sera obtained from neonatally bursectomized animals, collected at 3 weeks of age, a time when not even maternal antibodies were detectable by immunoelectrophoresis, displayed lytic activity on *T. cruzi* (Table 3). Similar results were obtained with the sera of chickens which had been either bursectomized and given cyclophosphamide or given cyclophosphamide alone (Table 3). The animals providing these sera had been injected intravenously with 0·5 ml of a 20 per cent (v/v) suspension of SRBC in saline at 14 days of age. Their sera showed no detectable anti-SRBC response by active haemagglutination 1 week later (Ivanyi, 1975). Instead, all of the untreated chickens of the same age showed a positive response. Also shown in Table 3, is the lytic capacity for *T. cruzi* of the sera of chickens immunosuppressed by neonatal infection with IBDV. These animals were also incapable of making antibodies to SRBC and their own sera were found by immunoelectrophoresis to contain only monomeric IgM of an anomalous type (Ivanyi and Morris, 1975). Here also, the trypomastigotes were lysed in the absence of free calcium ions.

Purified chicken IgG and fresh guinea-pig serum, either separately or together, failed to show any effect on the parasites.

Effect of normal chicken serum on the parasitaemia of mice infected with *T. cruzi*

The levels of parasitaemia of two infected mice were markedly decreased after the administration of 0·6 ml of normal chicken serum (0·4 ml i.v. and 0·2 ml i.p.). The values, $24{\cdot}9 \times 10^6$ and $75{\cdot}6 \times 10^6$ *T. cruzi*

per millilitre, measured 5 min before the treatment were reduced to $14{\cdot}1 \times 10^6$ and $28{\cdot}3 \times 10^6$, respectively, 30 min after.

DISCUSSION

The role of complement in both chicken resistance to *T. cruzi* infection and the destruction of the parasite *in vitro* by chicken sera has been established by these results. Decomplementation with CVF allowed the parasites to remain alive in the circulation for at least 24 h after intravenous infection. In contrast, in normal chickens the parasites were no longer detectable a few minutes after infection and disrupted organisms, missing their flagella and showing membrane damage, were frequently observed. Thus, the time needed by decomplemented chickens to accomplish complete elimination of the organisms from their blood is considerably lengthened when compared with the capacity of normal chicken serum to lyse large numbers of parasites within a few minutes.

The participation of complement in destruction of *T. cruzi* by chicken serum was demonstrated *in vitro* by sensitivity towards heat inactivation, addition of EDTA or incubation with CVF. Chicken complement-mediated lysis of antibody-sensitized red blood cells requires the presence of both calcium and magnesium ions (Gabrielsen, Pickering and Good, 1973); in these reactions complement is activated via the classical pathway since there is a requirement for C1 (Stolfi, Fugmann, Jensen and Sigel, 1971). However, lysis of *T. cruzi* occurred in the presence of 0·01 M EGTA or when adding excess magnesium ions to tubes containing EDTA, i.e. conditions under which calcium ions which are needed for complement activation via the classical pathway, are not provided. Hence, complement was apparently activated by a different mechanism, most likely analogous with the 'alternative pathway' described for mammalian complement. The reaction is not restricted to a given strain of the parasite since both Y and Tulahuén strains of *T. cruzi* (differing in their preferential cell tropism in susceptible hosts) were lysed by chicken sera. Thus, it is difficult to explain the negative results obtained by Warren (1958), who used trypanosomes from infected rats, on the basis of strain resistance towards lysis by chicken serum. Differences in the procedure used to demonstrate lysis may be the alternative explanation.

Complement-mediated lysis of *T. cruzi* by chicken serum was found to be antibody-independent. This was inferred from the fact that the parasites were readily lysed by sera of birds immunosuppressed by four different procedures which inhibited production of agglutinins against SRBC. There was no apparent difference between the extent of lysis caused by the sera of normal or immunosuppressed animals. Inhibition of complement in the sera of chickens immunosuppressed by infection with IBDV, accomplished by heating or by addition of EDTA or CVF, abrogated the reaction and, as in the case of normal chicken sera, lysis occurred in the absence of calcium ions. It should be noted that although chicken antibodies do not bind mammalian C1 (Stolfi *et al.*, 1973; Gabrielsen, Pickering, Linna and Good, 1973) it is not known whether they can activate the C3 shunt in mammalian sera. Considering this as a possibility, chicken IgG was tested for lytic activity on *T. cruzi* in the presence of guinea-pig complement but with negative results.

Avian embryos are known either to lack or have very low levels of serum complement (Sherman, 1919; McGhee, 1952; Gabrielsen *et al.*, 1973) which may explain the susceptibility of embryos towards the infection. This possibility is compatible with the fact that chickens become refractory to *T. cruzi* infection soon after hatching (Nery-Guimaraes and Lage, 1972), i.e. when complement levels suddenly appear to rise (Gabrielsen *et al.*, 1973).

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