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## **Antibody response to experimental *Trypanosoma rangeli* infection and its implications for immunodiagnosis of South American trypanosomiasis**

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### **Summary**

Differential immunodiagnosis of *T. rangeli* and *T. cruzi* infections in man poses a particular problem, not only because these parasites share antigenic determinants, as detected by immunofluorescence, but also because they have a similar geographical distribution, the same host range and often identical insect vectors. We show here that whereas mouse anti-*T. rangeli* sera have significant cross reactivity with *T. cruzi* by immunofluorescence, they are entirely specific when tested by ELISA, using apparently similar antigen preparations. Immunoprecipitation analysis detected relatively little cross-reactivity between heterologous antisera and parasite combinations. Intriguingly, immunization with *T. rangeli* epimastigotes was much more powerful than similar immunization with trypomastigotes, and the majority of the antibody was directed against a single polypeptide of apparent  $M_r$  73kDa.

**Key words:** *T. rangeli*; *T. cruzi*; serodiagnosis; humoral immunity; immunoprecipitation.

### **Introduction**

The diagnosis of protozoan diseases in Latin America and elsewhere relies heavily on the use of serological techniques as diagnosis is often required many months after initial infection when parasitaemia has subsided and parasites are rare. Thus, although *T. rangeli* infection in man is thought to be entirely non-pathogenic (D'Alessandro, 1976), it poses a serious problem to the diagnostic

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laboratory as it shares common antigenic determinants with *T. cruzi*, the causative agent of Chagas' disease (Guhl and Marinkelle, 1982). Both parasites have a similar geographical distribution, the same vertebrate hosts, and, in some regions, identical insect vectors.

Recently Schechter and his colleagues (1983) described an immunoassay using a purified glycoprotein (GP90) from *T. cruzi* epimastigotes which was able to detect clinical and experimental infections with high specificity and sensitivity. However, the cost of the production and isolation of this glycoprotein is prohibitive and would mitigate against its widespread use in Third World countries. Consequently, we have investigated other ways of increasing the discrimination of standard immunoassays and have found that ELISA, using epimastigote lysates and mouse antisera, is entirely specific for these two infections under a variety of experimental conditions. Immunoprecipitation analysis revealed a single immunodominant *T. rangeli* polypeptide of app  $M_r$  73 kDa, which showed a precisely similar migration position to GP72 (Snary et al., 1981) in the detergent extracts of *T. cruzi*.

## Materials and Methods

### *Parasite*

*T. rangeli* (San Augustin strain) parasites were maintained by cyclical passage in mouse, triatomine bug and Tobie's medium to maintain infectivity, as described previously (D'Alessandro, 1972). Epimastigotes used in these studies were harvested from in vitro culture, whereas metacyclic trypomastigotes were isolated from the salivary glands of insect hosts. Briefly, *Rhodnius prolixus* were inoculated intrafemorally with *T. rangeli* epimastigotes and the salivary glands removed 25 days later. Glands were disrupted by gentle shearing in a Potter homogenizer and washed by centrifugation (300 g for 15 min at room temperature).

### *Sera and immunoassays*

Groups of 5 mice were inoculated intraperitoneally once per week for at least 4 weeks with  $2 \times 10^7$  living epimastigotes, trypomastigotes or epimastigotes plus trypomastigotes of *T. rangeli* (details in Table 1). Two weeks after the last injection, mice were exsanguinated by cardiac puncture and blood from individual mice clotted at room temperature, prior to clarification of the expressed serum by centrifugation (450 g for 15 min at 4°C). Immunofluorescence and ELISA were carried out with fresh serum, whereas immunoprecipitation was performed with serum stabilized with 50% v/v glycerol for transport.

### *Indirect immunofluorescence and ELISA*

Epimastigote parasites were washed in phosphate buffered saline (PBS) by centrifugation and aliquots mixed with various dilutions of sera. Bound antibody was detected by an FITC conjugate of IgG rabbit anti-mouse immunoglobulin and visualized under a UV microscope with epifluorescent optics.

Microwell assay plates for ELISA were coated with epimastigote lysates (sonicated for 3 min at 0°C) and blocked with 3% w/v haemoglobin (Sigma) prior to immunoassay. Diluted sera were allowed to bind for 30 min at room temperature, after which plates were washed thrice with PBS and the bound antibody detected by incubation for 30 min with alkaline phosphatase conjugated IgG rabbit anti-mouse immunoglobulin. After washing, paranitrophenylphosphate was added as

Table 1. Immunoassay of anti-*T. rangeli* sera

Mice immunized with <i>T. rangeli</i>	Number boosts	Indirect IF* titre (epimastigotes)		ELISA titre* (epima- stigotes)	
		<i>T. rangeli</i>		<i>T. cruzi</i>	<i>T. rangeli</i>
<i>T. cruzi</i>					
Metacyclic trypomastigotes . . . . .	7	1:40	1:12	1:160	< 1:10**
Culture epimastigotes . . . . .	4	1:160	1:40	1:640	< 1:10**
Epimastigotes + trypomastigotes . . .	5	1:160	1:20	1:160	< 1:10**

\* average value of 5 mice per group

\*\* same optical density reading as background defined by normal mouse serum

substrate, the colour reaction allowed to develop over 30–60 min, and quantitated in an automatic micro-ELISA reader.

#### Immunoprecipitation

Epimastigotes of both parasites were washed by centrifugation in methione-free Eagle's modification of minimal essential medium with Earle's salts (EMEM) containing 2 mg·ml<sup>-1</sup> sodium bicarbonate, 2 mM glutamine, 100 µg·ml<sup>-1</sup> bovine serum albumin (BSA) and non-essential amino acid mix (Flow Laboratories, Irvine, Scotland). Radioactive labelling was achieved by incubating 3 × 10<sup>7</sup> organisms in 2.0 ml of the above medium containing 100 µCi·ml<sup>-1</sup> L-[35-S] methionine (>800 Ci·mmol<sup>-1</sup>) or pro rata. After 4.5 h at 37°C, parasites were washed three times in PBS and solubilized in 400 µl 1% w/v Renex 30 (Atlas Chemical Company, Leatherhead, UK) in the presence of protease inhibitors (Sigma) (1.10 phenanthroline, phenylmethanesulphonyl fluoride, p-chloromercuriphenyl sulphonic acid, N α-p-tosyl-L-lysine chloromethyl ketone (each at 2 mM final concentration) and aprotinin (10 units·ml<sup>-1</sup> final concentration) in 4 mM ethyleneglycol-bis (β amino-ether) N,N'-tetra acetic acid, pH 7.0. After clarification of the supernatant by high speed centrifugation, *T. cruzi* epimastigotes typically incorporated 2 × 10<sup>7</sup> total cpm radioactivity and *T. rangeli* epimastigotes 1.4 × 10<sup>7</sup> total cpm. In each case radioactivity given as cpm precipitated in cold 10% w/v TCA; these accounted for at least 85% of the radioactivity in the sample.

The techniques of immunoprecipitation, SDS-PAGE analysis and fluorography have been described in detail elsewhere (Wong et al., 1985). Briefly, aliquots of Renex solubilized epimastigotes containing 10<sup>6</sup> TCA precipitable cpm were reacted with the equivalent of 10 µl of serum and the immune complexes recovered with protein A-Sepharose for analysis on 10% SDS-PAGE gels. Internal molecular weight standards of a [14-C] methylated protein mixture (CFA 626, Amersham International plc) were included in each gel. The relative apparent molecular weight value (app M<sub>r</sub>) of each [35-S] labelled polypeptide was determined by linear regression analysis against a curve constructed using the internal molecular weight standards.

## Results

Morphological analysis of the contents of infected *R. prolixus* salivary glands showed that *T. rangeli* metacyclic trypomastigotes predominated over epimastigotes in a ratio of approximately 3:1 (full data not shown). Repeated immunization of mice with a similar artificial mixture of trypomastigotes and

culture-derived epimastigotes resulted in high titred anti-*T. rangeli* sera, which, when tested by immunofluorescence, showed an easily detectable cross-reaction with *T. cruzi* (Table 1). When similar immunizations were carried out with epimastigotes or trypomastigotes alone, both stages induced antibodies which cross-reacted with *T. cruzi*, however, trypomastigote immunization resulted in relatively low homologous and heterologous titres even though up to 7 immunizing boosts had been given.

Intriguingly, when similar comparisons were made using the ELISA technique, no such cross-reactivity was demonstrated. Even though the maximum positive titre of the anti-*T. rangeli* sera was greater than that determined by immunofluorescence with homologous antigen, the binding to heterologous antigens could not be distinguished from the background defined by normal mouse serum (Table 1).

SDS-PAGE analysis of the original preparations of detergent extracted *T. rangeli* and *T. cruzi* epimastigotes (Fig. 1, tracks Tr and Tc, respectively) showed a similar labelling efficiency of major polypeptides of app  $M_r$  85, 73 and 50 kDa, although there were other obvious and unique bands in each preparation, for example the 122 kDa doublet in *T. rangeli* (Tr Fig. 1a) and the 93 kDa band (probably GP90) in *T. cruzi* (Tc Fig. 1a). Sera of mice immunized with trypomastigotes and epimastigotes of *T. rangeli* precipitated a major polypeptide of app  $M_r$  73 kDa (tracks 1–4, Fig. 1a) when assayed with *T. rangeli* antigens. Although the same band was visible with immunoprecipitates of sera from mice immunized with trypomastigotes alone (Tracks 5–10, Fig. 1a) it was very weak. When the same sera were reacted with detergent extracts of radiolabelled *T. cruzi*, only a single mouse (track 1, Fig. 1b) showed a reaction with bands of  $M_r$  85, 73 and 50 kDa that was quantitatively different to identical bands bound by normal mouse sera (tracks 11 and 12 in Fig. 1a and b). These same polypeptides were by far the most intensely labelled proteins in the original *T. cruzi* detergent extract (Tc., Fig. 1b).

Immunoprecipitation of *T. rangeli* material with sera from epimastigote immunized mice showed a complex pattern of labelled polypeptides (tracks 1–

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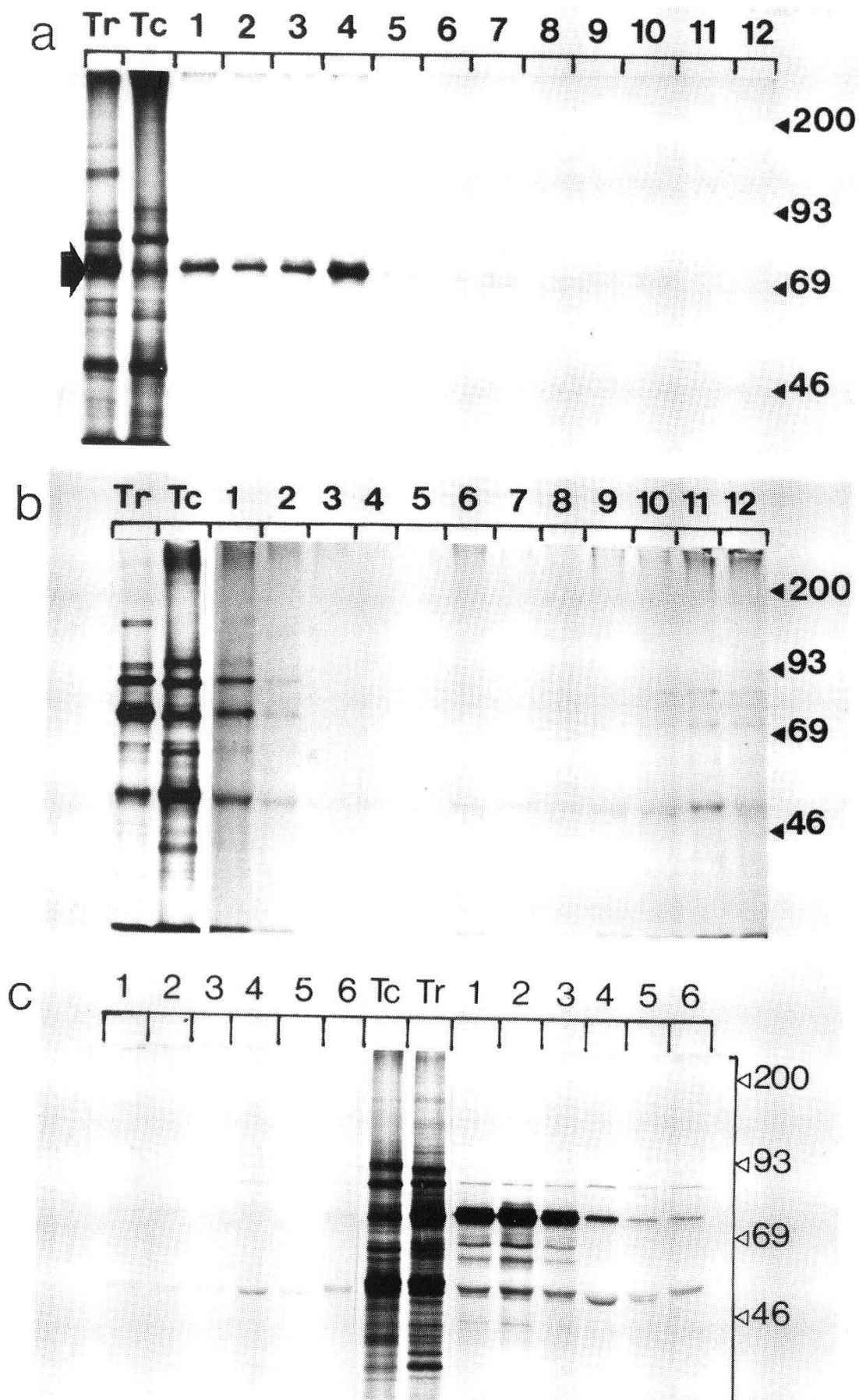
Fig. 1. Immunoprecipitation profile of radioactive polypeptides recognized by anti-*T. rangeli* sera. Epimastigotes of *T. rangeli* or *T. cruzi* were labelled with [<sup>35</sup>S] methionine, extracted with the non-ionic detergent Renex 30 and reacted with various anti-*T. rangeli* sera.

(a) *T. rangeli* polypeptides precipitated by sera from mice immunized with *T. rangeli* trypomastigotes and epimastigotes (1–4) or trypomastigotes alone (5–10). Sera 11 and 12 were from normal mice. Arrow indicates position of “GP72/73” in *T. cruzi* and *T. rangeli* epimastigotes.

(b) Polypeptides of *T. cruzi* precipitated by sera as in (a).

(c) Sera from mice immunized with *T. rangeli* epimastigotes (1–3) or normal mouse serum (4–6) reacted with *T. cruzi* antigens (left of figure) or *T. rangeli* antigens (right of figure).

In each case, Tc and Tr denote *T. cruzi* and *T. rangeli* detergent extract prior to immunoprecipitation, and migration position of internal molecular weight markers shown by arrowheads (molecular weight given in kDa).





3, Fig. 1c right) which contained bands of app  $M_r$  85, 73, 64, 60 and 50 kDa, in which the 73 kDa polypeptide was especially prominent. These sera gave a banding profile which was both qualitatively and quantitatively different from the banding pattern of normal mouse serum (tracks 4–6, Fig. 1c right), and showed immunoprecipitation with *T. cruzi* antigens which was only just above background defined by normal mouse sera (Fig. 1c, left).

## Discussion

The cross-reaction between *T. rangeli* and *T. cruzi* detected here by indirect immunofluorescence, is in accord with published reports that these two parasites share up to 50% of their immunogenic epitopes (Afchain et al., 1979; Guhl and Marinkelle, 1982). As a consequence, the specificity achieved by the ELISA technique was surprising as it was based on an unfractionated frozen/thawed lysate of the same epimastigotes used for immunofluorescence. The relatively trivial explanation of artefactual cross-reactivity produced by antibody trapping in whole, fixed organisms in the former technique can be discounted as normal mouse serum with fluorescent conjugate gave virtually no staining.

It seems possible that preparation of the antigen coated plates had resulted in an alteration of the antigen mixture due to (1) unrecognized fractionation of antigens through differential adsorption to the ELISA plate, (2) antigen loss due to disruption of conformational determinants (Celada, 1979) or (3) protein degradation due to the extremely active proteases released by epimastigote lysis (Itow and Plessman Camargo, 1977) and only incompletely inhibited by the complex cocktail of protease inhibitors used in these studies (detailed in Wong et al., 1985).

The immunoprecipitation studies suggest that the polypeptide of app  $M_r$  73 kDa is a major, immunodominant antigen in *T. rangeli* epimastigotes. This is intriguing, as it consistently migrated to precisely the same position as the epimastigote-specific GP72 component of *T. cruzi* when run on the same gel (for example Fig. 1a, Tr and Tracks 1–4 cf. Tc, arrowed). It seems possible that this component might have a similar role and structure in *T. rangeli* as that determined in *T. cruzi* (Ferguson et al., 1983). If the analogy was complete, then the very unusual structure of “GP73” would give it properties very different to the other cell surface proteins of *T. rangeli*, among which might be a different degree of adsorption to the plastic used for ELISA plates. Interestingly, not only could this explain the apparent paradox between our immunofluorescence and ELISA data but also it suggests that the immune response to GP72-like molecules might be the basis of *T. rangeli*/*T. cruzi* cross-reactivity. This molecule has been shown to mediate epimastigote to trypomastigote transformation in *T. cruzi* (Sher and Snary, 1982), thus as *T. rangeli* and *T. cruzi* epimastigotes

share the same insect vector (*Rhodnius prolixus*), it is perhaps not surprising that they might have surface regulatory molecules of similar structure.

The relative immunogenicity of the trypomastigote and epimastigote stages is extremely interesting. Even though the trypomastigote is thought to be the infective and proliferative stage in vivo, it is much less immunogenic than the epimastigote stage when tested by immunofluorescence or ELISA. These findings are precisely in accord with the immunoprecipitation studies, where sera from *T. rangeli* trypomastigote infected mice failed to react with any epimastigote antigens, and on mixed trypomastigote/epimastigote immunization, the character of the immune response was largely mediated by the epimastigote components. This is in direct contrast to *T. cruzi*, where trypomastigote and epimastigote share the majority of their major antigens (Snary and Hudson, 1979). The low immunogenicity of trypomastigotes is even more intriguing when one considers that, although this is the infective stage of the parasite, it produces only a very low level of parasitaemia. From immunological dogma it would have been reasonable to predict that a parasite which does not provoke a strong immune response might divide without control, to overwhelm its host.

Immunoprecipitation studies have evoked considerable interest recently, not only as a means of characterizing the "antigenic profile" of the immune response but also, in chagasic patients, as a means of defining the antigens that might be recognized by patients at different stages of this disease (for example, Araujo and Remington, 1981; Zingales et al., 1984; Martins et al., 1985). Consequently, it is reassuring to note that the cross-reactivity noted between immunofluorescent studies is not a significant feature of immunoprecipitation analysis. Work now completed from our laboratories has shown that ELISA and immunoprecipitation may be used to discriminate between sera of patients with *T. cruzi* and *T. rangeli* infections, either as single or mixed infections (Guhl F., Hudson L., Marinkelle C. J., Jaramillo C., to be published).

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