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Use of monoclonal antibodies for the differential detection of *Trypanosoma cruzi* and *T.rangeli* in epidemiological studies and xenodiagnosis

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Summary

T. cruzi and *T. rangeli* have the same insect and mammalian hosts, including man, and in addition share approximately half the antigenic determinants recognised by the humoral response. Thus serodiagnosis of *T. cruzi* infection in areas where *T. rangeli* is endemic may include an unknown rate of false positives due to this antigenic cross-reactivity. Similarly, the results of xenodiagnostic procedures and epidemiological surveys of insect vectors are prone to distortion because of the close morphological resemblance of the epimastigote stages. The description of a *T. cruzi* epimastigote specific monoclonal antibody, 2A2, which reacts with both culture and insect derived epimastigotes provides a more reliable basis for differential diagnosis of these two parasites.

Key words: *T. cruzi; T. rangeli;* monoclonal antibody; differential diagnosis; seroepidemiology; xerodiagnosis.

Introduction

The parasitic protozoan *T. cruzi* infects an estimated 10 million individuals in Latin America and in a variable proportion of patients can result in life threatening cardiac or digestive pathology recognised as Chagas' disease. In the majority of patients the parasitaemic phase of infection is transient and often goes unnoticed with the high background of general malaise in the socio-economic groupings usually affected.

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Consequently, diagnosis of the infection by direct microscopic examination is rarely possible and so routine diagnosis relies almost entirely on serological tests. However, these serological tests have several important disadvantages: 1. they show incomplete specificity due to shared antigens common to *T. cruzi, T. rangeli* and *Leishmania,* 2. they confirm that infection has taken place, but on a single serum sample, give no indication of the time for which the infection has been established, and 3. they give no estimate of the current parasitological status of the patient.

Leishmaniasis can usually be excluded because of obvious clinical differences on presentation, however, discrimination between *T. cruzi* and *T. rangeli* is more difficult. Both trypanosomes have the same mammalian reservoir hosts and are transmitted by the same triatomine vectors in many parts of Latin America (Hoare, 1972), but whereas *T. cruzi* causes Chagas' disease, *T. rangeli* infection is regarded as entirely non-pathogenic (D'Alessandro, 1976). Thus this cross-reactivity not only can result in clinical misdiagnosis but can also distort the findings of epidemiological surveys. Xenodiagnosis remains the most sensitive technique for the detection of *T. cruzi* or *T. rangeli* infection but epimastigotes of the two parasites appear too similar for easy diagnosis. Consequently our description of a monoclonal antibody, 2A2, which is entirely specific for insect and culture derived *T. cruzi* epimastigotes should facilitate discrimination between the two parasites whether used to examine laboratory or wild caught insects in xenodiagnosis or epidemiological surveys, respectively.

Materials and Methods

Host and parasites

T. cruzi epimastigotes of Y and Tulahuen strains were routinely maintained in monophasic culture using Warren's medium (Warren, 1960), whereas *T. rangeli* epimastigotes of Molino I strain were grown cyclically in bug, mouse and Tobie's biphasic medium (D'Alessandro, 1972). Amastigotes and trypomastigotes of *T. cruzi* were harvested from continuous in vitro culture with S2 cells (Hudson et al., 1984) or from batch culture with Vero cells (Sanderson et al., 1980), respectively.

Rhodnius prolixus were infected with *T. cruzi* by allowing the insect hosts to take a blood meal from infected mice, then, when mixed infections were required, *T. rangeli* epimastigotes were injected into the insect's haemocoel by intrafemoral inoculation. Homogenous infections were obtained by repeated feeding of *R. prolixus* on mice infected with either *T. cruzi* or *T. rangeli.*

Polyclonal and monoclonal antibodies

Hyperimmune mouse sera were produced in outbred mice by a total of 6 intraperitoneal injections of 5×10^7 epimastigotes given every 2 weeks. For monoclonal antibody production, BALB/c mice were immunised once with the same number of parasites and boosted 14 days later, 4 days before spleens were removed for fusion with X63.531 cells by the method described by Köhler and Milstein (1975). Positive wells from the primary plating were selected by indirect immunofluorescence and ELISA using epimastigotes as antigen, and the resulting hybridomas cloned at least 3 times by limiting dilution before monoclonal antibody was prepared by ascitic growth of the tumour in pristane treated mice. Two of the monoclonal antibodies in this study, 2A2 and 4G10, were isolated following immunisation with epimastigotes, whereas CE5 was raised against rat dorsal root ganglia and shown to cross react with *T. cruzi* (Wood et al., 1982).
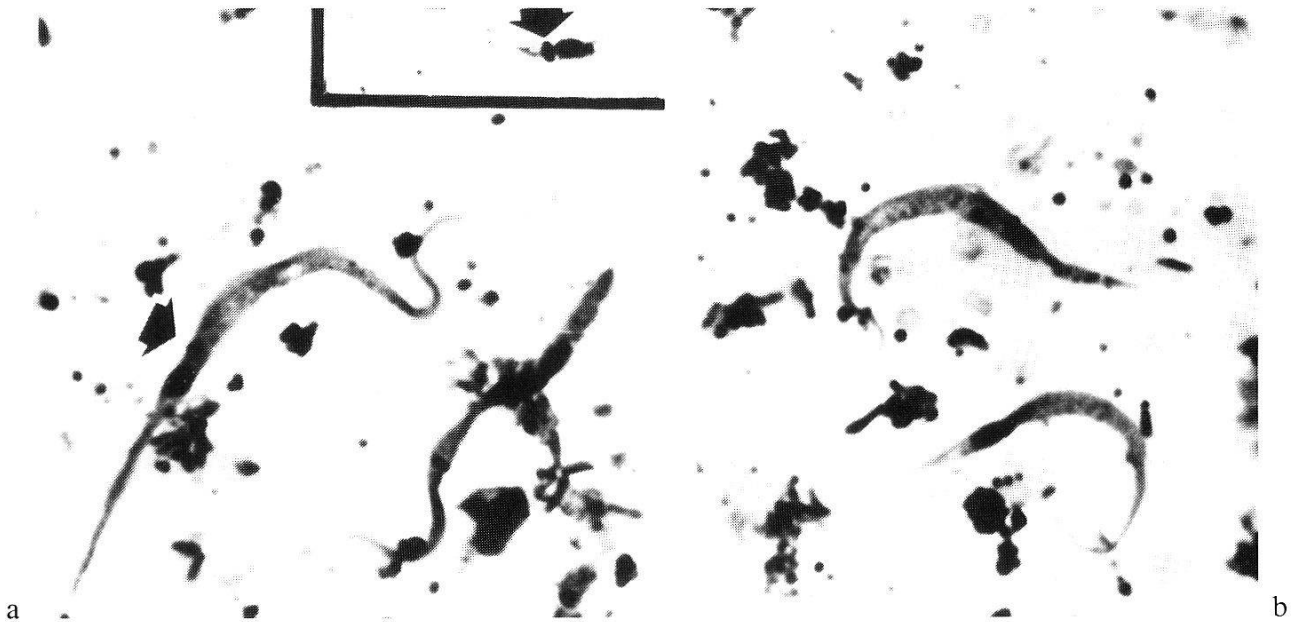
Fig. 1. Epimastigotes of *T. cruzi* and *T. rangeli* in faeces of *R. prolixus*. Faeces were air dried onto a microscope slide, fixed in methanol and stained with Giemsa's stain. a) Experimental infection showing the larger *T. rangeli* and smaller *T. cruzi* epimastigotes. The two parasites have kinetoplasts of very different shape and size, that of *T. rangeli* is punctiform (arrowed) whereas that of *T. cruzi* is bar-shaped (arrowed in insert). b) Under less ideal, and more usual, circumstances the two parasites appear almost identical, *T. cruzi* epimastigote (below) could only be distinguished from that of *T. rangeli* with difficulty in this natural, mixed infection from a wild caught insect. (Magnification in each main photograph ×2250).

Giemsa and immunofluorescent staining

Rectal ampulla contents were forced onto a slide by exerting slight pressure on the abdomen of the infected insect using forceps. After appropriate dilution to facilitate dispersion of rectal contents, preparations were air dried and fixed in absolute methanol for 30 sec prior to washing in 20 mM citric-phosphate buffer pH 5.75 and immersion for 15 min in Giemsa's stain diluted 1:5 in the buffer.

For indirect immunofluorescence, epimastigotes and metacyclic trypomastigotes were separated from ampulla contents by extensive washing and differential centrifugation. Faeces were washed thrice in phosphate buffered saline (PBS) by centrifugation at 640 g for 15 min at room temperature. This was followed by centrifugation of the resuspended pellet at 160 g for 2 min at room temperature, after which the top third of the supernatant contained a greatly enriched number of both parasites. Although fixation of insect derived parasites with formaldehyde, acetone or methanol was attempted, these procedures diminished the intensity of immunofluorescence, consequently these organisms were only air dried prior to antibody treatment. Culture derived parasites; epimastigotes, trypomastigotes or amastigotes were fixed for 30 min in 3.7% w/v formaldehyde prior to staining.

Mouse antibodies were diluted in PBS and applied to parasite material on slides for 30 min at room temperature. After extensive washing, bound antibodies were detected with FITC.IgG rabbit anti-mouse immunoglobulin serum diluted in PBS containing 1 μg/ml Evan's Blue dye. The specificity of immunofluorescent staining in mixed infections was confirmed by restaining antibody-treated parasites with Giemsa's stain followed by morphological identification.

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Table 1. Summary of antibodies tested by indirect immunofluorescence

Antibody	Immunogen-*	Isotope	Immunofluorescent titre on	
			T. cruzi	T. rangeli
Anti-T. cruzi	epimastigotes	HS	1:160	1:20
Anti-T. rangeli	epimastigotes	HS	1:80	1:320
2A2	epimastigotes	IgM,K	1:600	no reaction
4G10	epimastigote	IgM,K	1:100	1:20
CE5	rat neurons	IgM,K	1:100	1:20

* With exception of those used to raise anti-T. rangeli serum, all epimastigotes were T. cruzi, Y strain grown in Warren's medium.

H.S = hyperimmune serum

Results

Discrimination of T. cruzi and T. rangeli epimastigotes on morphological grounds can be straightforward under ideal conditions. Fig. 1a shows the characteristically larger T. rangeli epimastigote compared to that of T. cruzi, the distinctive punctiform appearance of the T. rangeli kinetoplast (arrowed) contrasts strikingly with the bar form kinetoplast shown by T. cruzi (arrowed in insert). However, when the faeces of wild caught insects are examined, as in Fig. 1b, the two species are usually more difficult to distinguish. Under these circumstances, identification requires an expert eye and painstaking microscopic examination.

Polyclonal sera showed the highest titre of positive fluorescence against homologous parasites, for example anti-T. cruzi serum reacted with all epimastigotes of this species even at a dilution of 1:160 but reacted with T. rangeli only up to a dilution of 1:20 (Table 1). Of the 3 monoclonal antibodies tested, only 2A2 reacted with T. cruzi but not T. rangeli (Fig. 2). When used as ascitic fluid this antibody reacted with T. cruzi epimastigotes (Fig. 2a) and trypomastigotes (Fig. 2d) from culture up to a dilution of 1:100, but reacted even more strongly with culture derived amastigotes up to a dilution of 1:600 (Fig. 2c). Significantly, although this antibody reacted with T. cruzi epimastigotes and metacyclic trypomastigotes from experimentally or naturally infected R. prolixus (Fig. 2e), it showed no binding to T. rangeli epimastigotes from in vitro culture, from R. prolixus faeces (epimastigotes) or salivary glands (metacyclic trypomastigotes) even at 1:20 dilution. CE5 and 4G10 monoclonal antibodies both reacted strongly with T. cruzi but also showed cross-reaction with T. rangeli (Fig. 2b) with a staining pattern identical to that shown by polyclonal sera (data not shown).

Naturally infected domicilliary R. prolixus were obtained from the Rio Negro Valley, Colombia (Guhl et al., 1987) and their faecal flagellates stained
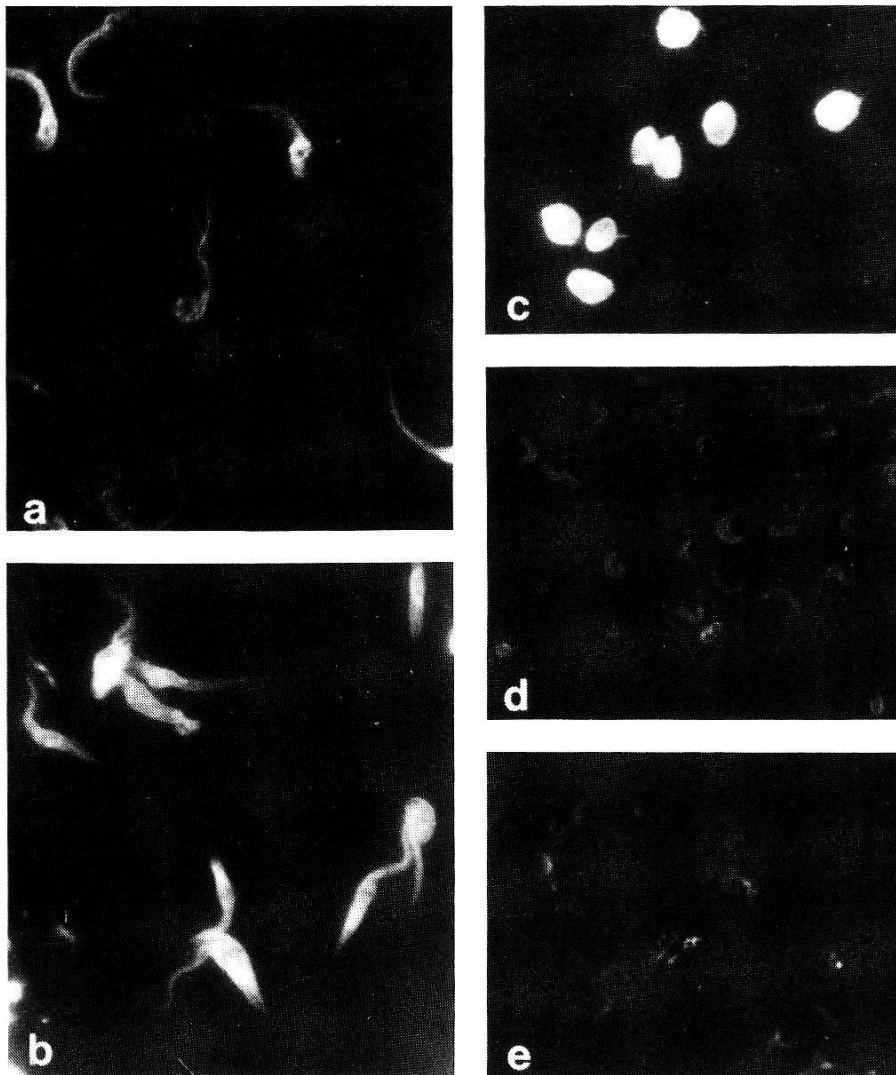
Fig. 2. Life cycle stages of each trypanosome stained with monoclonal antibodies by indirect immu-
nofluorescence. *T. cruzi* epimastigotes (a), amastigotes (c) and trypomastigotes (d) give clear positive
staining with 2A2 monoclonal antibody, whereas *T. rangeli* was negative (not shown). In each case
2A2 specific staining was limited to the surface membrane alone, although in the case of amastigotes
this was so bright that the parasite appeared homogeneously stained on photomicroscopy. Immuno-
fluorescence patterns shown by *T. rangeli* culture form epimastigotes (b) or insect derived epimasti-
gotes and metacyclic trypomastigotes (e) were very similar when stained with 4G10 (as here) and CE5
or hyperimmune polyclonal *T. cruzi* or *T. rangeli* sera.

with the monoclonal antibodies in pairs. The 4G10 antibody was used to detect
both *T. cruzi* and *T. rangeli* epimastigotes, and the former then specifically
identified with the 2A2 monoclonal. 80% of the insects examined carried
T. cruzi and of these 25% also carried *T. rangeli*.

Discussion

Immunological techniques can provide a convenient route for the devel-
opment of simple, sensitive and specific assays for the differential diagnosis of

parasitic diseases (WHO, 1975) and allow greater discrimination and sensitivity than microscopic examination. Immunological assays have the significant advantage that they can be carried out by personnel with minimal training and often may be automated. However, one major obstacle to the full exploitation of this approach is the known cross-reactivities which exist between parasite infections which carry profoundly different clinical implications. This is illustrated by *T. cruzi* and *T. rangeli,* both of which occur throughout Latin America but, whereas the former can produce serious disease and a life-threatening infection, the latter is considered to be totally non-pathogenic.

T. cruzi infection is usually diagnosed by indirect immunofluorescence or ELISA tests, but there is significant cross-reactivity with antibodies induced not only by *T. rangeli* but also *Leishmania* infections. Although this problem of specificity has been circumvented using a defined antigen from *T. cruzi* (Snary and Hudson, 1979), specificity testing against *T. rangeli* was carried out with sera from experimental mice and, in common with the majority of other serodiagnostic procedures, relied upon detecting the antibody response to infection rather than the parasite (Schechter et al., 1983). The definitive assay for determining infection status is still xenodiagnosis, wherein laboratory reared triatomine insects are allowed to feed on the patient and the insects' rectal contents examined after the parasite has passed through a long multiplicative phase. Although this assay is extremely sensitive and potentially can detect a single parasite in the blood meal (ca. 0.25 ml), it is still beset by the need for specific identification of the rectal flagellates.

Our finding that polyclonal sera showed a higher titre against homologous parasites by immunofluorescence confirms published data (Anthony et al., 1981) and is in accord with the estimate that these two parasites share about 40% of epitopes detected by conventional sera in immunoelectrophoresis (Afchain et al., 1979; Guhl and Marinkelle, 1982). Quantitative differences can be useful under carefully controlled conditions in the experimental laboratory, but would be totally unreliable criteria for routine application. In contrast, the monoclonal antibody 2A2 was totally parasite specific; it reacted with each of the life cycle stages of *T. cruzi,* whether grown in vivo or in vitro, up to a titre of 1:600, but failed to bind to *T. rangeli* even at 1:20. Two of the other monoclonals tested, 4G10 and CE5 reacted equally well with both parasites even though they had been prepared against very different immunogens. 4G10 was isolated from mice immunised with *T. cruzi* epimastigotes and it probably defines a common antigen similar to those described by Anthony and his colleagues (1981) who studied cross-reactivity between these parasites using monoclonal antibodies raised against *T. rangeli.* CE5 was isolated from mice immunised with rat dorsal root ganglion cells and recognises a common epitope shared by Purkinje neurons, heart muscle and *T. cruzi* (Wood et al., 1982), thus its surprising reactivity with the supposed non-pathogenic *T. rangeli* merits further investigation.

In this study, we made use of the cross-reactive monoclonal antibodies to identify all epimastigotes in the faeces of bugs with mixed infections (both experimental infections and wild caught insects from an area endemic for *T. cruzi* and *T. rangeli*) and then used 2A2 to identify those epimastigotes carrying the *T. cruzi* specific epitope. This approach provides a convenient tool for epidemiological studies. However, detection of minor populations of *T. rangeli* flagellates would require the positive identification only possible with a specific monoclonal antibody.

Experiments are now in progress to characterise the nature of the polypeptides carrying the 2A2 defined epitope and to isolate *T. rangeli* specific hybridomas from infected and immunised mice. The real potential of these reagents will only be realised once they have been fully validated in xenodiagnosis, and field studies for clinical diagnosis and epidemiological studies, respectively.

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