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# *Trypanosoma cruzi*: immunological consequences of parasite modification of host cells

R. RIBEIRO DOS SANTOS* & L. HUDSON *Department of Biochemistry, St George's Hospital Medical School, London*

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## SUMMARY

Parasite antigens released from *Trypanosoma cruzi*-infected cells were adsorbed to infected and uninfected mammalian cells thus rendering them susceptible to immune lysis by antibody and cell-mediated immunity directed against the parasite. BALB/c mice infected with *T. cruzi* for 15 days developed cytotoxic T lymphocytes specific for parasite antigens. At 60 days post infection, however, the mice developed an additional population of cytotoxic T lymphocytes that were able to kill normal syngeneic muscle or neurone-derived cell lines *in vitro*. These '60-day' T lymphocytes did not kill HeLa cells unless they were coated with *T. cruzi* antigens suggesting that the population of autoaggressive T lymphocytes was not an artefact due to an increase in natural killer cells.

## INTRODUCTION

Although the pathological findings in Chagas' disease have been well described (reviewed by Köberle, 1968) the mechanism of pathogenesis has not been fully elucidated. One of the most significant features of the pathology of this disease was first described by Vianna (1911). He observed that when a parasite pseudocyst ruptured and released *Trypanosoma cruzi* organisms, many of the surrounding uninfected cells were affected by degeneration, necrosis or lysis. Margarinos Torres (1929) and Chagas (1934) suggested that this may be due to a hypersensitivity reaction which, although elicited by *T. cruzi* products, involved uninfected cells by an unexplained mechanism. Later experimental evidence provided support for the notion that mammalian cells might share common antigens with *T. cruzi* (Santos-Busch & Teixeira, 1974) and so an anti-parasite response could give rise to an autoimmune reaction involving uninfected host cells. We believe that an alternative explanation may be provided by our demonstration that *T. cruzi* antigens can bind to mammalian cell surfaces (Ribeiro dos Santos & Hudson, 1980) thereby rendering them potential targets for the host's immune response against the parasite.

In this paper, we show that both antibodies and cells taken from mice 15 days after *T. cruzi* infection can kill parasite-modified mammalian cells *in vitro*. Later, at 60 days, a second population of lymphocytes were recovered from *T. cruzi*-infected mice that were able to kill unmodified syngeneic cells *in vitro*.

* Permanent address: Department of Parasitology, University of São Paulo, 14100 Ribeiro Preto, São Paulo Brasil.

Correspondence: Leslie Hudson, Department of Immunology, St George's Hospital Medical School, London SW17 0RE, UK.

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## MATERIALS AND METHODS

*Parasites.* BALB/c mice were infected with *Trypanosoma cruzi* (Y strain) by an intra-peritoneal injection of $10^3$ trypomastigotes per g bodyweight.

*Cell lines.* The following human and murine cell lines were maintained as described elsewhere (Ribeiro dos Santos & Hudson, 1980): A204—human rhabdomyosarcoma, HeLa—human mammary carcinoma, S2—murine fibrosarcoma, Neuro 2A—murine neuroblastoma. Briefly, the cells were grown in Dulbecco's modification of Eagle's minimal essential medium (DMEM) containing 10% v/v heat-inactivated foetal bovine serum (FBS) and diluted every 7 days with fresh medium at a ratio of 1:40 after a brief trypinization.

*Sensitization of cell lines with parasite antigens.* Culture-derived amastigotes (Hudson & Snary, 1979) or epimastigotes (Hudson, 1979) were suspended in distilled water and frozen and thawed three times. The lysate was made isotonic using 1·4 M sodium chloride solution and centrifuged at 30,000 *g* for 1 hr at 4°C. The supernatant was adjusted to a protein concentration of 500 $\mu g \cdot ml^{-1}$ (Lowry *et al.*, 1951) and used to sensitize cell lines as described previously (Ribeiro dos Santos & Hudson, 1980).

*$^{51}$Chromium labelling of cells.* Coverslip-adherent cells were incubated with 30 $\mu Ci \cdot ml^{-1}$, $^{51}CrO_4^{2-}$ (specific activity 100–300 $mCi \cdot mg^{-1}$ Cr; Radiochemicals Centre, Amersham, England) for 1 hr at 37°C and then washed five times with DMEM/FBS.

*Antibody plus complement cytolysis.* Anti-*T. cruzi* serum was prepared as before (Ribeiro dos Santos & Hudson, 1980) and absorbed with each cell line before use. $^{51}$Chromium-labelled cells were incubated with this absorbed antiserum (optimal final dilution 1:20) in the presence of guinea-pig serum (1:10 final dilution) as a complement source (total volume 400 $\mu$l). After incubation for 2 hr at 37°C, the supernatant was removed and 400 $\mu$l of saponin (10% w/v) added. Thus the total c.p.m. of $^{51}$Cr was determined for each culture well, and the percentage isotope release calculated as follows:

$$\% \text{ isotope release} = \frac{\text{c.p.m. supernatant}}{\text{c.p.m. supernatant} + \text{c.p.m. released by saponin}} \times 100.$$

Controls were as indicated in the legend to Fig. 1.

*Lymphocyte-mediated cytotoxicity.* Spleens were removed from *T. cruzi*-infected BALB/c mice and cell suspensions prepared (Ribeiro dos Santos & Hudson, 1980). These suspensions were enriched for T lymphocytes by filtration through nylon wool columns as described elsewhere (Gravely & Kreier, 1976). To 1-ml cultures of untreated or *T. cruzi* antigen-treated adherent cells were added $10^7$ lymphocytes and the mixture incubated for 18 hr at 37°C. A sample of the supernatant was removed and saponin added to release the remaining cell-associated $^{51}$Cr. The percentage isotope release was calculated as above. Controls were as described in the legend to Fig. 4.

*Antibody-dependent cell-mediated cytotoxicity.* A cell suspension was prepared by teasing apart spleens from normal BALB/c mice. An aliquot of cell suspension containing $10^7$ lymphocytes was added to each culture of $^{51}$Cr-labelled adherent cells to which anti-*T. cruzi* serum had also been added (final dilution 1:500). Each mixture was incubated for 18 hr at 37°C and the percentage of $^{51}$Cr released determined as in the previous section. Controls were performed as detailed in the legend to Fig. 3.

## RESULTS

Between 20–40% of cells were infected with *T. cruzi* parasites in long-term cultures of S2, A204, Neuro 2A or HeLa cells (Fig. 1). When these cultures were treated with anti-*T. cruzi* serum plus complement, the proportion of cells lysed (average of 62% for S2, A204 and Neuro 2A) was greater than the proportion infected (average of 27% for same cell lines). Only in the case of HeLa cell cultures, which showed the lowest isotope release and highest infection rate, did the percentage of $^{51}$Cr release (46%) approximate to the percentage of parasitized cells (38%).

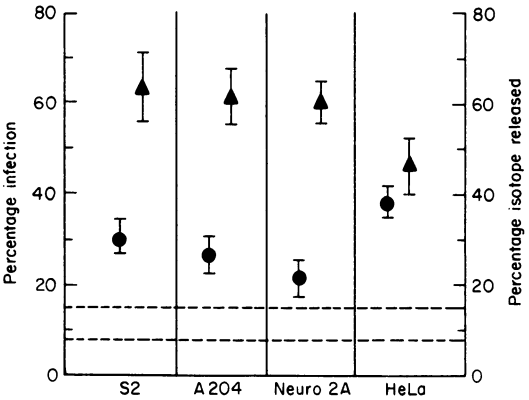


**Fig. 1.** Release of $^{51}Cr$ from *T. cruzi*-infected cell lines mediated by antibody and complement. (•) Percentage infection, (▲) percentage $^{51}Cr$ release. Each datum point represents the mean of six determinations ± s.e. The horizontal broken lines represent the limits of mean ± s.e. of the following controls for each cell line: spontaneous release from uninfected or infected cell lines, release from uninfected cells with anti-*T. cruzi* serum plus complement, release from uninfected or infected cells plus complement alone and release from uninfected or infected cells in the presence of normal rabbit serum plus complement.

Anti-*T. cruzi* serum plus complement was also able to lyse $^{51}Cr$-labelled cell lines treated with amastigote- or epimastigote-derived antigens (Fig. 2). Within each cell type, there was no significant difference in isotope release between cells sensitized with amastigote- or epimastigote-derived antigens (Fig. 2). Again, sensitized HeLa cells gave the lowest percentage release of isotope.

Anti-*T. cruzi* antibody, bound to antigen-sensitized S2 cells, was able to initiate antibody-dependent cell-mediated cytotoxicity by normal spleen cells (Fig. 3). The elevated isotope release (63%) was specific for *T. cruzi* antigens; unsensitized cells in the same system gave the same percentage $^{51}Cr$ release as S2 cells alone. Significantly, spontaneous release from S2 cells alone (25%) was not increased after the cells were treated with parasite antigen (24·5%; Fig. 3).

T lymphocyte-enriched spleen cell suspensions prepared from mice infected with *T. cruzi* for 15 days were able to release 70% of intracellular $^{51}Cr$ from syngeneic S2 cells sensitized with amastigote antigens (Fig. 4). When aliquots of the same lymphocyte suspensions were added to unsensitized S2

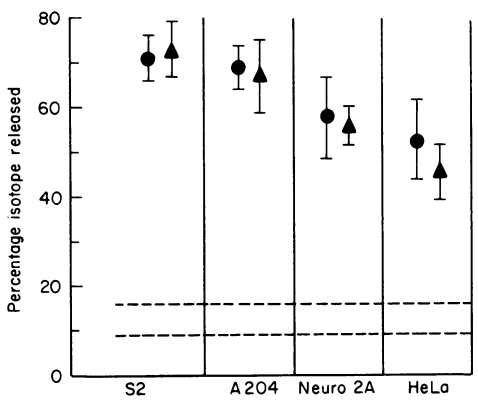


**Fig. 2.** Antibody plus complement-mediated release of $^{51}Cr$ from uninfected cell lines passively sensitized with *T. cruzi* antigens. (•) Percentage $^{51}Cr$ release from cells sensitized with 250 $\mu g \cdot ml^{-1}$ (final concentration) of amastigote-derived antigen, (▲) percentage $^{51}Cr$ release from cells sensitized with 250 $\mu g \cdot ml^{-1}$ (final concentration) of epimastigote-derived antigen. Each point represents the mean of six determinations ± s.e. The horizontal broken lines indicate the limits of mean ± s.e. of the following controls for each cell line used: spontaneous release from unsensitized cells, release from unsensitized cells in the presence of anti-*T. cruzi* serum plus complement, spontaneous release from antigen-sensitized cells, release from sensitized cells with normal rabbit serum plus complement and release from sensitized cells with complement alone.

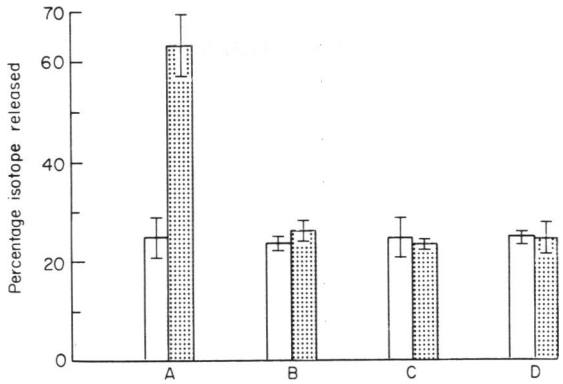


**Fig. 3.** Release of $^{51}Cr$ from S2 cells by antibody-dependent cell-mediated cytotoxicity. The following mixtures of S2 cells, either unsensitized (□) or sensitized with amastigote-derived antigens (▦), were incubated for 18 hr at 37°C: (A) S2 cells plus normal BALB/c spleen in the presence of 1 : 500 (final dilution) rabbit anti-*T. cruzi* serum, (B) S2 cells plus normal BALB/c spleen in the presence of 1 : 500 (final dilution) normal rabbit serum, (C) S2 cells plus normal BALB/c spleen or (D) S2 cells alone. Data shown as means of five determinations ± s.e.

cells only 30% of isotope was released; this was not significantly different from the spontaneous release from S2 cells alone (25%; Fig. 4).

In contrast, splenic T lymphocytes prepared from mice infected with *T. cruzi* for 60 days released 71% of isotope from unsensitized S2 cells. Isotope release was only slightly greater (80%) when aliquots of these lymphocytes were added to S2 cells coated with amastigote antigens.

Similar findings were obtained when T lymphocytes from mice infected with *T. cruzi* for 60 days were mixed with Neuro 2A cells; unsensitized Neuro 2A cells released 47·8 ± 2·8% (mean ± s.e.) of $^{51}Cr$ whereas antigen-sensitized cells released 68·0 ± 2·8% of intracellular isotope (full data not shown).

Direct host cell cytotolysis was not shown when 60-day lymphocytes were mixed with HeLa cells. Specific $^{51}Cr$ release was obtained only when the HeLa cells were coated with amastigote antigens; lymphocyte-mediated release from unsensitized cells did not differ significantly from the spontaneous release from HeLa cells alone (Fig. 4b).

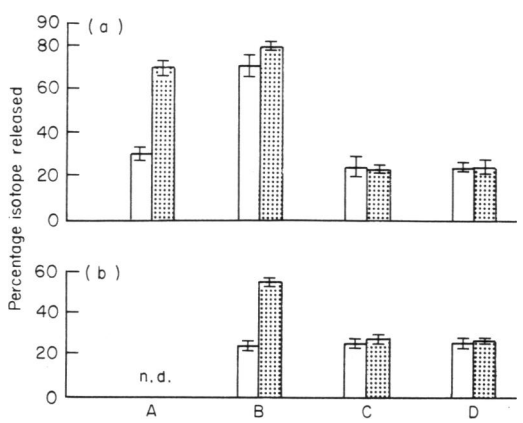


**Fig. 4.** Release of $^{51}Cr$ from (a) S2 or (b) HeLa cells mediated by T lymphocytes. The S2 or HeLa targets were either unsensitized (□) or sensitized with amastigote-derived antigens (▦). Splenic lymphocytes from either normal or *T. cruzi*-infected BALB/c spleen were filtered through nylon wool and mixed with the targets at ratios of 1 : 100. (A) T lymphocytes from BALB/c mice 15 days after infection with *T. cruzi*, (B) T lymphocytes from BALB/c mice 60 days after infection with *T. cruzi*, (C) normal BALB/c lymphocytes or (D) S2 cells alone. The cell mixtures were incubated for 18 hr at 37°C. Data shown as means of five determinations ± s.e.

## DISCUSSION

When cultures of S2, A204 or Neuro 2A cell lines infected with *T. cruzi* were labelled with $^{51}Cr$ and then treated with anti-*T. cruzi* serum plus complement there was a large specific release of intracellular isotope, indicating that the majority of cells had been lysed (Fig. 1). In these cultures, however, the percentage of *T. cruzi* infection did not exceed 30%. These observations may be explained by our previously published work showing that parasite antigens are adsorbed to the surface of uninfected and infected cells (Ribeiro dos Santos & Hudson, 1980).

In the case of HeLa cells alone, the percentage of infection approximated to the proportion of cells lysed as indicated by $^{51}Cr$ release. This was not due to an inability of HeLa cells to adsorb antigen, as all the cells tested here (S2, A204, Neuro 2A and HeLa) bound large quantities of antigen as detected by immunofluorescence (Ribeiro dos Santos & Hudson, 1980, and unpublished data). It is possible that the membrane of HeLa cells shed the antigen–antibody complex rapidly during the 2-hr incubation at 37°C or were simply less susceptible to complement-mediated lysis.

The sensitization of uninfected cells by parasite antigens was reproduced in the four cell lines tested using a 30,000-*g* supernatant of lysed amastigote or epimastigote organisms. Again, parasite-modified cells were susceptible to complement lysis mediated by anti-*T. cruzi* serum (Fig. 2). There was no difference in behaviour shown by lysates of either amastigotes or epimastigotes, suggesting that the sensitizing antigens were present in both culture forms.

In addition, no significant difference was found when the $^{51}Cr$ release from sensitized cells in the presence of complement was compared to that from sensitized cells alone (Figs 1 and 2). This indicates that these parasite antigens either cannot activate the complement bypass directly or are bound in insufficient amounts.

Normal syngeneic spleen cells in the presence of anti-*T. cruzi* serum were able to release $^{51}Cr$ from S2 cells sensitized with parasite antigens by antibody-dependent cell-mediated cytotoxicity (Fig. 3). However, the *in vivo* relevance of target cell lysis by K cells in this, as in other systems, has yet to be established.

Our finding that S2 cells sensitized with parasite-antigens were killed by T-enriched lymphocyte suspensions prepared from mice infected with *T. cruzi* for 15 days (Fig. 4a) is analogous to the published observations of Kuhn (Kuhn & Murnane, 1977) using *T. cruzi*-infected fibroblasts as target cells. The specificity of the reaction was shown by no significant increase in $^{51}Cr$ release from unsensitized S2 cells mixed with '15-day' T lymphocytes when compared with S2 cells alone (18-hr incubation period).

Parasite antigen-specific killing of sensitized S2 targets was not shown when $^{51}Cr$-labelled S2 cells were mixed with T-enriched lymphocytes from mice infected with *T. cruzi* for 60 days. S2 cells were killed to almost the same extent whether coated with parasite antigens or not (Fig. 4a). This direct cytolysis of a syngeneic muscle-derived cell line could either be due to an autoimmune reaction with 'self' tissue or a dramatic increase in natural killer cells. The latter explanation is considered unlikely in view of the observation that these '60-day' cytotoxic T lymphocytes only killed antigen-coated HeLa cells; uncoated HeLa cells gave only 'background' levels of release (Fig. 4b).

The development of autoaggressive T lymphocytes during the chronic stage of *T. cruzi* infection in mice is similar to other published findings in rabbit and human cell-mediated cytotoxicity systems (Santos-Busch & Teixeira, 1974; Teixeira & Santos-Busch, 1975; Teixeira *et al.*, 1978). It has been suggested that the development of autoaggressive T lymphocytes is due to the presence of antigens, common to the parasite and host tissue (Teixeira & Santos-Busch, 1975; Teixeira *et al.*, 1978). This seems unlikely, however, in view of the considerable time elapsing between the development of immunity to *T. cruzi* and 'self' components, 15 and 60 days respectively.

A second unexplained feature of Chagas' disease is the destruction of uninfected cells surrounding nests of parasites *in vivo*. Köberle (Köberle, 1974; Köberle & Alcântara, 1960) suggested that the parasite might produce a neurotoxin-like substance which could kill cells in the immediate vicinity of disrupted nests of parasites. We have been unable to find supportive evidence for this notion. For example, there was no increase in the spontaneous release of $^{51}Cr$ from S2 cells coated with parasite

antigen, either passively or in infected cultures (Fig. 4; Ribeiro dos Santos & Hudson, 1980), compared to the spontaneous release from S2 cells alone.

We suggest that during the acute phase of the disease parasite antigens are released following the disruption of infected host cells or due to immune lysis of parasites. From the present and previous data (Ribeiro dos Santos & Hudson, 1980) it seems likely that these parasite antigens could bind to host cells and thus render them susceptible to destruction by the host's own immune response against infection. If parasite antigen production and subsequent host cell death was sustained over a sufficiently long period then the chronic release of self components might elicit an autoimmune response.

The observation that about 60% of patients infected with *T. cruzi* are asymptomatic, in spite of the presence of anti-tissue autoantibodies (Cossio *et al.*, 1974) or self-reactive T lymphocytes (Teixeira *et al.*, 1978) suggests that a genetic predisposition (Fudenberg, 1978) might be required for the development of a self-sustaining autoimmune response which could perpetuate tissue damage.

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