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IMPROVED ENZYME-LINKED IMMUNOADSORBENT ASSAY (ELISA) FOR THE STUDY OF *TRYPANOSOMA CRUZI*-HOST CELL INTERACTION *IN VITRO*

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We herein present an improved assay for detecting the presence of Trypanosoma cruzi *in infected cultures. Using chagasic human sera (CHS), we were able to detect* T. cruzi *infection in primary cultures of both peritoneal macrophages and heart muscle cells (MHC). To avoid elevated background levels – hitherto observed in all experiments especially in those using HMC – CHS were preincubated with uninfected cells in monolayers or suspensions prior to being used for detection of* T. cruzi *in infected monolayers. Preincubation with cell suspensions gave better results than with monolayers, reducing background by up to three times and increasing sensitivity by to twenty times. In addition, the continuous fibroblastic cell line L929 was shown to be suitable for preadsorption of CHS. These results indicate that the high background levels observed in previous reports may be due to the presence of human autoantibodies that recognize surface and/or extracellular matrix components in cell monolayers. We therefore propose a modified procedure that increases the performance of the ELISA method, making it an useful tool even in cultures that would otherwise be expected to present low levels of infection or high levels of background.*

Key words: *Trypanosoma cruzi* – ELISA – infection in vitro

Studies on the *in vitro* invasion of mammalian cells by the flagellate protozoan *Trypanosoma cruzi,* the causative agent of Chagas' disease, are widely undertaken by groups seeking to identify pathways of adhesion and internalization of the parasite (Andrews & Colli, 1982; Piras et al., 1985) and/or to assess the effectiveness and mode of action of chemotherapeutic drugs (Brener, 1984). Usually, cultures of target cells are exposed to the parasite for different periods of time, washed, and then fixed immediately or maintained for longer periods to allow the development of intracellular forms of the parasite prior to fixation. To asses the nature and the levels of infection, the cultures, after appropriate staining, are subjected to microscopic examination to determine the number of parasites adherent to or inside the cells, percentage of infected cells as well as others quantitative parameters. Staining and counting are not only time consuming, but are also frequently affected by problems related to fixation or staining and to the inherent subjectiveness of counting. In addition, the number of parameters that can be tested in each experiment is strongly limited by the lengthiness of quantification procedures, since extensive microscopical examination of cultures is necessary if reliable results are to be obtained.

An Enzyme-Linked Immunosorbent Assay (ELISA) for *in vitro* evaluation of chemotherapeutic activity against *T. cruzi* has been described (De Tito & Araújo, 1988), clearly demonstrating that the number of internalized parasites in cell cultures correlates linearly with the optical density of an ELISA run in parallel. Based on the possibility to compare results obtained with both methods, these authors performed preliminar trials of chemotherapeutic drugs against *T. cruzi.*

Here we present an improved and simple procedure, in which preincubation of sera (before they are used to detect infection in monolayers) with uninfected cells decreases back-

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ground to significantly lower levels. Our modifications assure assay performance even under experimental conditions that induce a very low level of infection or a high degree of non-specific reaction with uninfected control cultures.

MATERIAL AND METHODS

Sera – Samples of normal goat sera were obtained from W. L. Immunochemicals (Rio de Janeiro, Brazil) and from Biomanguinhos (FIOCRUZ, Brazil). Human, goat and rabbit sera with positive serology for *T. cruzi* were obtained from Biomanguinhos (FIOCRUZ, Brazil).

Experimental animals – Adult male Swiss albino mice, weighing 18-20 g and bred at FIOCRUZ were used throughout this study, both as sources of normal mouse sera and for parasite maintainance.

Cells – Peritoneal macrophages (PM) were obtained from peritoneal washes of mice, as previously described (Araújo Jorge et al., 1989) and plated at a density of 5 x 10^4 cells per well in 96-well culture plates (Nunclon). The cultures of embryonic heart muscle cells (HMC) were obtained as described in detail elsewhere (Meirelles et al., 1986). Briefly, mouse embryos were dissected and the ventricular portion of the heart isolated. Then, after mincing, the tissue was dissociated with trypsin and collagenase, the dispersed cells were washed and plated either in 96-well multidishes at a density of 5 x 10^4 cells per well or in 15 cm^2 culture flasks (Nunclon) coated with gelatin. Cultures of HMC were mantained in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum (DME) at 37 °C, in a 5% CO_2 atmosphere. The L929 cell line was obtained from the American Type Culture Collection and maintained by weekly passages in DME.

Parasites – Blood trypomastigotes of *T. cruzi* Y strain were isolated from blood obtained by cardiac punction of mice at the seventh day post infection. Metacyclic trypomastigotes of the clone DM28c were obtained after metacyclogenesis in defined medium (Contreras et al., 1985).

Cell infection – Primary cultures of PM or HMC were washed three times with 0.1 M phosphate buffered saline (PBS) and a solution containing the desired numbers of parasites diluted in DME was added. Parasites and cells were left to interact for different periods of time (see results for details) after which the cultures were extensively washed with PBS to remove free swimming parasites. Infected cultures were fixed with 2% formaldehyde for 60 min, washed with PBS and processed for quantification of infection by ELISA. Experiments were run at least in triplicate.

ELISA for the evaluation of host cell infection by T. cruzi – Initially, ELISA tests were performed as described by De Tito & Araújo (1988), with minor modifications. Briefly, infected cell monolayers in 96-well culture plates were fixed and preincubated with normal mouse serum for 1 hr at room temperature to prevent non-specific or receptor-mediated binding of antibodies present in chagasic sera. Cultures were then washed with PBS containing 0.05% Tween 20 (PBS-T) and incubated with sera from chronic human chagasic patients with positive serology for *T. cruzi* (ELISA titres of 1/1280, Bio-Manguinhos, FIOCRUZ) for 2 hr at 37 °C, washed three times with PBS-T and incubated with an anti-human IgG peroxidase conjugated (W. L. Immunochemicals, Brazil) for 1 hr at 37 °C and again washed with PBS-T prior to revealing peroxidase activity (0.01% 3,5,3',5'-tetramethylbenzidine in 0.1 M citrate buffer pH 4.0 containing 50 µl of a 3% solution oh H_2O_2 for 20 min at 37 °C). Optical densities (O.D.) were read at 450 nm in a microELISA automatic reader (Titertek Plus – ICN/Flow).

In the modified procedures the human sera were preincubated either with monolayers of uninfected HMC or with suspensions of uninfected HMC or L929 cells. For preparation of cell suspensions, cells from confluent cultures were dispersed with 0.025% trypsin in PBS, washed, centrifuged at 15600 g/30 sec and the supernatant discarded. In the first protocol, sera were incubated with a confluent HMC monolayer in a 15 cm^2 culture flask for 1 hr at 37 °C, collected and centrifuged at 15600 g/5 min to remove detached cells and cell debris, and the supernatants set aside for ELISA. In the second protocol, 50 µl of sera were incubated for 30 min at 37 °C with 1 x 10^8 HMC or L929 in a conic bottom 1.5 ml centrifuge tube, centrifuged at 15600 g/30 sec, the supernatant collected, and the entire procedure repeated with another sample of dispersed cells of the same type. The preadsorbed sera were either used immediately for experimental

purposes or stored for several days (at 4 °C or frozen) prior to use. ELISA was carried out as described above. Results are expressed throughout as O.D. units at 450 nm.

RESULTS

We first tested the efficiency of using pooled chagasic human sera (CHS) in ELISA tests measuring *T. cruzi*-infection of PM and HMC cultures. As shown in Table, the sera were effective in the detection both of parasites, and of differences in infection levels in cultures exposed to increasing parasite/cell ratios. In addition, we found that the presence of gelatin, an important substrate for HMC adhesion, did not interfere with the assay, since similar values of O.D. were found for control and infected cultures in the presence or in the absence of gelatin substrate (not shown). After testing different batches of serum under dilutions ranging from 1:50 to 1:1000, we found that dilution of 1:200 was the most suitable and was used in all further experiments. Both rabbit and goat sera from *T. cruzi* infected animals were shown to be less effective than CHS in the detection of intracellular parasites.

TABLE

Infection of PM and HMC as detected by ELISA. Increasing parasite/cell ratios increases O.D. values of infected cultures

	Macrophages	
Parasites	3×10^4	9×10^4
none	1.089 ± 0.09[a]	1.322 ± 0.10
1.5×10^5	1.269 ± 0.09 (0.18)[b]	1.393 ± 0.01 (0.079)
3.0×10^5	1.274 ± 0.05 (0.19)	1.461 ± 0.09 (0.139)
7.5×10^5	1.382 ± 0.04 (0.29)	1.515 ± 0.08 (0.193)
	Heart muscle cells	
none	1.408 ± 0.030	
3×10^5	1.598 ± 0.007 (0.190)	

a: mean of triplicate readings ± 1 standard deviation.
b: number in parenthesis are the differences of the O.D. values for infected and control cultures.

The ELISA technique detected differences greater than 0.15-0.20 in O.D. readings between control and infected cultures which are described as statistically significant by De Titto & Araújo (1988). In adittion we have also noted that increases in the level of infection as monitored by microscopic counting leads to a proportional increase in O.D. values of infected cultures (not shown), confirming prior observations from De Titto & Araújo (1988). Although infection could be detected by such ELISAs, the high values of O.D. observed for non-infected control cultures (ranging from 1.0 to 1.4 D.O. units) decreased the sensitivity of the method.

Due to the high levels of non-specific backgrounds obtained with cultures of both HMC and PM (Table), different sera and/or protein rich solutions were used in order to reduce non-specific absorption of human antibodies to the cultures. Solutions of 5% bovine serum albumine (BSA) either alone or mixed with 10% normal mouse serum; 10 and 20% normal mouse serum; 10% powered milk and 10 and 20% goat sera, all these diluted in PBS, were alternatively tested as blockers. Although the background O.D. values for uninfected cultures remained high, 10% goat serum and 20% normal mice sera gave the most suitable results with both PM and HMC (not shown). Normal mouse serum was used a blocker in all the subsequent experiments.

In order to reduce the high background levels obtained in our initial assays, we have also made efforts to clear the CHS of antibodies that might recognize epitopes in HMC cultures. Sera were preincubated with non-infected HMC using two different procedures, and found to differ from non-incubated sera (Fig. 1). Preincubation of sera with HMC monolayers (Fig. 1, Experiment 1) led to a slight reduction in background O.D. values with noninfected cultures (0.456 and 0.375 for non-preadsorbed and preadsorbed sera, respectively), but did not increase the sensitivity of the assay (the difference in O.D. values between infected and control cultures using preadsorbed sera was 0.026, or roughly equivalent to the standard deviation for these readings). Preincubation of sera with HMC cell suspensions (Fig. 1, Experiment 2) on the other hand did increase the sensitivity of the system: background counts were reduced roughly threefold in control cultures, and O.D. values recorded with infected and non-infected cultures differed by 0.458. In addition, no significant differences was found when preadsorbed serum stored either at 4 °C or at –10 °C was used to detect infection in HMC monolayers (not shown).

Having obtained a significant reduction in background using preadsorbed sera, we carried out tests to establish whether or not this pro-

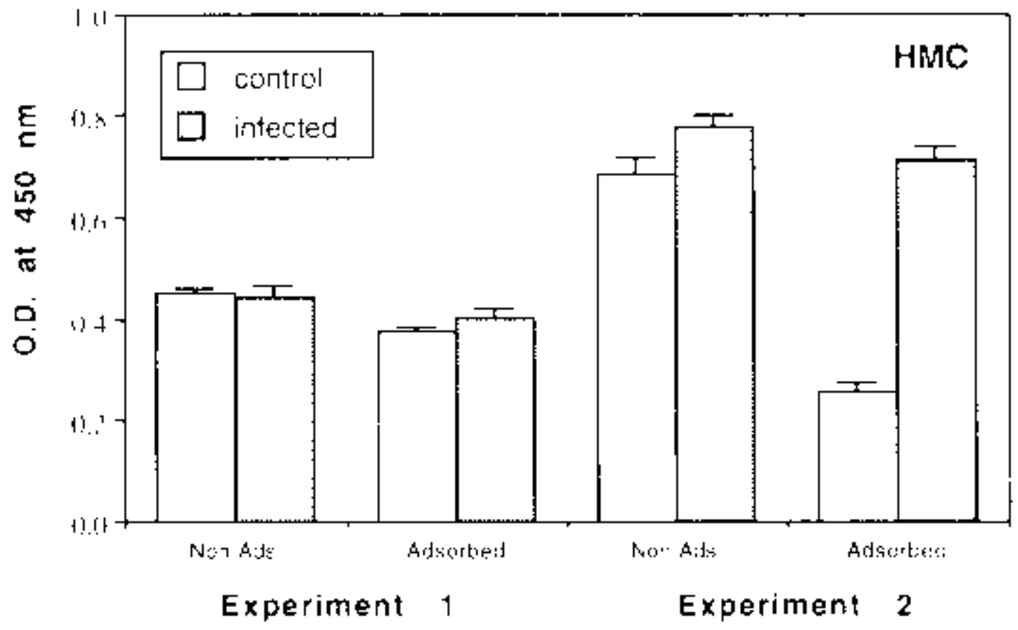

Fig. 1: effect of preadsorption of CHS on its sensitivity for the detection of *Trypanosoma cruzi* infection on HMC. HMC (5 x 10^4/well) in 96-well multidishes and exposed to parasites at a ratio of five blood trypomastigotes of *T. cruzi* Y strain per cell for 24 hr then fixed and processed for ELISA. In the Experiment 1, CHS was preadsorbed with HMC monolayers, and in Experiment 2 with HMC suspensions prior to be used in ELISA.

cedure might interfere with detection of infection using reduced parasite/host cell ratios. HMC cultures were incubated for 24 hr at 37 °C with increasing numbers of Y strain blood trypomastigotes and infection levels were evaluated. Infection in HMC cultures could be detected by the ELISA even at parasite/macrophage ratio as low as 2:1 (Fig. 2a). In a similar experiment in which PM were maintained in the presence of the parasites only for one hour (Fig. 2b), infection could be detected at an even lower parasite/host cell ratio, 0.5:1. In both cases, increasing the parasite/host cell ratio led to an accompanying increase in O.D. values (Figs 2a, 2b). Using a 10:1 parasite: cell ratio, yelds about 10-20% of infection in PM after 1 hr and 2-5% of infected HMC after 24 hr, in accordance with our previous observations (Araújo-Jorge et al., 1989; Meirelles et al., 1986), thus highlighting the increased sensitivity of the modified essay.

Attempts were made to ascertain if cell types other than primary HMC were also suitable for preadsorption of CHS, and to discover if the human chagasic sera could recognize other parasites from different sources. For these purposes, HMC cultures were incubated with metacyclic trypomastigotes from clone DM28c for 2 hr at 37 °C, with increasing parasite/host cell ratios. In half of the cultures, infection was subsequently assessed by ELISA using sera preadsorbed with suspensions of L929 fibroblastic cell line, and in the other half with sera preadsorbed with suspensions of HMC. Both sera proved to be equally effective in the detection of infection of HMC, although O.D.

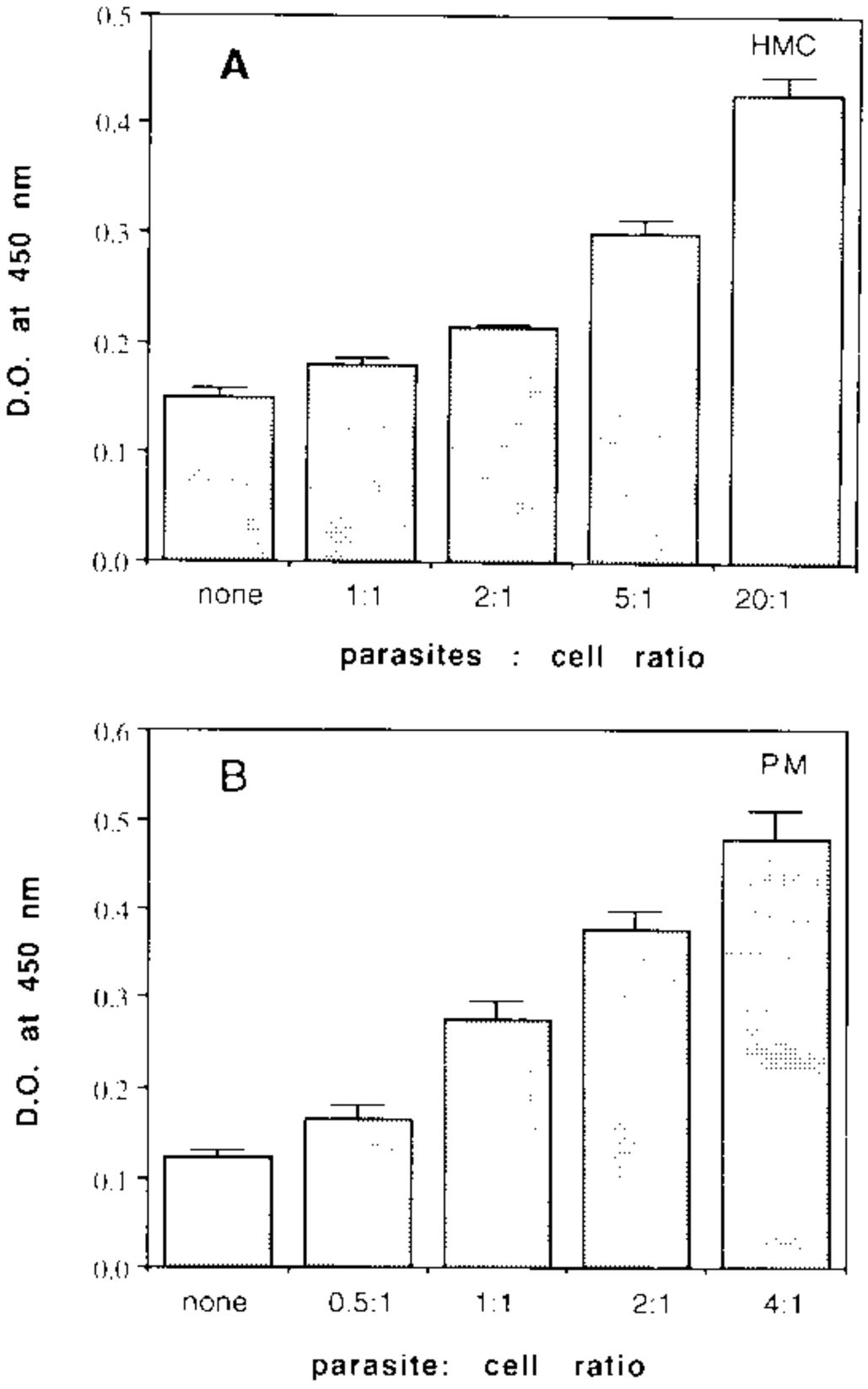

Fig. 2: detection of *Trypanosoma cruzi* infection. HMC (5 x 10^4/well) cultured in 96-well multidishes were esposed to increasing numbers of blood trypomastigotes of *T. cruzi* Y strain (A). PM (1 x 10^5/well) cultured in 96-well multidishes were treated in a similar way (B).

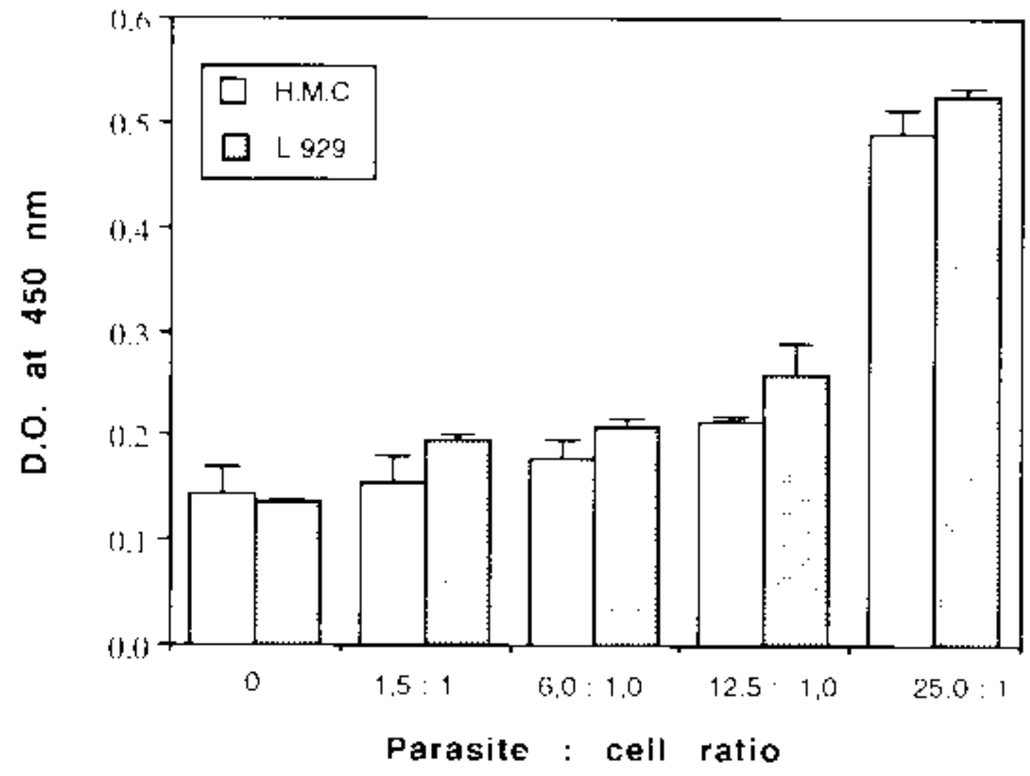

Fig. 3: effect of preadsorption of CHS either with HMC or L929 cells. Cultures of HMC (5 x 10^4/well) were exposed to increasing numbers of metacyclic trypomastigotes of Trypanosoma cruzi clone DM28c for 2 hr, fixed and processed for ELISA using CHS preadsorbed with HMC or with L929 cell suspensions.

values obtained with L929 pre-adsorbed sera were slightly higher than those observed with HMC preadsorbed sera (Fig. 3). We also noted

with considerable interest that both sera were capable of recognizing the intracellular parasites from clone DM28c even when the percentage of infected cells was in the range of 2 to 5% as determined by microscopical counting (parasite: host cell ratio of 5:1).

DISCUSSION

The interaction of the parasite *T. cruzi* with its host cell is a complex phenomenon, involving a wide variety of receptors (Araújo Jorge, 1989). Many types of molecules, such as carbohydrates (Araújo Jorge & de Souza, 1984) and glycoproteins (Ouassi et al., 1985) modulate this process either increasing or decreasing infection levels under controlled conditions. Assessing infection however, is complicated, since it is usually done by microscopic counts of the percentage of infected cells in a culture that has been exposed to the parasite and subsequently fixed. Statistically significant samples can only be obtained by counting a great number of cells for each experimental condition, thus limiting the number of variables that can be examined in each assay. The same limitations are also true for other intracellular parasites (Berman & Wyler, 1980). As a result of these problems, a variety of other procedures for evaluating infection of cultured cells by intracellular parasites have been proposed, using radioactive precursors (Cilliari et al., 1990), colorimetric reactions (Alcina & Fresno, 1987) and recently ELISAs – for at least two different parasites – *Toxoplasma gondii* (Merli et al., 1985) and *T. cruzi* (De Titto and Araujo, 1988).

The ELISA proposed by De Tito & Araujo (1988) to evaluate infection of cultured cells by *T. cruzi* has given us good results in cultures with relatively high parasite: host cells ratios (5-10:1) wether using PM or HMC (Table). It has been especially useful with HMC cultures since these are difficult to be counted microscopically, given that the membrane limit of each cell in a confluent culture is not always clear and that is common for the superimposition of cells in multilayers to occur in primary cultures of all connective tissue cells. Since they are major targets for *T. cruzi* invasion in infected animals, muscle cells provide an important tool in the study of parasite host-cell interaction (Meirelles et al., 1986; Araújo Jorge et al., 1986). However, the high background readings obtained in ELISA tests on infections of HMC have been problematical, since they sometimes mask low levels of infection (see Fig. 1 for instance). Similar problems have been reported in experiments with the fibroblastic cell line L929 (De Titto & Araujo, 1988). Initially, in order to block unspecific binding of human antibodies to HMC, we tried incubating the infected cultures with different antibodies and protein rich solutions. Although 20% normal mouse serum was shown to be the best solution for this step of the ELISA, any significant improvement in the sensitivity of the assay could be observed.

Since it is has been demonstrated that high levels of antibodies against components of the extracellular matrix (Gazzinelli et al., 1988) and muscle cell surface (Laguens et al., 1991) are found in the sera from chagasic patients, another possible explanation for the high backgrounds found in fibroblastic (De Titto & Araujo, 1988) and HMC cultures is that CHS may contain autoantibodies that recognize epitopes in plasma membranes and/or extracellular matrix of HMC.

We therefore employed two different procedures aimed at decreasing the binding of autoantibodies to HMC cultures. First we tried preadsorption of sera on homologue monolayers, and found that these approaches indeed reduce background O.D. values for infected cultures, but surprisingly did not increased the efficiency of the ELISA in detecting intracellular parasites (Fig. 1, Experiment 1). The reason for the reduction in background is not clear, it is unlikely that our procedure would lead to an uptake of large amounts of antibodies, because these cells do not present significant numbers of Fc receptors on their plasma membranes. Our second approach was to preadsorb CHS with cell suspensions of HMC. Using this method, the difference in O.D. counts between infected and control cultures was 0.458 (Fig. 1, Experiment 2), about 17 times that obtained with CHS preadsorbed with HMC monolayers. Thus not only were the backgrounds values reduced, but also the sensitivity of the ELISA was significantly enhanced. It is possible that the difference observed between the two protocols results from greater surface of contact between sera and cells in the case of suspensions or to rearrangements of cell surface components, leading to facili-

tated access of antibodies both to membrane epitopes, and to those components of the extracellular matrix that are closely associated with plasma membranes, such as fibronectin (Beppu et al., 1989) and glycosaminoglycans (Mourão et al., 1986).

A noteworthy feature of the procedure that used HMC suspensions was that it was able to detect increasing levels of infection both in PM and HMC cultures (Figs 2a, b, respectively), indicating that there was no important loss of sensitivity in the assay due to eventual uptake or degradation of large amounts of anti-*T. cruzi* antibodies during the process of preadsorption with cell monolayers.

Although reduced background was achieved in ELISAs using HMC-preadsorbed CHS, the utilization of primary cultures – wich demands long manipulations for relatively low numerical yields – remains a limiting factor. A further disadvantage would be that the production of HMC primary cultures is not a usual procedure in most laboratories working with *T. cruzi* infection *in vitro*. We therefore decided to try substituting primary cultures of HMC with another cell type, preferably for a permanent cell line that would resemble HMC in a number of important features, such as synthesis of considerable amounts of extracellular matrix and elevated rates of proliferation, with the later making it possible to obtain large numbers of cells for use in preadsorption of CHS. The fibroblastic L929 cell line was chosen since it is routinely used by many laboratories working on *T. cruzi*.

L929 gave similar results to those obtained using dispersed HMC. Both HMC and L929-preadsorbed sera were capable of detecting infection in HMC cultures that had previously been incubated with increasing numbers of metacyclic trypomastigotes from *T. cruzi* DM28c clone (Fig. 3) for a period of 2 hr. Cultures of a continuous fibroblastic cell line were therefore shown to be at least as efficient as primary cultures in increasing the efficiency with which ELISAs detect parasites in infected cells. In addition our results shown that preadsorbed CHS can be used to detect parasites from more than one source (Figs 2, 3).

The present study confirms that ELISA techniques can give satisfactory results when used to detect cell infection by *T. cruzi,* both in cultures of mononuclear cells and in primary connective tissue cultures. The major drawback of these assays has been that high background levels in control and infected cultures have hitherto made it difficult to detect infection. However, as we have shown, the sensitivity of the assays can be greatly enhanced by a simple procedure that reduces background by submitting sera to preadsorption with noninfected control cells, taken either from a continuous cell line or from primary cultures.

The ELISA technique presented here is time saving, since the entire procedure of infecting cultures and quantifying infection can be performed in 8 hr, specially if parasites samples with relatively high index of infectivity are used (such as the DM28c clone). The use of the 96 well plates for culturing cells results in reasonable economy of materials such as culture media and sera, as well as it makes possible to work with smaller numbers of parasites and cells due to decreased surface of the ELISA plates in comparison with 24 wells plates or petri dishes, where cells are routinely cultured over coverslips prior to microscopic examination. This improved assay thus should be of use for most laboratories working on *T. cruzi*-host cell interaction *in vitro*.

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