APPLICATION OF TWO TYPES OF *TRYPANOSOMA CRUZI* AMASTIGOTES OF DIFFERENT VIRULENCE TO ELISA FOR CHAGAS' DISEASE

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Abstract: *Trypanosoma cruzi* amastigotes derived from fibroblast cultures and from cell-free liquid medium were comparatively evaluated as solid-phase antigens of ELISA for Chagas' disease. Amastigotes were obtained from the high- and low-virulent clones of the Tulahuen strain and from the G-1 strain. They were sonicated and directly applied into wells of a microplate to use insoluble particles as antigens. Fifty-one sera from patients of Chagas' disease including one acute case (with Romana's sign), 10 sera from patients of cutaneous leishmaniasis including one serum of mixed infection with Chagas' disease and 10 sera from patients of toxoplasmosis including three of mixed infection with either Chagas' disease or leishmaniasis were examined. All amastigotes from different sources and from different clones or strains showed considerable correlation with each other. Slightly higher sensitivity was found in amastigotes of high-virulent clones and slightly higher specificity in those from cell-free culture. One acute case was detected as positive by these methods although it was negative by the usual ELISA using epimastigotes.

INTRODUCTION

Chagas' disease caused by *Trypanosoma cruzi* (*T. cruzi*) is still a major public health problem in South America (Moncayo-medina, 1987). Various serological diagnostic methods have been developed to detect patients and to examine the epidemiological condition (Meirvenne and Ray, 1985). The immunofluorescence antibody test with formaldehyde-fixed culture forms (epimastigotes) seems to be most sensitive and specific among those (Camargo,

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1974). However, the enzyme immunoassay has been getting popular because of its easiness and rapidity (Anthony *et al.*, 1974). Some reports indicated that the use of amastigotes as antigens gave better sensitivity than that of epimastigotes (Gam and Nevo, 1977; Araujo and Guptill, 1984; Cossio *et al.*, 1985) but this advantage was limited to the use of whole bodies of fixed amastigotes, suggesting that some important antigens were insoluble in water. Kitagawa *et al.* (1987) reported the successful use of insoluble antigens in water for enzyme immunoassay. In the present work, we attempted to use insoluble antigens from amastigotes of two clones and one strain of *T. cruzi* in enzyme immunoassay to detect patients of Chagas' disease. Furthermore, two sources of amastigotes, namely derived from fibroblast cell cultures and from cell free tube cultures (Brenner and Chiari, 1965) were comparatively examined for their usefulness as solid-phase antigens.

MATERIALS AND METHODS

Parasite

The high-virulent clone (H-23) and the low-virulent clone (L-38) were derived from the single strain Tulahuen (Kanbara *et al.*, 1987) which had been obtained from NIH, Bethesda, U.S.A. through Keio University. The strain G-1 was obtained from Prof. I. Tada, Kumamoto University Medical School, Japan as an isolate from a patient in Guatemala in 1986.

Preparation of amastigote antigen

1. Amastigotes in cell cultures

Culture forms of each clone or strain were inoculated into fibroblast cultures from ICR mouse skins and cultured in MEM with 10% newborn bovine serum at 37°C in 5% CO₂ atmosphere. Medium change was done every 2-3 days. When most of fibroblasts were infected, clusters of amastigotes appeared in overlaid medium with free trypomastigotes. They were collected and washed in saline by low-speed centrifugation at 1,200 rpm for 5 min to remove trypomastigotes. The clusters were broken by repeated passing through a needle with 23 G, and the number of amastigotes was counted on a hemocytometer and suspended in saline at concentration 10⁸/ml.

The suspensions were sonicated with 60 W for 2 min and diluted with 10 mM Tris buffer (pH 8.6) upto the concentration of 5 × 10⁸/ml. The volume of 200 µl dilution was applied to each well of a microplate and incubated at 37°C for 4 h to prepare a solid-phase antigens. The solid-phase antigens were fixed in 3% formalin and unused spaces were blocked by 1% casein.

2. Amastigotes in cell-free tube cultures

Trypomastigotes of each clone or strain developed in infected fibroblast cultures were isolated by passing through a CM-cellulose column (Kanbara and Nakabayashi, 1983), inoculated into test-tubes containing LIT media with a slight modification and incubated at 27°C. They were transformed to amastigotes within 120 h and proliferated mainly as amastigotes during several passages. The amastigotes were processed in the same manner as described above.

Sera

Fifty one patient antisera of Chagas' disease including one antiserum obtained from an acute phase patient showing Romana's sign, ten antisera from patients of cutaneous leishmaniasis including one antiserum of mixed infection with Chagas' disease and ten antisera

Figure 1 Correlation between serum titers against amastigotes of G-1 strain derived from fibroblast cultures (G-1-C) and those derived from cell-free tube cultures (G-1-T).

Figure 2 Correlation between serum titers against amastigotes of H-23 clone derived from fibroblast cultures (H-23-C) and those derived from cell-free tube cultures (H-23-T).

Figure 3 Correlation between serum titers against amastigotes of L-38 clone derived from fibroblast cultures (L-38-C) and those derived from cell-free tube cultures (L-38-C).

from toxoplasmosis patients including three of mixed infection with Chagas' disease and leishmaniasis were used enzyme-linked immunosorbent assay (ELISA) technique. Each 50 μ l of sera at 1/100 dilution was put into each well and the microtiter plates were incubated at 37°C, 1 h. After washing with PBS-Tween three times, 100 μ l of 1:4,000 dilution in 1% BSA-PBS of goat anti-human IgG conjugated with peroxidase was added and incubated at 37°C for 1 h. After washing with PBS-Tween three times, 100 μ l of substrate solution containing 0.5 mg/ml of o-phenylene diamine and 0.02% of H₂O₂ in 0.05 M citrate-phosphate buffer, pH 5.0 was added and incubated at 37°C for 30 min. The stop solution (10 μ l of 8 N H₂SO₄) was, then, added and the optical density at 492 nm was read.

Statistical analysis of differences of mean values and correlations in ELISA titer were done by t-test.

RESULTS

In the comparison of ELISA titers between the two methods of antigen preparation in G-1 strain and two clones of Tulahuen strain H-23 and L-38, there were significant correlations between two methods for antigen preparation, namely, $r=0.7953$ ($p<0.001$), 0.4338 ($p<0.01$) and 0.9956 ($p<0.001$), respectively (Figs. 1, 2 and 3). Though the significances were not found in the differences of mean values between the two methods in two strains (G-1 and H-23), a significance was found in the difference of mean value in L-38 strain ($p<0.005$) (Table 1). It means that the microtiter plate coated with amastigote antigens from tube cultures as an antigen was more reactive than with amastigote antigens from cell cultures. Analyzing the relationship between virulent and low virulent clones (H-23-C and L-38-C), there was a significant correlation between two clones, namely, $r=0.6653$, $p<0.001$ (Fig. 4). The mean value of the ELISA titers in H-23-C was higher than that of L-38-C and its difference is statistically significant ($p<0.001$) (Table 1). It is suggested that the antigenicity of virulent strain against patient antisera is more reactive than that of low virulent strain. In comparison of ELISA titers between G-1 and each Tulahuen strain, there were also significant correlations, namely, $r=0.6270$ ($p<0.001$), $r=0.8741$ ($p<0.001$) (Figs. 5, 6). The mean value of ELISA titer in G-1 strain was lower than that of virulent clone and higher than of low virulent clone (Table 1). Each difference of the mean value was statistically significant ($p<$

Table 1 The comparison of ELISA titers in different strains of amastigotes

Strains	Chagas' disease	Negative	No. of patients less than mean $\pm$ 2SD
G-1-C	0.850 $\pm$ 0.191	0.396 $\pm$ 0.098	3
H-23-C	0.942 $\pm$ 0.153	0.491 $\pm$ 0.127	5*
L-38-C	0.752 $\pm$ 0.155	0.302 $\pm$ 0.088	1*
G-1-T	0.876 $\pm$ 0.159	0.380 $\pm$ 0.084	0
H-23-T	0.904 $\pm$ 0.218	0.315 $\pm$ 0.069	0
L-38-T	0.772 $\pm$ 0.162	0.300 $\pm$ 0.094	1*

Values are presented as means $\pm$ SD.

*: No. of positive includes an acute case.

Figure 4 Correlation between serum titers against amastigotes of H-23 and L-38 clones derived from fibroblast cultures (H-23-C and L-38-C).

Figure 5 Correlation between serum titers against amastigotes of G-1 strain and H-23 clone derived from fibroblast cultures (G-1-C and H-23-C).

Figure 6 Correlation between serum titers against amastigotes of G-1 strain and L-38 clone derived from fibroblast cultures (G-1-C and L-38-C).

0.001, $p < 0.001$, respectively).

It means that the antigenicity of G-1 strain against patient antisera is less reactive than that of virulent clone and more reactive than that of low virulent clone. As an antiserum obtained from a patient in acute phase was not screened as a positive case in the ELISA system which used epimastigote as an antigen for solid-phase, we tried to analyze whether this new system can be applied to screening. Nine antisera from cutaneous leishmaniasis patients and seven antisera from toxoplasmosis patients without mixed infection with Chagas' disease was used as a negative control.

The ELISA system using G-1-T or H-23-T were satisfactory if more than mean value $\pm$ 2SD was considered to be positive. In these system an antiserum from a patient in acute phase was also evaluated to be positive.

DISCUSSION

Antigenicity of *T. cruzi* has a wide variation among developmental stages and also among isolates or strains (Nussenzweig *et al.*, 1963; Kloetzel *et al.*, 1975; Bongertz and Dvorak, 1983; Morgado *et al.*, 1985; Okanla *et al.*, 1982; Flint *et al.*, 1984; Arango and Remington, 1981). Moreover, antibodies produced in infected mammals have a big diversity according to the course of infection and to the individual difference (Grögl and Kuhn, 1985). Therefore, serological diagnostic methods depend on detection of common antigens in prepared specimens for antigens, which are mainly derived from culture forms (epimastigotes) of a certain strain. In this point, the higher sensitivity and specificity in the immunofluorescein assay is quite reasonable because it uses total antigens including insoluble antigens. Kitagawa *et al.* (1987) introduced the method to apply insoluble antigens to ELISA for quantification of some bacterias. Since some works (Gam and Neva, 1977; Arango and Guptill, 1984; Cossio *et al.*, 1985) revealed that the use of amastigote antigens was more sensitive than that of epimastigote antigens, we used insoluble antigens of amastigotes of different clones or strain which were cultured in different ways and compared their efficiency as solid-phase antigens.

The results demonstrated good correlation between amastigotes from two sources and also between different strain or clones.

The first interesting point was that amastigotes derived from tube cultures gave better specificity than those from cell cultures because of lower level of titers among negative controls. This might be due to purity of antigens and meant that amastigotes in tube cultures kept the common antigens with those in cell-cultures as far as the ELISA test used in the present work was concerned. The second point was the high-virulent clone showed better sensitivity and the G-1 strain, which was not cloned and kept medium virulence, showed middle sensitivity. This indicated that the additional antigen which were commonly detected by patients existed in high-virulent amastigotes. Some of the additional antigens in high-virulent amastigotes were probable to be recognized by an acute patient although only one case was available in the present work. Since we prepared solid-phase antigens in Japan, the fixation step which generally reduced antigenicity was essential because of long transportation at unfavorable temperature. The present work revealed that amastigotes grown in cell-free system were available and more useful than those in cell cultures. Amastigotes in cell-free media are possible to continuously propagate in devised media (Pan, 1978) and to be used

as easily as epimastigotes. Therefore, various trials to examine the suitable quantity and quality of amastigotes antigens, effects of various fixatives and the suitable blocking solutions will develop the sero-diagnosis for Chagas' disease.

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Trypanosoma cruzi から得た 2 種の Amastigote を抗原として 用いた ELISA 法の試み

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マウス線維芽細胞内で増殖した Amastigote (Am-C) および cell-free の液体培地中で増殖した Amastigote (Am-T) を固相抗原とした ELISA 法を用いて、患者血清に対する反応性の検討を行った。Amastigote は G-1 株および Tulahuen 株の強毒、弱毒クローンから得られたものを用いた。抗原として用いた Amastigote は超音波破碎した後、不可溶性部分を含んだままで、マイクロタイタープレートに固相として用いた。血清は 1 例の急性期患者を含む 51 例のシャーガス病患者血清、1 例のシャーガス病との混合感染患者血清を含む 10 例の皮膚リーシュマニア症患者血清、および 3 例のシャーガス病、もしくは皮膚リーシュマニア症との混合感染患者を含む 10 例のトキソプラズマ症患者血清を用いた。その結果、調整法の異なるそれぞれの Amastigote の間、およびクローン間、株間でもそれぞれにおいてかなり高い相関が見られた。強毒株を固相抗原として用いた ELISA において高い反応性が、また cell-free の液体培地中で増殖した Amastigote を用いた ELISA において高い特異性が見られた。通常の Epimastigote を用いた ELISA 法で陰性であった 1 例の急性期患者血清は、G-1-T, H-23-T において陽性と判定された。

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