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Monoclonal Antibodies to *Trypanosoma cruzi*: Characterization of Specific Antigens in Epimastigote Stage

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Introduction

The protozoan hemoflagellate *Trypanosoma cruzi* (*T. cruzi*), causative agent of Chagas' disease, displays a morphologically distinct form in the midgut of reduviid bugs or in either the bloodstream or the tissue of mammalian host. It is well known that there are common antigens and specific antigens among these developmental forms (Kanbara *et al.*, 1974; Kloetzel *et al.*, 1975; Fruit *et al.*, 1978; Snary and Hudson, 1979; Repka *et al.*, 1980; Snary, 1980; Araujo and Remington, 1981; Nogueira *et al.*, 1981 and 1982; Okanla *et al.*, 1982; Zingales *et al.*, 1982; Scharfstein *et al.*, 1983). Moreover, it has been demonstrated that cross-reactive antigens exist among the parasites of Trypanosomatidae family (Afchain *et al.*, 1979). However, a definite analysis of these antigens has not yet been performed except a few major antigens.

Recently, analysis of antigen by means of monoclonal antibody has been reported in several parasites such as *Schistosoma* (Norden *et al.*, 1982; Strand *et al.*, 1982; Zodda *et al.*, 1983), *Plasmodium* (Perrin *et al.*, 1980; Schofield *et al.*, 1982; Hall *et al.*, 1983), *Toxoplasma*(Handman and Remington, 1980; Johnson *et al.*, 1983), *Leishmania* (De Ibarra *et al.*, 1982; Fong and Chang, 1982; Handman and Hocking, 1982; McMahon-Pratt and David, 1982) or *Trypanosoma* (Anthony *et al.*, 1981; Lyon *et al.*, 1981; Pearson *et al.*, 1981; Snary *et al.*, 1981; Araujo *et al.*, 1982; Wood *et al.*, 1982; Alves *et al.*, 1983; Crowe *et al.*, 1983). In *T. cruzi*, stage-specific monoclonal antibodies have been produced(Snary *et al.*, 1981; Araujo *et al.*, 1982; Alves *et al.*, 1983). However, molecular weight of the antigen recognized by monoclonal antibody has been clarified about only one surface glycoprotein of epimastigotes (Snary *et al.*, 1981). In this study, we planned to produce monoclonal antibodies recognizing other specific antigens in epimastigote form and to examine the character of the corresponding antigens.

Materials and Methods

Parasites: Epimastigotes of three strains of *T. cruzi* (Tulahuen, Y and Berenice) were grown in liver-infusion tryptose (LIT) medium containing 5 % calf serum at 28°C. Trypomastigotes and amastigotes of *T. cruzi* Tulahuen strain were obtained from cultures of infected HeLa cells by means of differential centrifugation and column chromatography (Kaneda and Nagakura, 1983). HeLa cells were maintained in Eagle's minimum essential medium containing 10 % fetal calf serum at 37°C. Promastigotes of *Leishmania donovani* 1S 2 Dwyer and *Leishmania braziliensis* were also grown in LIT medium. Trypomastigotes of *Trypanosoma gambiense*

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Wellcome and *Trypanosoma rhodesiense* were recovered from the blood of infected mice. These parasites were harvested and washed three times with phosphate buffered saline (PBS) by means of centrifugation.

Preparation of monoclonal antibody: Epimastigotes of *T. cruzi* Tulahuen were fixed in 3 % formalin diluted with PBS for 30 min and washed wih PBS. Female Balb/c mice were inoculated intraperitoneally with 10^8 fixed epimastigotes and Freund's complete adjuvant. This was followed by a similar injection after 3 weeks. Three weeks later, 10^8 parasites were inoculated again without adjuvant. Three days after the last inoculation, 2×10^8 spleen cells from two immunized mice were fused with 10^8 Sp2/o mouse myeloma cells which had been cultured in Dulbecco's modified Eagle's medium containing 10 % fetal calf serum, according to the method of Köhler and Milstein (1975). Hybridomas producing the desired antibodies were screened by light-microscopic, enzyme-labeled antibody method or enzyme immunoassay (see below) and were cloned by limiting dilution. Hybridoma cells (2×10^7) were injected intraperitoneally into Balb/c mice primed with pristane. Ascites fluid was collected 10–14 days later and clarified by centrifugation.

Determination of immunogloblin isotype: The immunogloblin subclass of monoclonal antibodies secreted by hybridomas was determined by double diffusion in agar employing ten-fold concentrated culture media and sera of goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 (Nordic Immunological Laboratories).

Light-microscopic, enzyme-labeled antibody method (ELAM): Fixed parasites were smeared on slides, air-dried and stored at −20°C until use. The slides were incubated with 50 μl 3 % bovine serum albumin in PBS (BSA-PBS) to block the nonspecific binding sites. Fifteen minutes later, the fluid was removed and replaced with 50 μl sera or ascites diluted 1 : 20 in BSA-PBS or culture supernatants. Sera of mice immunized or infected with *T. cruzi* Tulahuen strain and sera of non-immunized mice were used as positive and negative controls, respectively. After 30 min incubation at room temperature (RT), the slides were washed three times with PBS, additionally with BSA-PBS and then incubated with 50 μl of 1 : 100 diluted horseradish peroxidase-labeled IgG fraction of rabbit anti-mouse IgG (Miles Yeda Ltd.) for 30 min at RT. After PBS washing, they were incubated with 0.02 % 3, 3′-diaminobenzidine : 4 HCl (DAB) and 0.01 % H_2O_2 in 50 mM Tris-HCl (pH 7.6) for 10 min at RT. Finally, they were washed, dehydrated and mounted. The intensity of immunochemical staining was semiquantitatively evaluated by microscopy.

Enzyme immunoassay (EIA): Antigens prepared by three ways were used for EIA. Formalin-fixed epimastigotes of *T. cruzi* were suspended in DW (2.5×10^8 cells/ml). Forty μl aliquots of the suspension were added to wells of microplate and dried at 4°C. This was used as whole antigen. Intact Tulahuen epimastigotes of *T. cruzi* were incubated in 10 mM phosphate buffer (pH 7.4) for 10 min at 4°C. The suspension was sonicated in ice-bath and centrifuged at 10,000 g for 30 min. The supernatant was diluted in carbonate-bicarbonate buffer (pH 9.6) and used as water-soluble antigen. The 200 μl of antigen containing 4 μg protein was added to each well of microplate and incubated overnight at 4°C. These microplates were washed with three changes of PBS containing 0.05 % Tween 20 (PBS-Tween) and incubated with 350 μl BSA-PBS for 1 hr. After BSA-PBS was removed, 200 μl of ascites or sera diluted 1 : 100 in PBS-Tween or culture supernatants diluted 1 : 10 in PBS-Tween was added to the microplates, which were incubated for 2 hr at RT and washed with PBS-Tween three times. A 200 μl amount of alkaline phosphatase-labeled goat antibody to mouse IgG (Kirkegaard and Perry Laboratories, Inc.), diluted 1 : 1,000 in PBS-Tween, was applied and incubated for 2 hr at RT, followed by three times washing. Subsequently, 200 μl of para-nitrophenyl phosphate in 10 % diethanolamine buffer (1.7 mg/ml), pH 9.8 was added. After 30 min incubation at

Table 1 Reactivity of monoclonal and polyclonal antibodies to various parasites of Trypanosomatidae in ELAM

Antigen		Antiboby					
Species	Stage	SNI	TCE 04	TCE 13	TCE 40	SIM	SIF
Trypanosoma cruzi Tulahuen	E	−	⫴	⫲	⫲	⫴	⫴
	T	−	−	−	−	⫲	⫴
	A	−	−	−	−	⫴	⫴
Trypanosoma gambiense Wellcome	T	−	−	−	−	+	⫲
Trypanosoma rhodesiense	T	−	−	−	−	+	⫲
Leishmania braziliensis	P	−	−	−	−	+	⫲
Leishmania donovani 1S 2 Dwyer	P	−	−	−	−	+	⫲

The intensity of color reaction is indicated as: −, negative; +, weakly positive; ⫲, moderately positive; ⫴, strongly positive. Abbreviations: SNI, sera of non-immunized mice; TCE 04, TCE 13 and TCE 40, monoclonal antibodies against *T. cruzi* Tulahuen epimastigotes; SIM, sera of mice immunized with *T. cruzi* Tulahuen epimastigotes; SIF, sera of mice infected with *T. cruzi* Tulahuen; E, epimastigotes; T, trypomastigotes; A, amastigotes; P, promastigotes.

RT, the reaction was stopped by the addition of 50 μl 3 N NaOH. The optical density of each well was read in spectrophotometer at 405 nm.

To examine the binding of antibodies with external membrane only of epimastigotes, cells fixed in formalin were used as surface antigen. The procedure was essentially the same as described above except for V bottom tubes used instead of flat bottom microplates, and cells were collected by centrifugation at 400 g for 10 min after each incubation.

Pronase or periodate treatment: Whole antigens for EIA method were pretreated with the following procedure. Epimastigotes were incubated with 0.1 % pronase E (Sigma Chemical Co.) for 20 min at RT or 25 mM sodium metaperiodate for 20 min at 4°C. The control cells were incubated with PBS. After washing by centrifugation, organisms were fixed in formalin and dried out on the microplate.

Immunoblotting procedure: Epimastigotes were lysed with sample buffer containing SDS without mercaptoethanol. After boiling for 3 min, lysates were analyzed by electrophoresis in 10 % polyacrylamide gels according to the method of Laemmli (1970). Protein bands were electrophoretically transferred to nitrocellulose membrane by using the method of Towbin *et al.* (1979). Electroblotting was carried out at 20 V for 15 hr in cold room. After transfer, the nitrocellulose sheet was washed in PBS-Tween and was sliced off into vertical strips. One of the strips was stained with amidoblack to detect molecular weight standards. The remaining strips were incubated with 500 μl BSA-PBS for 1 hr at RT and with 500 μl ascites or sera diluted 1 : 50 in PBS-Tween for 2 hr at RT followed by PBS-Tween washing. After blocking by BSA-PBS was repeated, they were incubated with 500 μl horseradish peroxidase-labeled rabbit IgG (anti-mouse IgG) diluted 1 : 300 in PBS-Tween for 2 hr and washed in PBS-Tween. For the color reaction, the strips were incubated for 30 min with 0.01 % DAB, 0.01 % H_2O_2 in 50 mM Tris-HCl (pH 7.6) at RT. After the final washing, the strips were dried.

Results

Specificity of monoclonal antibodies

By using ELAM, sera either of mice immunized with epimastigotes of *T. cruzi* (SIM) or of mice infected with *T. cruzi* (SIF) showed intense color reactions against all stages of *T. cruzi* (Table 1, Fig. 1). On the other hand, three monoclonal antibodies against *T. cruzi* Tulahuen epimastigotes (TCE 04,

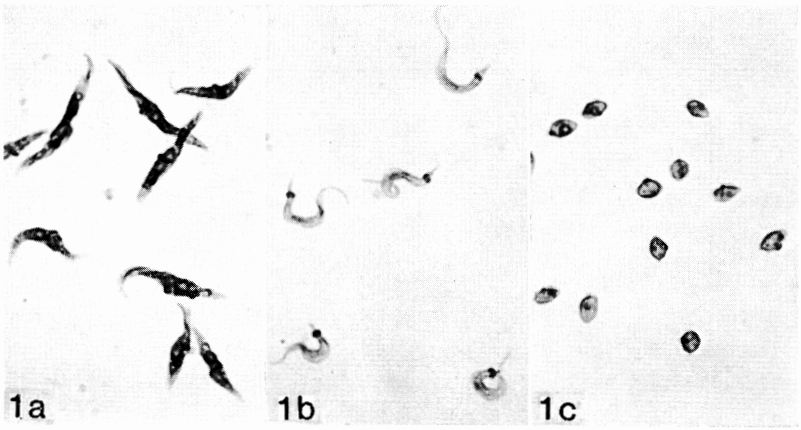

Fig. 1 Immunochemical staining of *T. cruzi* by polyclonal antibody (SIM). a, epimastigotes; b, trypomastigotes; c, amastigotes. ×1,000.

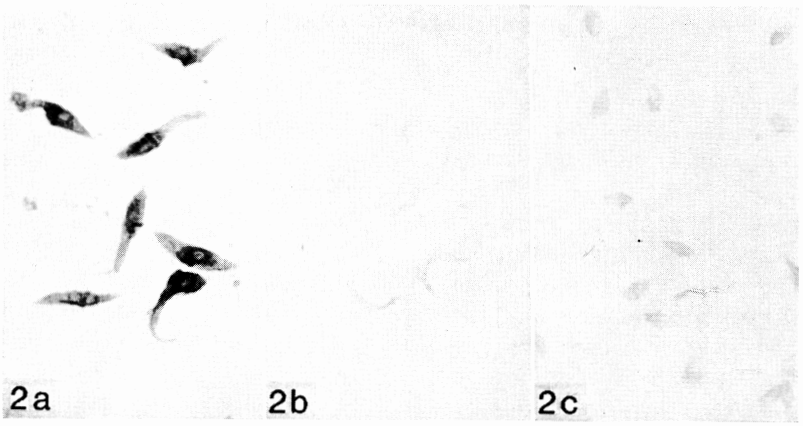

Fig. 2 Immunochemical staining of *T. cruzi* by monoclonal antibody (TCE 40). a, epimastigotes; b, trypomastigotes; c, amastigotes. ×1,000.

TCE 13 and TCE 40) were shown to be specific to the epimastigotes, but to neither the trypomastigotes nor amastigotes (Table 1, Fig. 2). Although these color reactions against epimastigotes were detected in all cells, the reaction by TCE 04 was most intensive at equivalent dilution, and a small number of epimastigotes were stained more intensely with TCE 40.

In double immunodiffusion tests with IgG subclass-specific antisera, we demonstrated that TCE 04 and TCE 13 belonged to the IgG 1 subclass, while TCE 40 was included in IgG 2b subclass (Fig. 3).

Furthermore, SIM and SIF showed cross-reaction to trypomastigotes of *Trypanosoma gambiense* and *Trypanosoma rhodesiense* or promastigotes of *Leishmania braziliensis* and *Leishmania donovani*, whereas three monoclonal antibodies never reacted to the parasites of other species (Table 1).

To determine whether these stage-specific monoclonal antibodies were specific to the strain of *T. cruzi*, reactivities of these monoclonal antibodies were compared with EIA by using whole epimastigote antigens obtained from three strains, Tulahuen, Y and Berenice. Any apparent difference was not observed in the relative reactivity of TCE 40 (Fig. 4). However, reactivities of TCE 04 and TCE 13 to Y or Berenice strain were quite different from those to Tulahuen. That

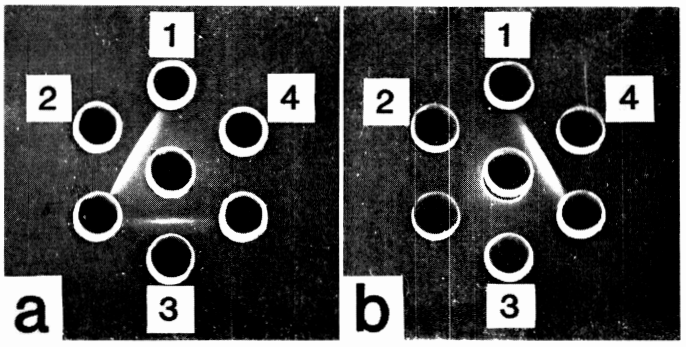

Fig. 3 Double immunodiffusion tests of monoclonal antibodies (concentrated culture media) using goat antisera against mouse IgG subclasses.
Peripheral well : 1, culture medium of myeloma cells ; 2, TCE 04 ; 3, TCE 13 ; 4, TCE 40. Center well : a, anti-mouse IgG 1 serum ; b, anti-mouse IgG 2b serum.

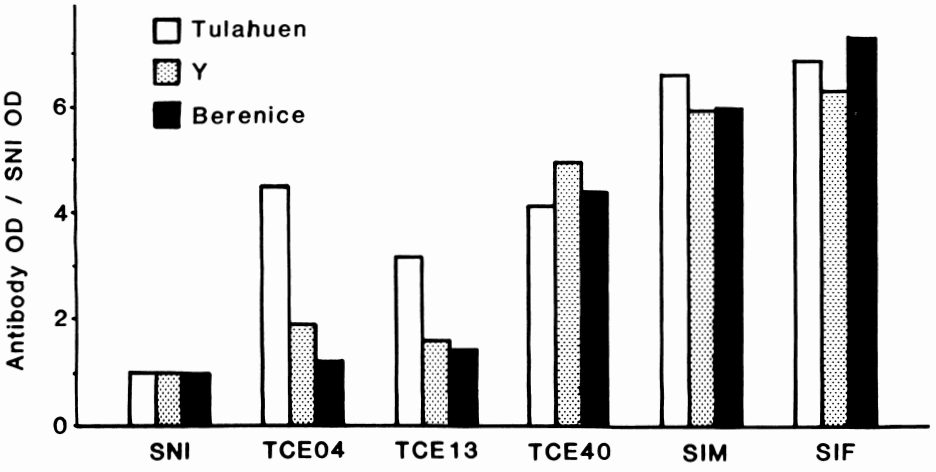

Fig. 4 Relative reactivity of monoclonal and polyclonal antibodies in EIA using whole epimastigote antigens from three strains of *T. cruzi*. Abbreviations are reffered to Table 1.

is, bindings of TCE 04 and TCE 13 to Y epimastigotes were smaller than to Tulahuen epimastigotes ; bindings of those to Berenice epimastigotes were more poor (Fig. 4).

Characterization of antigens recognized by monoclonal antibodies

In order to investigate whether antigenic determinants recognized by the monoclonal antibodies are present on the surface of the organism, we observed reactivities of the antibodies against two different antigens and effects of the antibodies on the agglutination of the parasites.

As shown in Fig. 5, the polyclonal sera, SIM and SIF showed high reactivities against both soluble antigen and cell surface antigen of the epimastigotes. In contrast, three monoclonal antibodies were strongly reactive against cell surface antigen as compared with soluble antigen (Fig. 5). Moreover, addition of three monoclonal antibodies to the suspension of live epimastigotes of *T. cruzi* caused the agglutination of cells (data was not shown).

When epimastigotes were treated with pronase E or periodate, reactivity of three mono-

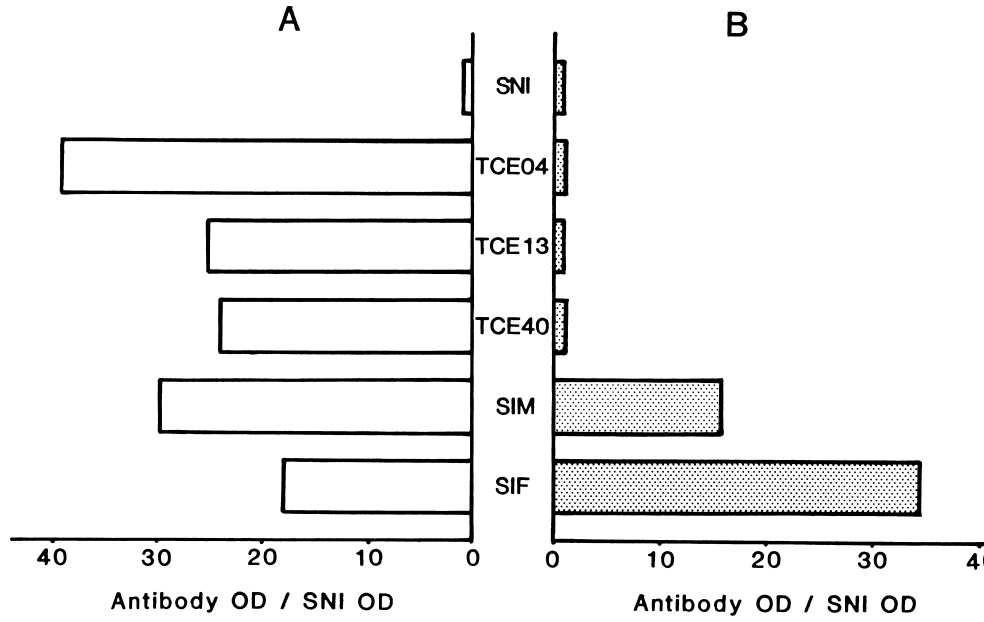

Fig. 5 Relative reactivity of monoclonal and polyclonal antibodies to *T. cruzi* epimastigotes in EIA using cell surface (A) or soluble (B) antigen.

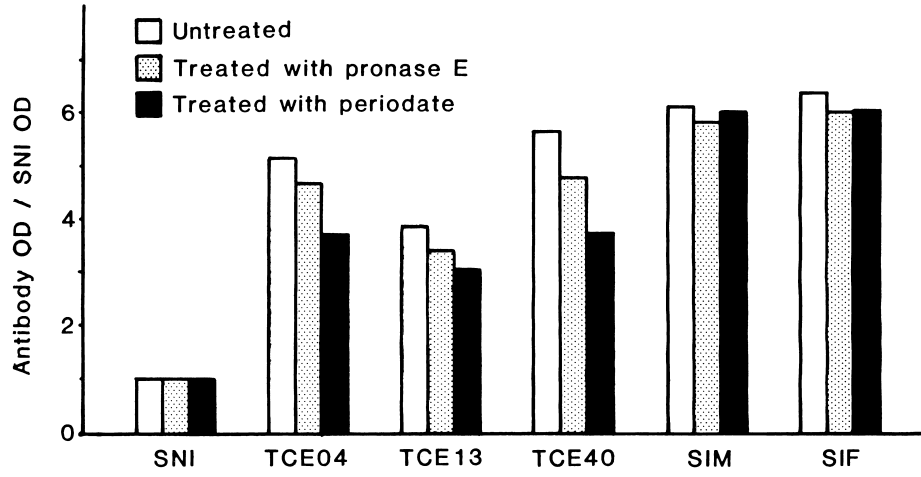

Fig. 6 Effect of pronase E or periodate treatment on the relative reactivity of monoclonal and polyclonal antibodies to the epimastigotes of *T. cruzi* in EIA using whole antigen.

clonal antibodies was reduced as compared with that of untreated epimastigotes (Fig. 6). This fact indicated that antigens corresponding to three monoclonal antibodies were composed of protein and carbohydrate.

Determination of apparent molecular weight (MW) of antigens recognized by monoclonal antibodies was carried out by immunoblotting method. Specific proteins were detected by the treatment with monoclonal antibodies, while incubation with SIM produced the pattern of many different bands (Fig. 7). TCE 04 bound to the component of MW 43,000. The binding of TCE 13 was directed against both bands MW 36,000 and 52,000, and immunoreaction of TCE 40 was

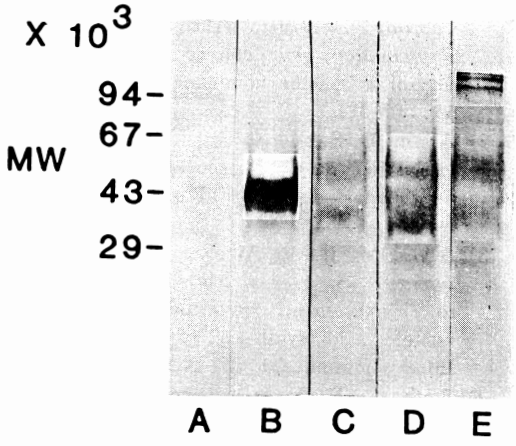

Fig. 7 Immunoblotting patterns from electrophoresis of epimastigotes of *T. cruzi*. Details of sample preparation and staining are given in Materials and Methods. The antibodies are indicated as follows : A, SNI ; B, TCE 04 ; C, TCE 13 ; D, TCE 40 ; E, SIM.

relevant to MW 32,000 and 50,000. These monoclonal antibodies did not react to the component of MW 72,000 under these experimental conditions.

Discussion

It has been reported that each developmental form of *T. cruzi* is composed of a complex series of components possessing common or stage-specific proteins. Snary and Hudson (1979) have demonstrated that the presence of the major glycoprotein (MW 90,000) on the cell surface is detected during the stages of life cycle, while Nogueira *et al.* (1981) have reported that this antigen is found in the mammalian stage only. Zingales *et al.* (1982) have also shown that antigens of MW 95,000 and 80,000 are shared by cultured epimastigotes and bloodstream trypomastigotes. More recently, it has been observed that a glycoprotein of MW 25,000 which is defined by sera of chagasic patients is represented at the parasite surface, appearing at all developmental stages (Scharfstein *et al.*, 1983). On the other hand, a major glycoprotein specific to the insect stage has been identified and apparently estimated as MW 72,000 (Snary *et al.*, 1981) or 75,000 (Nogueira *et al.*, 1981 and 1982).

Our results indicated that three monoclonal antibodies obtained were specific to cultured epimastigote form, indicating different glycoproteins of MW range between 32,000 to 52,000. Although the presence of many different surface components on *T. cruzi* has been reported, the present study produced further evidence for the presence of low molecular stage-specific antigens on the epimastigote cell surface. Thus, monoclonal antibodies are considered to make contributions to the elucidation of not only major but minor antigenic components and to the analysis of the relevant antigen characteristics.

According to Afchain *et al.* (1979), four-tenths of soluble antigens from *T. cruzi* are shared by *T. brucei* and three-tenths are shared by *L. donovani* or *L. mexicana*. The cross reactivity between *T. cruzi* and *Leishmania* species is an especially important problem of serodiagnosis in co-endemic area, when unpurified antigens are used (Camargo and Rebonato, 1969 ; Allain and Kagan, 1974 ; Anthony *et al.*, 1980). We observed that three monoclonal antibodies were specific to species, and the antigens recognized by these antibodies existed on the cell surface. Our observations in *T. cruzi* are in accord with those by other workers showing that species-specific antigenic determinants in *Leishmania* are derived from the cell surface structure (De Ibarra *et al.*, 1982).

It is well known that antigenic constitutions are different among the strains of *T. cruzi* which are morphologically indistinguishable (Nussenzweig *et al.*, 1963 ; Nussenzweig and Goble, 1966 ; González-Cappa and Kagan, 1969). The present observation indicated that the antigenic difference in strain was detected by the property of TCE 04 or TCE 13. The distinct reactivities of monoclonal antibodies, therefore, may be used effectively for the identification of strains.

Recently, Bongertz and Dvorak (1983) have shown immunoelectrophoretically that *T. cruzi* strain isolated from patients is composed of an antigenically heterogeneous population of organism. Difference in reactivity of TCE

40 against Tulahuen epimastigotes was observed in this experiment. This fact suggests that the population of Tulahuen strain used as antigens is not uniform.

It is of interest that the role of the antigens relevant to monoclonal antibodies may be established in the host-parasite relationship. Sher and Snary (1982) have demonstrated that a cell surface glycoprotein of MW 72,000, recognized by monoclonal antibody, may be a receptor controlling parasite differentiation in the vector. Further studies are in progress to examine the biological function of cell surface antigens identified by three epimastigote-specific monoclonal antibodies.

Summary

In the present study, monoclonal antibodies were produced against epimastigote form of *Trypanosona cruzi* Tulahuen strain, and their properties were examined. Three monoclonal antibodies (TCE 04, TCE 13 and TCE 40) specific to epimastigotes but not to trypomastigotes and amastigotes were demonstrated by using light-microscopic, enzyme-labeled antibody method. These monoclonal antibodies were never reacted with trypomastigotes of african trypanosoma or with promastigotes of *Leishmania* species. When epimastigotes from three different strains (Tulahuen, Y and Berenice) of *T. cruzi* were examined by enzyme immunoassay, no conspicuous differences were observed in immunoreactivity against TCE 40, but the reactivities of TCE 04 and TCE 13 to Y or Berenice were markedly lower than those to Tulahuen. Immunogloblin subclasses of a group of TCE 04 and TCE 13 and TCE 40 belonged to IgG 1 and IgG 2b, respectively. Antigens recognized by three monoclonal antibodies appeared to be glycoproteins existing on the cell surface. Apparent molecular weights of these epimastigote-specific antigens were 43,000 (TCE 04), 36,000 and 52,000 (TCE 13) and 32,000 and 50,000 (TCE 40) as judged by the immunoblotting method.

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Trypanosoma cruzi に対するモノクローナル抗体：Epimastigote 型特異抗原の解析

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細胞融合法を用いて，*Trypanosoma cruzi* Tulahuen 株の epimastigote 型虫体に対するモノクローナル抗体を作製し，それらの性質を検討した．光顕酵素抗体法によつて観察すると，3種類のモノクローナル抗体（TCE 04, TCE 13, TCE 40）は epimastigote 型に対して特異的に反応し，trypomastigote 型および amastigote 型には反応しなかつた．また，他種トリパノソーマの trypomastigote 型やリーシュマニア属の promastigote 型にも，観察した限りでは反応が見られなかつた．さらに，酵素免疫測定法を用いて，*T. cruzi* 3株（Tulahuen, Y および Berenice）の epimastigote 型虫体に対する，モノクローナル抗体の反応性を比較した．その結果，TCE 40 は3株において同様に高い反応性を示したが，TCE 04 と TCE 13 のY株，Berenice 株に対する反応性は，Tulahuen 株に対してよりも著しく低かつた．また，TCE 04 と TCE 13 の免疫グロブリンサブクラスは IgG 1 であり，TCE 40 のサブクラスは IgG 2b であつた．3種のモノクローナル抗体によつて認識されている抗原は，虫体表面に存在する糖蛋白質であると思われ，免疫ブロッティング法によると，これら epimastigote 型特異抗原のおおよその分子量は，それぞれ4.3万（TCE 04），3.6万と5.2万（TCE 13）および3.2万と5万（TCE 40）であつた．