Production and Characterization of Monoclonal Antibodies against *Trypanosoma cruzi*-Associated Antigens

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TAKASU, N., MASUKO, T., SUGAHARA, K. and HASHIMOTO, Y. *Production and Characterization of Monoclonal Antibodies against Trypanosoma cruzi-Associated Antigens*. Tohoku J. Exp. Med., 1989, **159** (4), 313-321 — Nine monoclonal antibodies (mAb) recognizing characteristic antigens in *Trypanosoma* (*T.*) *cruzi* were obtained from hybridomas which had been established by a fusion between mouse myeloma cells and the spleen cells of a mouse immune to the epimastigote form of *T. cruzi* (Tulahuen strain). Antigen specificities of these mAb were assessed by an indirect immunofluorescence (IIF) method using *T. cruzi* in different life cycles (amastigote and trypomastigote) and other members of the Trypanosomatidae. The mAb were classified into 3 groups from their reaction patterns to different parasites: 1) The strain specific mAb that reacted only with *T. cruzi* Tulahuen epimastigote, 2) the species specific mAb that reacted with all *T. cruzi* strains but not with other species of parasites, and 3) the mAb that were cross-reactive with other species of Trypanosomatidae. Most mAb were specific to epimastigote form of *T. cruzi*, but some reacted weakly with trypomastigote and amastigote form of the parasites. Immunoblotting and glycolipid analyses of the membrane fraction of homogenized parasites using the mAb identified at least 3 distinct antigenic molecules; those of protein nature having Mr. 43,000 and 58,000 and Mr. 43,000 and 62,000 and molecule(s) of glycolipid nature. ———
Trypanosomas: *Trypanosoma* antigens; monoclonal antibodies

Chagas' disease caused by *Trypanosoma cruzi* (*T. cruzi*) infection arises a serious problem in Latin America. Ten to twenty million people are believed infected and present chemotherapy is unsatisfactory (Gutteridge 1985). *T. cruzi* has a complex life cycle and exists in at least four distinct morphological forms: Epimastigotes and an infective form of metacyclic trypomastigotes are found as the insect vector, and trypomastigotes are present in the blood stream of vertebrate hosts, while amastigotes live as intracellular parasites (Brenner 1973). The evidence for antigenic differences between the different stages of the parasite life-cycle as well as antigenic differences between strains and cloned stocks of *T. cruzi* have been reported (Araujo and Remington 1981; Bongertz and Dvorak 1983; Chapman et al. 1984).

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A major cell-surface antigen detected by sera from patients with Chagas' disease is a 90 kDa glycoprotein (Nogueira et al. 1981; Snary 1983). This glycoprotein has been found on epimastigotes, amastigotes and blood trypomastigotes. A 72 kDa glycoprotein specific for the insect stages of the life cycle of *T. cruzi* has been identified by cell-surface labelling and mAb immunoprecipitation studies using anti-parasite antibodies (Nogueira et al. 1981; Sher et al. 1983). A 25 kDa glycoprotein has been isolated from soluble extracts of epimastigotes. This glycoprotein, which is also present in trypomastigotes, reacts strongly with sera from infected animals, and, despite being isolated as a soluble component, is associated with the surface membrane of the parasite (Scharfstein et al. 1983).

Although a variety of monoclonal antibodies have been produced against this parasite, the biochemical properties or biological significance of the antigens are yet unclear, as compared with antigens of mammalian cells. In order to understand the nature of *T. cruzi*-associated antigens and to undertake diagnostic or therapeutic applications, we have tried to produce monoclonal antibodies specific to this parasite.

MATERIALS AND METHODS

Parasites

The Tulahuen, Y, and Berenice strains of *T. cruzi*, *T. gambiense*, *Leishmania donovani* and *Leishmania braziliensis* used in this study were donated by Dr. Y. Kaneda, the Department of Parasitology, School of Medicine, Tokai University, Kanagawa, Japan.

The epimastigote form of *T. cruzi* and promastigote form of *Leishmania* were grown in liver infusion tryptose (LIT) medium containing 5% calf serum at 28°C. Trypomastigotes and amastigotes of *T. cruzi* Tulahuen were obtained from cultures of HeLa cells infected with the parasites. Trypomastigotes of *T. gambiense* were obtained from the peripheral blood of infected mice, and washed 3 times with PBS after treatment with (0.83%) ammonium chloride solution.

P3x63Ag8.653 mouse myeloma cells were grown in RPMI 1640 medium containing 10% FCS at 37°C, and used for cell fusion.

Immunization of mice

T. cruzi epimastigotes (5×10^7 cells) were washed 4 times with PBS and suspended in 1 ml PBS. After killing by freezing in liquid nitrogen, the parasites were mixed with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI, USA) for the first immunization or Freund's incomplete adjuvant (Difco Laboratories) for the booster injections. BALB/c mice of 6 weeks of age were immunized first by an s.c. injection and then received two booster i.p. injections of the parasite preparation. The final booster immunization was performed by i.p. and i.v. injections of the parasites without adjuvant.

Preparation of hybridomas

Hybridomas were prepared according to the method of Masuko (Masuko et al. 1985). In brief, three days after the final immunization, spleen cells of an immunized mouse were fused with P3x63Ag8.653 mouse myeloma cells in the presence of polyethylene glycol 1540. The cells were then suspended in RPMI 1640 medium containing 10% FCS and distributed into Falcon 96-well plates. One day after the fusion, hybridomas were selected by culture of the cells in medium containing hypoxanthine, aminopterin and thymidine (HAT medium).

Reactivity of antibodies in culture of hybridomas was checked by the enzyme-linked immunosorbent assay (ELISA) and the indirect immunofluorescence assay (IIF). Selected hybridomas were cloned by the limiting dilution method using mouse thymocytes as the feeder cells. Cloned hybridoma cells were injected i.p. into syngeneic mice which had been treated with pristane (Aldrich Chemical Co., Milwaukee, WI, USA).

IIF

IIF was performed by means of a micro-immunofluorescence assay developed in our laboratory. (Detail for the method is reported in an accompanied paper; Takasu et al. 1989). *T. cruzi* and others parasites were washed twice with PBS and suspended in PBS at a concentration of 5×10^5 parasites/ml. Aliquots ($10 \mu\text{l}$) of the suspension were distributed in wells of a Nunc plate (Terasaki's plate) and centrifuged at $1,800 \times g$ for 10 min. After removing the supernatants, the parasites in wells were air-dried, fixed with cold acetone-methanol (1:1) and treated with mAb (hybridoma culture supernatants) for 30 min at 37°C , washed with PBS for 3 times, and then treated with fluoresceineisothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulins. After 3-washings with PBS, the samples were observed under an Olympus fluorescence microscope.

Isolation of parasite membranes

T. cruzi epimastigotes were collected from culture by centrifugation at $750 \times g$ for 10 min at 4°C and washed 4 times with PBS. PBS containing 1mM phenylmethylsulfonyl fluoride was added to the cell pellet and the suspension was sonicated for 30 min. After centrifugation at $500 \times g$ for 15 min at 4°C , the supernatants were further centrifuged at $105,000 \times g$ for 60 min. The resultant precipitates were used as a crude membrane fraction.

ELISA

Aliquots ($50 \mu\text{l}$) of a suspension of parasite membranes in PBS (equivalent to 10^6 parasites) were added to wells of polyvinyl chloride plates and incubated overnight at 4°C to permit the passive adsorption of the antigen to the plate. The plates were washed once with PBS and blocked with each $100 \mu\text{l}$ of PBS containing 1% bovine serum albumin (BSA) for 1 hr. Following incubation with $50 \mu\text{l}$ of hybridoma culture supernatants for 1 hr, the plates were washed 3 times with PBS, and then $50 \mu\text{l}$ of 1:200 diluted rabbit anti-mouse immunoglobulins (Dako, Glostrup, Denmark) in PBS containing 1% BSA was added to each well. After 1-hr incubation and 3-washings with PBS, $50 \mu\text{l}$ of 1:1,000 diluted horseradish peroxidase-conjugated protein A (HRP) (Zymed Laboratory Inc., San Francisco, CA, USA) was added to wells and incubated for 1 hr. After washing, residual HRP in wells was assayed by developing the color with $100 \mu\text{l}$ of 0.05% 2,2'-azinobis (3-ethylbenzthiazoline sulfonic acid) (ABTS) and 0.01% hydrogen peroxide and measuring absorbance at 414 nm using an InterMed NJ-2000 Immunoreader.

Extraction of glycolipids from *T. cruzi* epimastigotes

Epimastigotes (3×10^8 cells) were collected from a liver infusion tryptose (LIT) culture and washed three times with saline. Glycolipids in the parasites were extracted by treating them ultrasonically in 10 ml of chloroform-methanol (2:1) (Saito and Hakomori 1971). The resulting extracts were used as antigens for ELISA, where the extracts corresponding to 10^7 parasites were distributed to each well of an ELISA plate and assayed an amount of antigen recognized by mAb.

Immunoblotting

T. cruzi epimastigote membranes were solubilized in sample buffer containing 2% sodium dodecyl sulfate (SDS) and subjected to polyacrylamide-gel electrophoresis (PAGE) in slab gels of 1.5 mm thick according to the method of Laemmli (1970). Separated proteins were transferred to nitrocellulose paper according the method of Towbin (Towbin et al.

1979), and assayed by ELISA for the immunoreactivity against anti-parasite mAb.

RESULTS

Hybridomas

Following screening of culture supernatants by IIF and ELISA, it was confirmed that many hybridomas produced antibodies against *T. cruzi* epimastigotes. Nine hybridoma cultures were selected for their immunoreactivities to trypomastigotes and amastigotes of *T. cruzi* Tulahuen, Y and Berenice strains and cloned by the limiting dilution method. Monoclonal antibodies produced by these nine cloned hybridomas were named ACE-1, -2, -3, -4, -5, -6, -7, -8 and -9. Immunoglobulin isotype of ACE-1, -2, -3 and -5 was of IgM and that of ACE-4, -6, -7, -8 and -9 was of IgG1, and all contained k light chains.

Antigen specificity of mAb

The antigen specificity of mAb was analyzed by IIF using the acetone-fixed *T. cruzi* epimastigotes, amastigotes, and trypomastigotes as target cells. The results are summarized in Table 1. All showed positive reaction against epimastigotes. ACE-3, -4, -5 and -6 also reacted with amastigotes, and ACE-7 and -9 with trypomastigotes. Reactivity of the mAb with amastigotes and trypomastigotes was weak, as compared with their reactivity with epimastigotes.

Table 1 also shows the reactivity of the mAb with the epimastigote form of *T. cruzi* Y and Berenice strains, other members of the Trypanosomatidae such as *T. gambiense*, *L. donovani* and *L. brazilienses*, and HeLa cells (human cervix carcinoma). ACE-1, -2, -3 reacted only with *T. cruzi* Tulahuen among the parasites tested but ACE-2 also reacted strongly with HeLa cells. ACE-4, -5 and

TABLE 1. Reactivity of anti-*T. cruzi* mAb against various parasites and HeLa cells

Parasites	mAb (ACE-)								
	1	2	3	4	5	6	7	8	9
<i>T. cruzi</i> Tulahuen									
Epimastigote	+	+	+	+	+	+	+	+	+
Trypomastigote	-	-	-	-	-	-	+	-	+
Amastigote	-	-	+	+	+	+	-	-	-
<i>T. cruzi</i> Berenice	-	-	-	-	-	-	+	+	+
<i>T. cruzi</i> Y	-	-	-	+	+	+	+	-	+
<i>T. gambiense</i>	-	-	-	-	-	-	-	+	+
<i>L. braziliense</i>	-	-	-	-	-	-	-	-	-
<i>L. donovani</i>	-	-	-	-	-	-	-	-	-
HeLa (human)	-	+	-	-	-	-	-	-	-

Acetone-fixed parasites or HeLa cells were analyzed for their reactivity with mAb by IIF as described in Materials and Methods.

Fig. 1. Immunofluorescence pattern of acetone-fixed parasites stained with anti-*T. cruzi* mAb. Acetone-fixed parasites were stained with mAb and FITC-labeled rabbit anti-mouse immunoglobulins. A-C, epimastigote form of *T. cruzi*. A, ACE-8 mAb; B, ACE-9 mAb; C, ACE-3 mAb. D, Amastigote form of *T. cruzi* in HeLa cells stained with ACE-4 mAb.

-6 reacted with *T. cruzi* Y strain, ACE-8 with Berenice strain, and ACE-7, and -9 with both *T. cruzi* Y and Berenice strains. The IIF staining in an epimastigote form of parasites fixed in cold acetone revealed bright stain over the entire organism (ACE-2, -6, -7, and -8) or bright stain of superior part of the parasites (ACE-1, -4, -5 and -9). ACE-3 stained partially the superior and flagellum parts of the parasites (Fig. 1). ACE-4 also stained an amastigote form of *T. cruzi* resided in HeLa cells.

The reactivity of living parasites was positive for ACE-2 and ACE-5, where the parasites agglutinated with the mAb and were immunofluorescently stained as clots (data not shown).

Biochemical characterization of T. cruzi-associated antigens defined by mAb

To assess biochemical characters of antigens recognized by mAb, reactivities of the membrane fractions of parasites with mAb were assayed by ELISA. As

TABLE 2. Reactivity of anti-*T. cruzi* mAb against *T. cruzi* epimastigote membranes, by ELISA

Absorbance (at 414 nm) with mAb (ACE-)								
1	2	3	4	5	6	7	8	9
0.99	0.99	0.54	0.92	1.04	1.20	1.76	0.64	1.04

shown in Table 2, all mAb reacted with the membrane fractions. We then assessed the molecular weight of the mAb-defined antigens by SDS-PAGE and Western blotting. The results are depicted in Fig. 2. ACE-8 detected the components of apparent molecular weights of 43,000 and 58,000 from *T. cruzi* Tulahuen epimastigote, but it did not react with the components of *T. cruzi* Y. ACE-9 detected the components of molecular weights of about 43,000 and 62,000 from both *T. cruzi* Tulahuen and *T. cruzi* Y.

Reactivity of the nine mAb with epimastigote glycolipids are shown in Fig. 3. ACE-1, -4, -5, -7 and -9 reacted with the glycolipids fraction extracted from a epimastigote form of *T. cruzi* but other mAb did not react with the glycolipids.

Fig. 2. Molecular weights of the mAb-defined antigens determined by SDS-PAGE and Western blotting. Molecular weights of standard materials are shown as K, kilo dalton. A, ACE-8 mAb; B, ACE-9; T, *T. cruzi* Tulahuen; Y, *T. cruzi* Y.

Fig. 3. Reactivity of mAb with a glycolipid fraction extracted from epimastigote form of *T. cruzi*. Glycolipid fractions were extracted from the parasites and their reactivity with anti-*T. cruzi* mAb was assayed by ELISA.

DISCUSSION

In this work, we have produced nine mAb against the epimastigote form of *T. cruzi* Tulahuen and characterized the mAb-defined antigens present in *T. cruzi* and the related parasites. Based on the reactivity with various parasites, the mAb are classified to 3 groups; 1) specific to the epimastigote form of *T. cruzi* Tulahuen (ACE-1, -2 and -3); 2) selective to parasites of *T. cruzi* species (ACE-4, -5, -6 and -7); and 3) cross-reactive with other species of parasites such as *T. gambiense* (ACE-8 and -9).

As for the biochemical character of parasite antigens defined by the mAb, two mAb definitely reacted with glycoprotein antigens having Mr. of 43,000 and 62,000 (ACE-9), and Mr. 43,000 and 58,000 (ACE-8). An epitope recognized by ACE-9 mAb appeared to be present on a sugar(s), because this mAb reacted not only with the glycoprotein antigens but also reacted with glycolipid(s) extracted from *T. cruzi*.

MAb to *T. cruzi*-associated antigens have also been prepared in other laboratories and molecular weight of the antigens recognized by these mAb are reported; 75, 82 and 90 kDa antigens present in metacyclic trypomastigotes (Teixeira and Yoshida 1986); 85 kDa antigen present in trypomastigotes (Alves et al. 1986); 40 kDa antigen (Segura et al. 1986), 43 kDa, 36/52 kDa and 32/50 kDa antigens (Tachibana and Kaneda 1984), and 72 kDa antigen (Sher et al. 1983) present in epimastigotes; 25 kDa antigen (Scharsfstein et al. 1983), 24 kDa, 43 kDa and 51 kDa antigens (Orozco et al. 1984), 58/60 and 68 kDa antigens (Manas et al. 1986) and 85 and 90 kDa antigens (Segura et al. 1986) present in multiple forms of *T. cruzi*. Considering from the molecular weight of antigens, the mAb from other laboratories and several of our mAb may define the same or similar parasite

antigens. However, different panels of parasites have been used in the assays for antigens, except the experimental system of, Tachibana and Kaneda (1984). Therefore, it is unclear yet whether our and other mAb recognize the same antigens or not.

In contrast to studies on protein antigens of *T. cruzi*, the informations about glycolipid structure of this parasite are extremely scarce. We examined the reactivities of mAb against the glycolipid fraction extracted from the parasites, since mAb reactive to glycolipids components of *T. cruzi* have not been reported. We found that ACE-1, -4, -5, -7 and -9 reacted with the glycolipid fraction from *T. cruzi*.

As for the subcellular localization of the mAb-defined antigens, the antigens mainly distributed in intracellular components of parasites (see Fig. 1). However, ACE-2 and -5 appeared to recognize also the cell-surface antigens of *T. cruzi* because they could agglutinate unfixed living parasites and showed cytotoxicity to *T. cruzi* in the presence of rabbit complement (data not shown).

MAB prepared in this work will be useful for classification of trypanosomas.

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