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A Monoclonal Antibody Against Blood Forms of *Trypanosoma cruzi* Lyses the Parasite In Vitro and Inhibits Host Cell Invasion

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ABSTRACT

Monoclonal antibodies (mAbs) of the IgM isotypes were produced from mice immunized with blood forms of *Trypansoma cruzi* Y strain. Characterization of the epitope recognized by one of the mAbs, 164C11, as well as the effects of this mAb on complement-mediated lysis and host cell invasion are reported. Immunocytochemical analysis showed that the mAb was reactive with various strains of *T. cruzi* (Y, WSL, and Colombiana) as well as other trypanosomatids. The mAb 164C11 demonstrated a high complement-mediated lytic activity against bloodstream trypomastigotes, being more effective than chronic mouse serum. A protein with an apparent molecular weight of 72 kDa was detected by this mAb on all developmental stages of *T.*

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cruzi. Studies using periodate and endoglycosidase treatments suggested that the epitope is not a carbohydrate and seems to be located on the parasite membrane. In addition, preliminary results are presented, suggesting that the 72-kDa protein is involved in adhesion/or internalization of bloodstream trypomastigotes.

Index Entries: *Trypanosoma cruzi;* monoclonal antibodies; epitope characterization; lytic activity; host cell invasion.

INTRODUCTION

Trypanosoma cruzi, the causative agent of Chagas' disease, infects 20 million people in Central and South America (*1*), and evokes both cellular and humoral immune responses in its host. It is well established that *T. cruzi* has a complex antigenic constitution, yet there are many common antigens among Trypanosomatidae (*2*).

Both humoral and cellular immunologic responses have been analyzed in experimental infections in an attempt to identify antigens relevant to protective immunity (*3,4*). These studies have been extended by the use of hybridoma technology (*5*) to produce monoclonal antibodies (mAbs) against different developmental stages and fractions of *T. cruzi* (*6–10*).

The present work reports the establishment and preliminary characterization of murine hybridoma cell lines that secrete mAbs against bloodstream trypomastigote antigens of *T. cruzi*. The epitope specificities of one of these mAbs were characterized, and the lytic properties to trypomastigotes from cell culture and bloodstream were evaluated. In addition, it was found that this mAb inhibits parasite infectivity and may be implicated in the host cell recognition by *T. cruzi*.

MATERIALS AND METHODS

Parasites

Epimastigotes of three strains of *T. cruzi*, Y, WSL, and Colombiana, classified as type I, II, and III, respectively (*11–13*), were grown in diphasic Maekelt medium (*14*). Trypomastigotes and amastigotes of *T. cruzi* Y strain were obtained from cultures of infected Vero cells (*15*). Y strain blood trypomastigotes were obtained by histopaque centrifugation as previously described by Gomes et al. (*16*). Promastigotes of *Leishmania amazonensis* (IFA/BR/67/PH8) and *Leishmania chagasi* (MHOM/BR/74/PP75) were grown in RPMI 1640 medium containing 2 m*M* L-glutamine, 15% FBS, and antibiotics (penicillin 100 U/mL) and streptomycin (100 μg/mL) at 26°C.

Immunization of Mice

Y strain bloodstream trypomastigotes were used to immunize three female 1-mo-old Balb/c mice. The inoculations were performed 20 d apart on three occasions subcutaneously with 1×10^6 freeze-thawed/sonicated parasites. The first injection was performed with complete Freund's adjuvant, the second and third with incomplete Freund's adjuvant. Mice were boosted intraperitoneally 4 d before cell fusion.

Production of Hybridomas

Spleen cells from trypanosome immunized mice were mixed with immunoglobulin-nonsecreting, 8-azaguanine-resistant Sp2/0 myeloma cells (*5,17*). These cells were fused with 50% polyethylene glycol following the technique described by Galfre et al. (*18*). Hybridomas producing the desired antibodies were screened by ELISA (enzyme linked immunosorbent assay) (*19*) and were cloned by limiting dilution. Hybridoma cells were injected intraperitoneally into pristane-primed Balb/c mice. Ascites fluid was collected 10–14 d later and clarified by centrifugation.

Monoclonal Antibody Characterization

ELISA

Freeze-thawed and sonicated bloodstream trypomastigotes were used as antigens for ELISA according to Voller (*19*).

ELIA

The antibody isotype was determined by enzyme labeled anti-isotype assay (ELIA) essentially as in Gomes et al. (*20*).

ELAM

The enzyme-labeled antibody method (ELAM) was performed according to Tachibana and Kaneda (*7*). As a specificity control, Vero and macrophage (Mϕ) cells were used.

Lysis Assay

Bloodstream trypomastigotes or cell-culture derived trypomastigotes were suspended in 0.1*M* PBS, pH 7.3 to a cell concentration of 3×10^6 cell/mL. The complement mediated lysis was carried out according to Krettli et al. (*21*).

Host Cell Invasion Assay

Growth of Vero and Mϕ cells were performed according to Andrews and Colli (*15*). Y strain bloodstream trypomastigotes, were preincubated for 1 h at RT with 164C11 mAb supernatant. As a control, trypomastigotes preincubated with supernatant of Sp2/O cell culture were used.

After the preincubation period and washing, the parasites were suspended in DME (Dubbecco's modified Eagle's medium) containing 5% fetal calf serum and added to the cell monolayer at a parasite:cell ratio of 3:1. The preparations were then incubated for 6 h at 37°C, 5% CO_2. The monolayers were washed with DME to remove the remaining parasite and incubated further for 24, 48, and 72 h. After the incubation the cells were washed with PBS, fixed, and stained in Giemsa. The percentage of infected cells and the number of parasites/cells was evaluated by randomly counting 100 cells at x1000 magnification. The Student's *t*-test was used in the statistical analysis. In a parallel experiment, the cells were incubated with the 164C11 mAb and infected with the parasites incubated with supernatants of Sp2/O cell cultures.

Antigen Characterization

Western Blot

Parasites in the presence of 1 m*M* phenylmethylsulfonyl fluoride (PMSF) and 0.1 m*M* disodium ethylenediamine tetraacetic acid (EDTA) were solubilized with sample buffer (0.0625*M*, Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, β-mercaptoethanol, and 0.001 bromophenol blue). After boiling for 3 min, lysates were analyzed by electrophoresis in 10% polyacrylamide gels according to the method of Laemmli (22). The proteins were then blotted onto nitrocellulose sheets-NTC (0.45 μ pore size; Bio-Rad, Richmond, CA) and the immunoassay performed essentially as described by Towbin et al. (23). The strips were incubated overnight at room temperature with 164C11 ascites, immune mouse sera, or with sera of nonimmunized mice, each diluted 1:100, and 164C11 culture supernatants, that were used neat. After incubation with primary antibody and washing, the blot was then incubated with horseradish peroxidase-labeled IgM sheep (antimouse IgM, μ chain specific, Sigma, St. Louis, MO). For the color reaction, the strips were incubated with 0.01% DAB, 0.01% H_2O_2 in 50 m*M* Tris-HCl (pH 7.6) at RT.

Deglycosylation Experiments

Two kinds of periodate treatment were carried out. In the first treatment, epimastigote lysates were separated by SDS-PAGE, blotted to nitrocellulose (NTC) and then treated as described by Woodward et al. (24). The second periodate treatment was performed according to Maizels et al. (25). Epimastigotes were treated with sodium periodate and submitted to Western blot analysis.

Glycosidase treatment of antigens was accomplished by treating aliquots with *N*-glycanase (Genzyme, Boston, MA), according to the instructions of the supplier, followed by Western blot analysis.

Isolation of Parasite Membranes

Epimastigotes Y of strain *T. cruzi* suspended in 0.1*M* PBS, pH 7.4, 0.1 m*M* EDTA, and 1 m*M* PMSF were ruptured by sonication and centrifuged

at 30,000*g* for 60 min. The pellet (insoluble protein), and the supernatant (soluble proteins) were analyzed by Western blot analysis. Protein concentration was determined by the method of Lowry et al. (*26*).

RESULTS

Selection of Hybrid Cell Lines and Isotype Characterization

One fusion was performed with Sp2/O myeloma cells and spleen cells from mice immunized with bloodstream Y strain *T. cruzi*. The efficiency of the fusion was 100%. Antitrypanosomal activity was detected by ELISA in 3% of the wells (B9, C11, and D5) containing hybrid cells. Clones were selected by limiting dilution and 13 cell lines were established. Analysis of immunoglobulin isotypes by ELIA showed that all the mAbs were IgM. The mAb 164C11 showed strong reactivity to bloodstream trypomastigotes by ELISA and was selected for this study.

Specificity of the Monoclonal Antibody

The specificity of the mAb 164C11 evaluated by the ELAM method showed the presence of common antigens on the amastigotes, epimastigotes, bloodstream, and cell-culture derived Y strain *T. cruzi* trypomastigotes (Fig. 1). Reactivity was observed on specific body areas of the amastigotes, epimastigotes, and trypomastigotes. The mAb was also found to react with other strains (WSL and Colombiana) of *T. cruzi* and other trypanosomatids (*L. amazonensis* and *L. chagasi*). Vero and Mϕ cells are not reactive with the mAb (data not shown).

Western Blot Analysis

The mAb recognized a 72 kDa protein on the amastigotes, epimastigotes, bloodstream, and cell-culture derived Y strain trypomastigotes of *T. cruzi* (Fig. 2A). The difference in the intensity of the recognized bands is probably owing to the relative amounts of antigens present in different stages of the parasite as also suggested by Fig. 1. In addition, 80- and 110-kDa bands were recognized in bloodstream trypomastigotes, suggesting some degree of homology. Western blot analysis also was carried out using epimastigote antigens of other *T. cruzi* strains (*see* Material and Methods) as well as promastigotes of *L. amazonensis* and *L. chagasi*. A 72-kDa protein was also recognized on the epimastigotes of the WSL and Colombiana strains, whereas proteins of slightly different molecular weights were observed on *L. amazonensis* and *L. chagasi* (Fig. 2B). As the 72-kDa protein is present on all developmental stages of *T. cruzi* Y strain, epimastigote forms were selected to characterize the epitope recognized by the mAb.

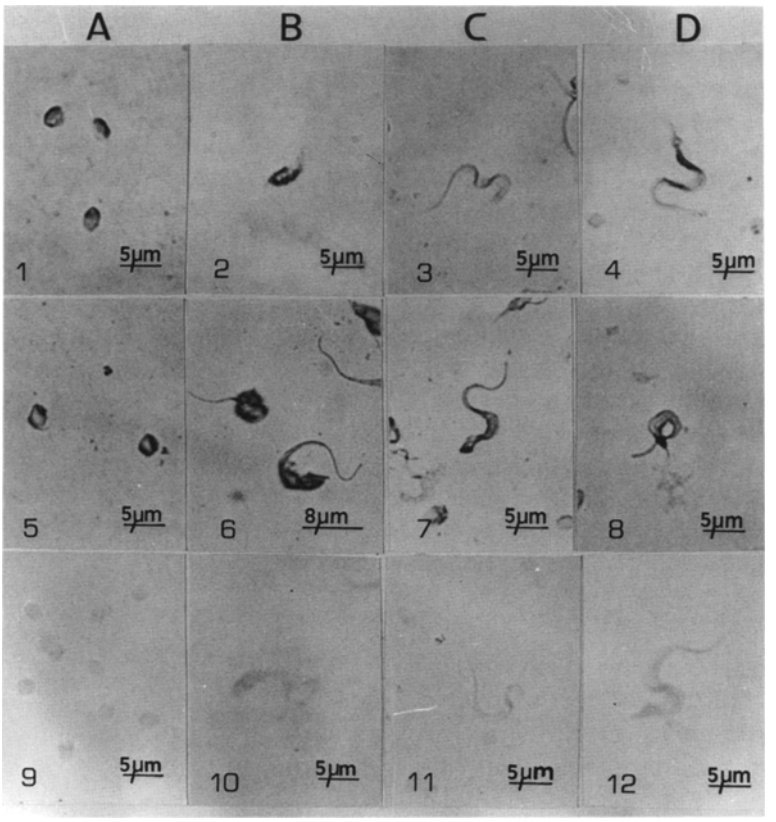

Fig. 1. Immunoperoxidase staining reaction by ELAM method on all developmental stages of *T. cruzi* (Y strain) with 164C11 mAb. A, amastigotes; B, epimastigotes; C, cell-culture derived trypomastigotes; D, bloodstream trypomastigotes. 1–4, 164C11 mAb reactivity; 5–8, infected mouse serum reactivity; 9–12, normal mouse serum reactivity.

Epitope Characterization

To evaluate if the epitope recognized by the mAb was a peptide or carbohydrate periodate oxidation and endoglycosidase F treatment was performed. Two kinds of periodate oxidation assays were used. First, strain Y epimastigote lysates were electrophoresed, blotted onto NTC, and then treated with periodate (*24*). As shown in Fig. 3A, the periodate treatment did not abolish the ability of the 72-kDa protein to bind the mAb. Second, the periodate treatment was performed followed by Western blot analysis (*25*), resulting in a 60-kDa protein recognized by the mAb (Fig. 3B), suggesting that although the 72-kDa antigen is a glycoprotein, the epitope recognized is a peptide. Finally, digestion with endoglycosidase F neither abolished the reactivity nor changed the migration of the antigen, indicating that the carbohydrate moiety is not N-linked (Fig. 3C).

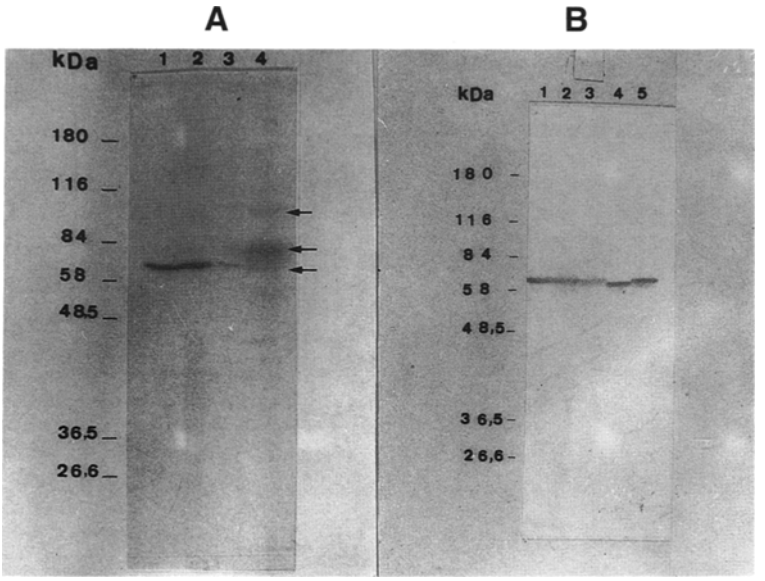

Fig. 2. Reactivity of 164C11 mAb with *T. cruzi* Y strain antigens by Western blot. (**A**) 1, Amastigotes; 2, epimastigotes; 3, cell-culture derived trypomastigotes; 4, bloodstream trypomastigotes (arrows indicate the bands of interest (110, 80, and 72 kDa). (**B**) Different strains of *T. cruzi* and *Leishmania*. 1, 2, and 3, epimastigotes of Y, WSL, and Colombiana strain of *T. cruzi*, respectively; 4, promastigotes of *L. amazonensis;* 5, promastigotes of *L. chagasi*. 1×10^7 parasites were used per lane.

Association of the 72-kDa Antigens with the Membrane

The detection of the 72-kDa antigen by Western blot in the insoluble pellet (Fig. 4) after ultracentrifugation of *T. cruzi* homogenates may indicate that the antigen is part of the parasite membrane.

Lytic Effect of the Monoclonal Antibody

164C11 demonstrated the highest complement-mediated lysis (CML) activity directed against bloodstream trypomastigotes (among the mAbs tested) and was more effective than the chronic mouse serum-CMS. CML activity was very weak when the mAb was directed against cell-culture-derived trypomastigotes (Table 1). This finding may be related to different amounts of the target antigen in different stages of the parasite.

Effect of Monoclonal Antibody 164C11 on Trypomastigote–Host Cell Interaction

To determine whether the 72-kDa glycoprotein could be associated with parasite entry into professional and nonprofessional phagocytic

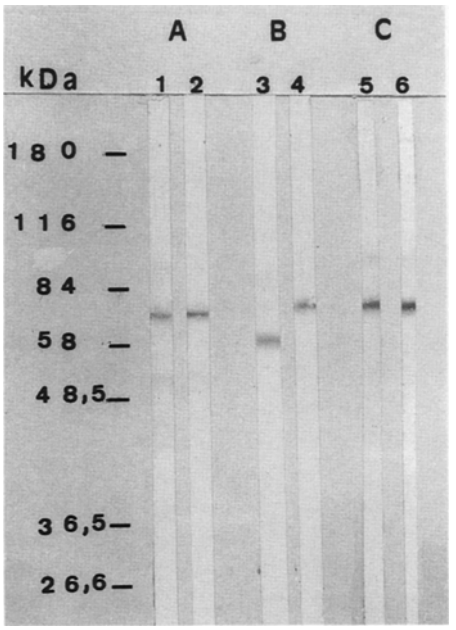

Fig. 3. Effect of periodate and glycosidase treatments on the 164C11 mAb-reactivity. Epimastigote forms of *T. cruzi* Y strain were used as antigen. A, parasite proteins were separated by SDS-PAGE, blotted onto NTC, and treated with periodate: treated (lane 1), untreated (lane 2). B, parasites were treated with periodate following SDS-PAGE and Western blot: treated (lane 3), untreated (lane 4). C, parasites were treated with endoglycosidase F treated (lane 5), untreated (lane 6).

cells, we examined the interaction of blood forms (Y strain) with Mϕ and Vero cells, in the presence of mAb 164C11. As shown in Tables 2 and 3, after an incubation of 72 h the percent infection inhibition by 164C11 on Mϕ and Vero cells was 82.8 and 75.7%, respectively. The intracellular multiplication of parasites was also inhibited. The number of parasites/100 Mϕ or /100 Vero cells was 3.3 ± 0.5 and 7.3 ± 1.5, respectively (X ± SD). In constrast, in the control the cellular infection was elevated (42.6 ± 8.3 parasites/100 Mϕ and 43.6 ± 21.3/100 Vero cells). These differences are significant ($p < 0.05$). No inhibition was observed when the Mϕ and Vero cells were incubated with the mAb before the infection with the nontreated parasites (data not shown). These results suggest that the 72-kDa glycoprotein may be one of the trypomastigote surface components implicated in the process of the host cell invasion.

DISCUSSION

Hybridomas secreting mAbs were produced using spleen cells from mice immunized with Y strain bloodstream trypomastigotes. The results

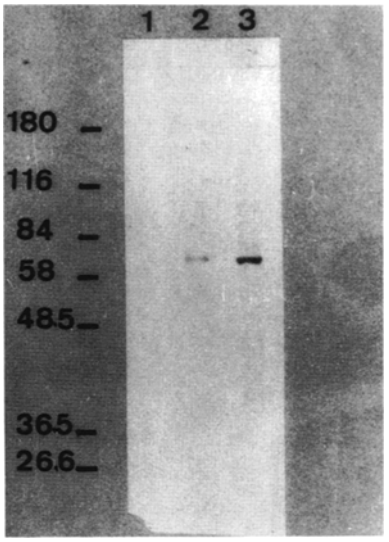

Fig. 4. Western blot analysis of soluble and insoluble proteins of epimastigote forms of Y strain *T. cruzi*. Parasites were sonicated and centrifuged at 30,000*g* for 60 min. 1, Supernatant (soluble proteins, 10 μg); 2 pellet (insoluble proteins, 10 μg); and 3, total epimastigotes antigens (20 μg). The reaction was done using 164C11 mAb.

Table 1
Complement-Mediated Lysis of Trypomastigotes by Monoclonal Antibody Against Blood Forms of *Trypanosoma cruzi*

	CML of trypomastigotes, %[a]	
mAb	Bloodstream	Cell-culture derived
164C11	83.0 ± 6.0	56.0 ± 1.5
Chronic serum[b]	54.0 ± 4.5	54.0 ± 5.8

[a]The results are the average of three experiments performed with mAb supernatant followed by standard deviations.
[b]Chronic chagasic mouse serum.

presented in this report show that 100% of the selected hybridomas secreted IgM isotype immunoglobulins. The reason for the high prevalence of hybridomas producing antibodies of this isotype is not clear. However, this seems to be a common finding when mAbs are produced against *T. cruzi* (*8,27,28*). According to Araujo et al. (*27*), the predominance of IgM isotype may be caused by the antigen employed to immunize the mice or to the immunization protocol. In addition, the fact that the spleen is the major site of IgM synthesis in the early mice may explain the predominance of the IgM isotype after fusions (*29*).

Table 2
Effect of Monoclonal Antibody 164C11 on the Invasion of Macrophage by *T. cruzi* Bloodstream Trypomastigotes

Culture, h		Infected cells, %[a]	Parasites/ 100 cells[a]	Inhibition, %[b]
24	C[c]	11.0 ± 7.0	16.6 ± 12.6	0
	mAb	2.0 ± 1.0	2.0 ± 1.0	82.0
48	C[c]	15.3 ± 7.5	39.0 ± 27.2	0
	mAb	2.0 ± 0	2.6 ± 1.1	87.0
72	C[c]	12.0 ± 5.0	42.6 ± 8.3	0
	mAb	1.0 ± 1.2	3.3 ± 0.5	89.6

[a]The values are the means followed by the standard deviations of triplicate samples.
[b]Percentage of inhibition refers to infected cells per 100 cells.
[c]C, Control.

Table 3
Effect of Monoclonal Antibody 164C11 on the Invasion of Vero Cells by *T. cruzi* Bloodstream Trypomastigotes

Culture, h		Infected cells, %[a]	Parasites/ 100 cells[a]	Inhibition, %[b]
24	C[c]	12.3 ± 8.5	19.6 ± 8.5	0
	mAb	3.0 ± 1.7	3.3 ± 1.5	75.7
48	C[c]	18.0 ± 7.0	44.0 ± 24.0	0
	mAb	4.3 ± 4.1	7.3 ± 5.1	76.2
72	C[c]	16.6 ± 5.6	43.6 ± 21.3	0
	mAb	7.6 ± 0.5	7.3 ± 1.5	78.4

[a]The values are the means followed by the standard deviations of triplicate samples.
[b]Percentage of inhibition refers to infected cells per 100 cells.
[c]C, Control.

The 164C11 mAb showed reactivity with a 72-kDa polypeptide present on all develomental stages of *T. cruzi* Y strain, as well as on epimastigotes of WSL and Colombiana strain. On the other hand, the epitope is not species specific as it is present in *Leishmania*. As judged by the photomycrographs in Fig. 1, there may be a variation in the relative abundance of the antigen on different stages of the parasite.

Epitope analysis of epimastigote antigens was evaluated by periodate and glycosidase treatments. Periodate breaks hexose rings and therefore disrupts carbohydrate epitopes. Endoglycosidase F selectively removes N-linked oligosaccharides. Pretreatment of epimastigotes with periodate before Western blot analysis resulted in the recognition by the mAb of a 60-kDa polypeptide. This finding suggests that a carbohydrate moiety was broken off the 72-kDa polypeptide. On the other hand, the periodate

treatment of parasites after transfer to NTC or glycosidase treatment did not modify the reactivity pattern of the 72-kDa polypeptide. Taken together, these data suggest that the 72-kDa polypeptide is a glycoprotein (not N-linked) but the epitope recognized is a peptide.

A mAb, WIC 29.26, has been described that binds to the carbohydrate portion of a 72-kDa glycoprotein (GP 72) on the surface of *T. cruzi* epimastigotes and metacyclic trypomastigotes (*6*). Further characterization of this GP 72 has been based on assays using both mAb WIC 29.26 and other independently derived mAbs with the same or similar specificities. Such studies using radioimmunoassays showed that the GP 72 epitope recognized by WIC 29.26 and a second GP 72-specific antibody (8G2 B9) is expressed in a zymodeme-specific fashion (*30,31*). However, zymodeme associated differences in GP 72 expression were not apparent in SDS-PAGE of surface-labeled epimastigote antigens immunoprecipitated with WIC 29.26 (*31*). In addition, ELISA with WIC 226.4 raised against periodate-treated GP 72 (*31*) confirmed that a GP 72 polypeptide was expressed in all *T. cruzi* strains examined. The characterization of a gene for GP 72 described by Snary et al. (*6*) was performed by Cooper et al. (*32*). An amino acid sequence was determined from a peptide identified by WIC 29.26 in a Western blot, providing a direct link between the mAb and the gene. This protein has a very high proportion of serine and threonine residues, some of which are clustered together with proline. Although no consensus signal for *O*-glycosylation is known, protein domains destined for *O*-glycosylation are rich in proline, threonine, and serine (*33*). This amino acid bias is consistent with the earlier observation that GP 72 is heavily *O*-glycosylated (*34*). In addition Cooper et al. (*32*) did not detect any change in mobility of GP 72 after treatment with *N*-glycanase.

As there are some similarities between the antigen recognized by our mAb and the 72-kDa glycoprotein characterized by Snary et al. (*6*) and Cooper et al. (*32*), it is not clear that they are unrelated. We are addressing this question currently.

There is preliminary evidence that the antigen recognized by the mAb described in the present paper is membrane associated. Parasite cell surface antigens are logical candidates for subunit vaccines because they are exposed to the host's immune system (*35*).

The mAb 164C11 presented CML activity and was prepared using spleen cells of mice immunized with the sonicated bloodstream trypomastigotes without any challenge. This is in conflict with the experiments of Krettli and Brener (*36*) that did not detect lytic antibodies in the sera of mice immunized with dead parasites, or with a purified glycoprotein isolated from epimastigotes. The presence of lytic antibodies may be a good marker for protection. Lytic antibodies in the serum of mice immunized with the flagellar fraction of epimastigotes (Tulahuen strain) were able to confer a high degree of protection (*9,10*). Because trypomastigotes are resistant to complement in the absence of lytic antibodies and the 72-kDa glycoprotein is a target antigen for the mAb 164C11, the 72-kDa

glycoprotein may play an important role in complement resistance of the parasite that is abrogated by specific antibodies. Currently we are investigating the protective potential of the 164C11 mAb in passive immunization experiments. This mAb is also being used to affinity purify the 72-kDa antigen, to be used subsequently in vaccination experiments.

The 72-kDa antigen described in this paper, may be one of the bloodstream trypomastigote surface molecules involved in host cell recognition, based on the fact that the penetration of bloodstream trypomastigotes in Vero and Mϕ cells is partially inhibited by mAb 164C11. These findings suggested that the 72-kDa antigen is implicated in adhesion and/or internalization of bloodstream trypomastigotes. One possibility is that the host cells recognize and bind a portion of the 72-kDa molecule containing the 164C11-reactive epitope, which is likely to be a polypeptide sequence rather than a carbohydrate. The latter assumption is based on the fact that the treatment with periodate does not alter the reactivity of 72-kDa antigen with mAb 164C11. Another possibility is that oligosaccharide side chains of the 72-kDa antigen could bind to some lectin-like receptor molecule on the host cell surface. In this case, 164C11 might impair binding of 72-kDa antigen to the host cell through steric hindrance of oligosaccharide side chains. However, further experiments are needed to completely clarify the participation of the 72-kDa antigen in parasite–host cell invasion.

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