

## Passive Transfer of a Monoclonal Antibody Specific for a Sialic Acid-Dependent Epitope on the Surface of *Trypanosoma cruzi* Trypomastigotes Reduces Infection in Mice

GIOVANNI FRANCHIN,<sup>1</sup> VERA LUCIA PEREIRA-CHIOCCOLA,<sup>1,2</sup> SERGIO SCHENKMAN,<sup>1</sup>  
AND MAURICIO M. RODRIGUES<sup>1\*</sup>

Department of Microbiology, Immunology, and Parasitology, Federal University of São Paulo—Escola Paulista de Medicina, São Paulo, S.P. Brazil 04023-062,<sup>1</sup> and Laboratory of Xenodiagnostic, Instituto Dante Pazzanese de Cardiologia do Estado de São Paulo, São Paulo, S.P. Brazil 04012-180<sup>2</sup>

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*Trypanosoma cruzi*, the parasite that causes Chagas' disease, proliferates in the cytosol of mammalian cells. When the trypomastigote forms exit the infected cell, they become extensively sialylated because the parasite contains an enzyme called *trans*-sialidase. This enzyme efficiently catalyzes the transfer of bound sialic acid residues from host glycoconjugates to acceptors containing terminal  $\beta$ -galactosyl residues on the parasite surface. The sialic acid acceptors are developmentally regulated mucin-like glycoproteins that are extremely abundant on the trypomastigote surface. In the present study, we determined whether passive transfer of monoclonal antibodies specific for sialic acid acceptors could reduce the acute infection induced by *T. cruzi* in a highly susceptible mouse strain. We found that passive transfer to naive mice of an immunoglobulin G1 monoclonal antibody directed to a sialylated epitope of these mucin-like glycoproteins significantly decreased parasitemia and the number of tissue parasites as measured by a DNA probe specific for *T. cruzi*. Upon challenge with trypomastigotes, mice which received this antibody also had a significant increase in survival. A statistically significant reduction in parasitemia could be accomplished with relatively small doses of immunoglobulin, and Fab fragments alone could not mediate protective immunity. The precise mechanism of parasite elimination is unknown; however, this monoclonal antibody does not lyse trypomastigotes *in vitro* in the presence of human complement or mouse spleen cells.

After contact with trypomastigotes of *Trypanosoma cruzi*, mammalian hosts develop the acute phase of Chagas' disease, characterized by a patent parasitemia that lasts for a few weeks. The chronic phase initiates when the parasitemia declines significantly, becoming subclinical. Most individuals carry this infection for life. During the chronic phase of Chagas' disease in humans, mice, and rats, reinfection with trypomastigotes, even at large doses, fails to generate a second patent parasitemia or casualties (6, 12). More than half a century ago, it was suggested that acquired immunity in rats was due to antibodies, since passive transfer of serum from chronically infected animals could protect naive rats from acute-phase infection with bloodstream trypomastigotes of *T. cruzi* (6). In subsequent years, it became well established that like the immunoglobulins in these rats, immunoglobulins obtained from chronically infected humans and mice could passively transfer immunity against experimental acute-phase infection (14). In spite of the long-held knowledge that antibodies alone are extremely effective in passively protecting mammalian hosts against acute infection, the antigens that are the target of protective antibodies could not be defined because these polyclonal antibodies recognize multiple proteins on the surface of parasites. Most important is the fact that to date, to our knowledge, not a single monoclonal antibody (MAb) could reproduce the protection mediated by polyclonal antibodies.

Bloodstream trypomastigotes are extracellular forms re-

sponsible for disseminating the infection in mammalian hosts, and it is likely that they are the main target of protective antibodies. When trypomastigotes rupture the mammalian cell, they become extensively sialylated. The process of sialic acid acquisition is mediated by an enzyme called *trans*-sialidase (TS). This enzyme efficiently catalyzes the transfer of bound sialic acid residues to acceptors containing terminal  $\beta$ -galactosyl residues (4, 5, 23). In *T. cruzi*, the sialic acid acceptors are developmentally regulated mucin-like glycoproteins that are extremely abundant in the trypomastigote surface (1, 7, 23). The exact role of the acquisition of sialic acids remains unknown. However, it has been suggested that TS and the sialic acid acceptors participate in invasion of mammalian cells by trypomastigotes (3, 9, 17, 19, 21, 24, 27). In the present work, we studied whether MAbs specific for sialic acid-dependent epitopes on the surface of bloodstream trypomastigotes could passively transfer immunity to acute infection elicited by *T. cruzi* in a highly susceptible mouse strain.

### MATERIALS AND METHODS

**Parasites and mice.** Trypomastigotes from Y strain (28) were obtained from culture supernatants of LLCMK<sub>2</sub> cells grown in low-glucose Dulbecco's modified Eagle's medium (Life Technology, GIBCO BRL, Grand Island, N.Y.) with penicillin, streptomycin, and 10% fetal bovine serum at 37°C, under 5% CO<sub>2</sub>, as described previously (26). Bloodstream trypomastigotes were obtained from 7-day-infected A/Sn mice. The blood was collected from the axillar vein and transferred to a tube containing heparin. After centrifugation, the parasites were collected with plasma, centrifuged, and washed twice in phosphate-buffered saline (PBS). Female 5- to 8-week-old A/Sn and BALB/c mice, purchased from the University of São Paulo, were used in this study. A/Sn mice are highly susceptible to infection with trypomastigotes of the Y strain since 100% of animals die after intraperitoneal (i.p.) infection with as few as 250 trypomastigotes per mouse. In comparison, a dose 20 times higher (5,000 parasites) is required to kill 100% of BALB/c mice, and C57Bl/6 mice are resistant to a dose as high as 10,000 bloodstream trypomastigotes per animal.

\* Corresponding author. Mailing address: UNIFESP—Escola Paulista de Medicina, Rua Botucatu, 862, 6º andar, São Paulo, S.P. Brazil 04023-062. Phone: 55 11 576-4532. Fax: 55 11 571-1095. E-mail: rodriguesm.dmp@epm.br.

TABLE 1. MAbs used

MAb	Specificity <sup>a</sup>	Antigen	Isotype	Reference
3C9	Sialic acid-dependent epitope	Mucin-like glycoproteins (~50–210 kDa)	IgG1	2
46	Sialic acid-dependent epitope	Mucin-like glycoprotein(s) (~120 kDa)	IgG2A	24
39	C-terminal repeats	<i>trans</i> -Sialidase	IgG2b	25

<sup>a</sup> All three MAbs recognize epitopes expressed on the surface of cell culture or blood trypomastigotes of *T. cruzi* Y.

**MAbs and passive transfer experiments.** Three MAbs were used in the experiments. Details on their names, specificities, and isotypes are given in Table 1. Hybridomas were grown in RPMI medium (Life Technologies, GIBCO-BRL) containing 10% fetal calf serum (Cultilab, Campinas, Brazil). BALB/c mice were injected i.p. with 0.5 ml of pristane (Sigma, St. Louis, Mo.), and 1 day later, they were inoculated i.p. with  $5 \times 10^6$  hybridoma cells collected from *in vitro* cultures or from ascitic fluids. Ten to 20 days later, the ascitic fluid was drained from the peritoneal cavity with the help of a needle. The fluid was collected into a 15-ml tube containing 0.1 ml of heparin. After centrifugation at 1,500 rpm in a tabletop centrifuge for 10 min, the supernatant was collected and frozen at  $-20^\circ\text{C}$ . The cells in the pellet were washed twice in RPMI medium, the number of hybridoma cells was estimated, and  $5 \times 10^6$  cells were reinoculated into pristane-treated mice. MAbs were purified essentially as described in reference 11. After centrifugation at  $10,000 \times g$  for 30 min, ascitic fluids were diluted twice in PBS and filtered (Millipore Corp. [Bedford, Mass.] filters; 22- or 45- $\mu\text{m}$  pore size). The ascitic fluids were passed through a protein G-agarose column (Sigma) previously equilibrated in PBS. The column was washed with 10 column volumes with PBS and another 10 volumes of PBS–0.5 M NaCl. Subsequently, the antibodies were eluted with 0.2 M glycine (pH 3.0). Each fraction contained 1 ml and was immediately neutralized by adding 0.2 ml of 1 M Tris-HCl (pH 8.0). The material eluted was dialyzed twice against 1 liter of PBS. Normal mouse immunoglobulin G (IgG) was obtained from the sera of BALB/c mice. The sera were diluted twice in PBS, and the IgG was purified in protein G-agarose exactly as described above. The amount of protein was estimated by the absorbance at 280 nm. One optical density unit was equal to 0.8 mg of antibody. The purity of the antibody preparations was determined by vertical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and nonreducing conditions. The gel was stained with Coomassie blue, and no band other than the IgG was seen in the gel.

Fab fragments were generated by digestion with papain followed by purification with columns of protein G-Sepharose exactly as described in reference 11. The purity of these fragments was estimated by SDS-PAGE. To determine whether these Fab fragments were still capable of binding to the antigen, we performed an immunofluorescence assay. Briefly, trypomastigotes obtained from LLCMK<sub>2</sub> cells were washed in medium, resuspended to a concentration of  $10^7$  parasites/ml, and then fixed with 10 ml of 4% paraformaldehyde in PBS. The fixative was removed by centrifugation. The parasites were washed with PBS and added to immunofluorescence slides. After drying, the slides were treated with 1% bovine serum albumin in PBS and then incubated with several concentrations of MAb 3C9 or Fab fragments. Bound antibodies were detected with a fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Boehringer, Einheiten, Germany). Fab fragments stained trypomastigotes at concentrations as low as 2  $\mu\text{g}/\text{ml}$ . Intact MAb 3C9 gave a positive reading until a concentration of 0.2  $\mu\text{g}/\text{ml}$  was reached.

For passive transfer experiments, groups of three to five A/Sn mice received the indicated doses of purified MAbs or IgG purified by protein G from normal mouse serum. The antibodies were injected intravenously (i.v.) in the tail vein. One to two hours later, the animals were inoculated i.p. with  $5 \times 10^2$  blood-form trypomastigotes or i.v. with  $2 \times 10^6$  cell culture-derived trypomastigotes.

**Estimation of parasites in the blood and in the tissues.** Parasitemia was monitored in blood collected daily (5  $\mu\text{l}$ ) from the tail tip of each mouse. Fifty microscopic fields were counted (see Fig. 1). When the parasitemia was very high, the concentration of trypomastigotes in the blood was estimated with a hemacytometer-Neubauer chamber (see Fig. 2, 3, 4, and 7). In some experiments, the number of parasites in blood and tissues was estimated by hybridization with a DNA probe specific for *T. cruzi* as described in reference 26. Briefly, blood and tissues were homogenized in 1% SDS–50 mM Tris-HCl (pH 7.4) and treated with 50  $\mu\text{g}$  of proteinase K per ml for 3 h at  $65^\circ\text{C}$ . The total DNA was extracted once with phenol-chloroform and once with chloroform-isoamyl alcohol, precipitated with ethanol, resuspended in water, boiled, and adjusted to  $16 \times \text{SSC}$  on ice ( $1 \times \text{SSC}$  is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]). Each sample was then filtered through wet disks of nitrocellulose membranes (HATF, 0.45- $\mu\text{m}$  pore size; Millipore Corp.). The filters were washed in  $6 \times \text{SSC}$ , dried, and incubated at  $80^\circ\text{C}$  under vacuum. The prehybridization and hybridization were done at  $42^\circ\text{C}$  in 50% formamide– $5 \times \text{SSC}$ –1 mM EDTA–1% SDS–0.2% nonfat dry milk with the minisatellite DNA probe of *T. cruzi*, kindly provided by

A. Gonzalez (10), labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham, Little Chalfont, England) by random-primed synthesis (Pharmacia Biotech, Uppsala, Sweden).

The standard curve consisted of a serial twofold dilution of DNA extracted from known amounts of culture-derived trypomastigotes. The parasite DNA was diluted in tubes containing 1 ml of Tris-EDTA and 200  $\mu\text{g}$  of mouse liver DNA. The first dilution contained 1,285 ng of *T. cruzi* DNA (equivalent to  $10^6$  parasites), and the last contained only 64.25 ng per ml (equivalent to 50 parasites). The samples of the standard curve were blotted onto wet nitrocellulose filters, and they were hybridized in parallel to experimental samples as described above. The number of counts per minute (cpm) was estimated by liquid scintillation counting. The cpm obtained was proportional to the log of the number of parasites in the standard curve. The presence of up to 5 mg of normal mouse DNA did not reduce the cpm detected for a certain amount of parasite DNA. Due to the variable recovery of DNA from different tissue samples, the cpm was initially corrected for 100  $\mu\text{g}$  of total DNA. This number was then plotted in the standard curve, and the amount of parasite DNA equivalents was estimated per 100  $\mu\text{g}$  of input DNA.

The number of parasites in the tissues was also estimated microscopically in spleen histological sections. Fragments of spleens from mice injected with PBS or monoclonal antibodies before challenge with trypomastigotes were fixed overnight in PBS containing 10% formaldehyde, dehydrated through a graded series of ethanol (70, 90, and 100%) and 100% chloroform, and embedded in paraffin. Paraffin blocks were sectioned at a 5- $\mu\text{m}$  thickness and mounted onto glass slides. Deparaffination was performed with xylene and subsequent rehydration with a graded series of ethanol (100, 90, and 70%) and, finally, tap water. The sections were stained with hematoxylin-eosin by use of standard protocols, and the number of amastigote nests was estimated by light microscopy.

**Agglutination, complement-mediated lysis, and antibody-mediated cellular toxicity (ADCC).** Agglutination reactions were made with tissue culture-derived trypomastigotes in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The parasites, at  $10^7/\text{ml}$ , were incubated for 30 min at  $37^\circ\text{C}$  with different amounts of antibodies in Terasaki microplates. Complement-mediated lysis was assessed with trypomastigotes which were washed and resuspended to a concentration of  $10^7$  parasites/ml in RPMI medium containing 1% bovine serum albumin and 10% normal mouse serum. Twenty-five microliters of the parasite suspension was added to each well in a 96-well flat-bottom plate. Subsequently, the same volume of a solution containing the desired antibody in different concentrations or of medium only was added to each well in quadruplicate. After 60 min at  $37^\circ\text{C}$ , two wells of each sample received 50  $\mu\text{l}$  of normal human serum as a source of complement. The other two wells received 50  $\mu\text{l}$  of normal human serum inactivated at  $56^\circ\text{C}$  for 30 min. After another 60 min at  $37^\circ\text{C}$ , the number of live (motile) parasites was estimated with the aid of a hemacytometer. Lysis was calculated as follows: the number of parasites in cultures containing inactivated component was subtracted from the number of parasites in cultures containing active complement, and this number was divided by the number of parasites in cultures containing inactivated complement and multiplied by 100.

ADCC reactions were made with tissue culture-derived trypomastigotes in RPMI medium containing 10% fetal bovine serum. Fifty microliters of parasite suspension containing  $10^7$  trypomastigotes/ml was added to each well in a 96-well flat-bottom plate. Subsequently, 100  $\mu\text{l}$  containing  $2 \times 10^8$  spleen cells from A/Sn mice was added per well. The final spleen cell/parasite ratio was 40 to 1. The required antibody, in different concentrations, was added to each well in 50  $\mu\text{l}$ . Triplicate cultures were incubated for 5 h at  $37^\circ\text{C}$ . The number of live (motile) parasites was counted with the aid of a hemacytometer, and the percent lysis was calculated as follows: the number of parasites in cultures containing medium only was subtracted from the number of parasites in cultures containing spleen cells and the indicated antibody, and this number was divided by the number of parasites in cultures containing medium only and multiplied by 100.

**SDS-PAGE and immunoblotting.** Trypomastigotes were purified from the blood of infected mice as described above, and their number was estimated by counting with a hemacytometer. After centrifugation, the parasites were resuspended in SDS-PAGE sample buffer (2% SDS, 50 mM Tris-HCl [pH 6.8], 5% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue) and boiled for 5 min. The amount of liquid corresponding to  $3 \times 10^6$  trypomastigotes was loaded onto a 10% polyacrylamide–0.1% SDS gel. After electrophoresis, the samples were transferred to nitrocellulose membrane for 18 h at 30 V and  $4^\circ\text{C}$ . The membranes were treated with 5% nonfat dry milk in PBS for at least 2 h, washed with PBS, and incubated with MAb 3C9 or 46 at a concentration of 50  $\mu\text{g}/\text{ml}$  in 5% nonfat dry milk–PBS. Bound antibodies were detected by incubating the membranes with anti-mouse IgG coupled to peroxidase diluted 1:4,000 (Life Technologies, GIBCO-BRL) followed by development of the chemiluminescent reaction with enhanced chemiluminescence reagent (Amersham) and exposure to X-ray films (Kodak, Rochester, N.Y.).

**Statistical analysis.** Student's *t* test and log rank test were performed with the True Epistat software package.

## RESULTS

Earlier studies have demonstrated that Fab fragments of MAbs that recognize sialic acid-dependent epitopes on the surface of trypomastigotes inhibited invasion of nonphagocytic

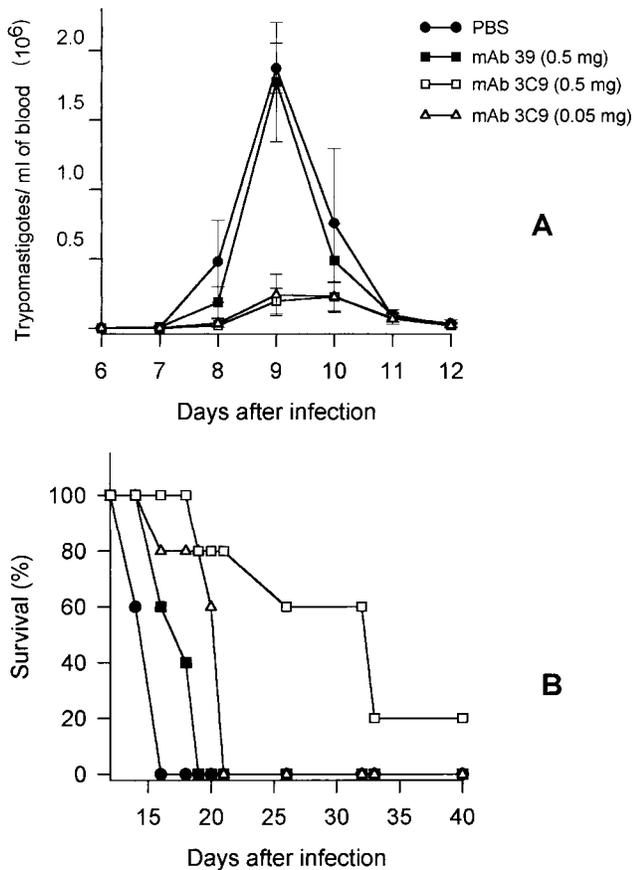


FIG. 1. Passive transfer of MAb 3C9 reduces parasitemia and delays mortality of mice challenged with bloodstream trypanosomes. (A) A/Sn mice were passively immunized i.v. with 0.5 or 0.05 mg of MAb 3C9, 0.5 mg of MAb 39, or PBS. Two hours later, they were challenged i.p. with 500 bloodstream trypanosomes. Parasitemia was estimated by direct counting of blood smears and represent the mean value  $\pm$  standard error of the mean obtained from five mice. At the peak of infection at day 9, a significant reduction in parasitemia was observed in mice that received 0.5 or 0.05 mg of MAb 3C9 when compared to that of control animals injected with PBS ( $P < 0.0001$  and  $P = 0.0001$ , respectively; Student's  $t$  test). There was no statistically significant difference in the parasitemias of mice injected with MAb 39 and control animals. (B) Survival rate of mice passively immunized with the indicated MAb or PBS. A statistically significant delay in mortality was found among mice that received 0.5 or 0.05 mg of MAb 3C9 and control animals injected with PBS (two-tailed  $P = 0.022$  and  $0.023$ , respectively; log rank test). No statistically significant difference was observed when we compared the survival rates of mice injected with MAb 39 and control animals ( $P > 0.05$ ). There were five mice per group.

cells by *T. cruzi* (22, 27). To investigate whether one of these MAbs could decrease acute infection in vivo, passive transfer of MAb 3C9 was performed in naive mice that, 1 to 2 h later, were challenged with trypanosomes of *T. cruzi*. Mice that received doses of 0.5 or 0.05 mg of purified MAb 3C9 before challenge with 500 bloodstream trypanosomes displayed a significantly lower level of parasitemia at the peak of infection (day 9) than mice that received PBS (Fig. 1A). This inhibition was specific since passive transfer of a MAb that recognizes the repeat region of TS (MAb 39) failed to reduce the parasitemia when as much as 0.5 mg was injected per mouse. Most relevant was the fact that protective immunity conferred by 0.5 mg of MAb 3C9 significantly delayed mortality (Fig. 1B).

Protective immunity conferred by passive transfer of MAb 3C9 was also achieved upon challenge with large doses of cell culture-derived trypanosomes. As shown in Fig. 2A, a strong reduction in the peak of parasitemia (day 4 after infection)

could be observed in mice that received 0.25 mg of MAb 3C9 and were challenged i.v. with  $2 \times 10^6$  tissue culture-derived trypanosomes (Fig. 2A). In addition, while mice injected with purified normal mouse IgG died, 100% of animals that received MAb 3C9 survived the infection. This model was then selected for subsequent studies because the level of parasitemia increases fast and it is extremely synchronized, reducing the individual mouse variation at the peak of infection.

The reduction in parasitemia caused by passive transfer of MAb 3C9 was dependent on the dose of antibody transferred per mouse, and statistically significant inhibition could be achieved with doses as small as 0.005 mg (Fig. 3A). A significant increase in survival time could be obtained with 0.05 mg of antibody per mouse ( $P = 0.034$ , log rank test); however, only the highest dose of IgG protected three of four animals.

We then studied the trypanocidal activity of a second antibody, MAb 46, which also recognizes a sialic acid-dependent epitope in the trypanosome surface (24). Although present in the same group of mucin-like molecules, the epitope recognized by MAb 46 is distinct from the one recognized by MAb 3C9. As shown in Fig. 4A, MAb 3C9 recognizes, in the immunoblot, a wide range of proteins that migrate from 50 to 210 kDa in extracts of blood-form trypanosomes. In contrast, MAb 46 recognizes a protein(s) with an apparent molecular mass of 120 kDa. Although both MAbs recognize sialylated epitopes on the surface of trypanosomes, passive transfer of MAb 46 was unable to reduce the parasitemia after challenge with trypanosomes (Fig. 4B). Also, no difference in survival was observed (data not shown). This result suggests that not all antibodies to sialic acid-dependent epitopes on the surface trypanosomes are protective.

The reduction of the number of trypanosomes in the blood did not necessarily mean that infection had diminished in organ tissues. To detect the amount of *T. cruzi* in major organ tissues, we hybridized the DNA extracted from each tissue with a probe corresponding to *T. cruzi* minisatellite DNA (10). By using this method, it was possible to detect as few as 200 parasites with up to 5 mg of host DNA loaded per filter. Detection was optimized when infections were performed i.v.

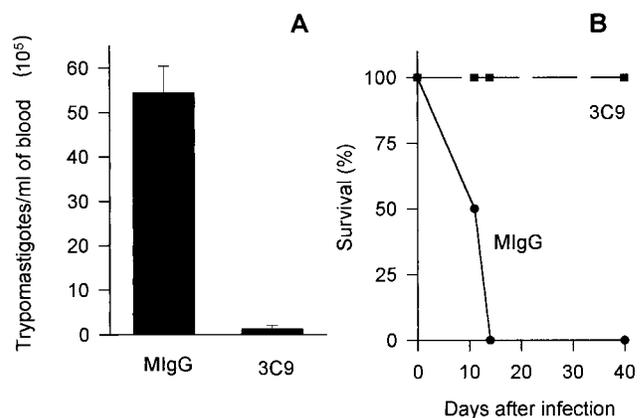


FIG. 2. Passive transfer of MAb 3C9 completely protects mice against a challenge with cell culture-derived trypanosomes. A/Sn mice were passively immunized i.v. with 0.25 mg of protein G-purified normal mouse IgG (MIgG) or MAb 3C9. One hour later, they were challenged i.v. with  $2 \times 10^6$  culture-derived trypanosomes. (A) Parasitemia was estimated at the peak of infection (day 4), and the results represent the mean value obtained from four mice  $\pm$  standard error of the mean. A significant reduction in parasitemia was observed in mice that received 0.25 mg of MAb 3C9 when compared to that of control animals injected with MIgG ( $P < 0.0001$ , Student's  $t$  test). (B) Survival rates of mice passively immunized with MIgG or MAb 3C9 are shown. The statistical difference between the two groups was  $P$  of 0.0009 (log rank test).

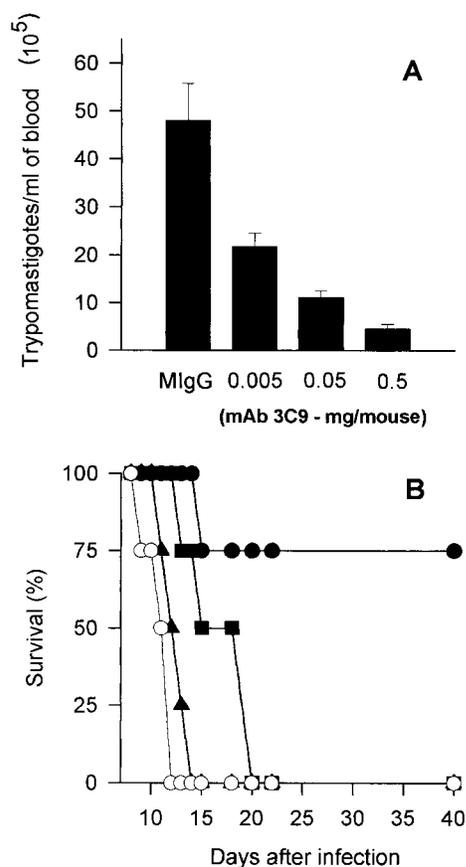


FIG. 3. Dose dependence of passive immunity transferred by MAb 3C9. A/Sn mice were passively immunized i.v. with the indicated dose of MAb 3C9 or 0.5 mg of normal mouse IgG (MlgG). One hour later, they were challenged i.v. with  $2 \times 10^6$  tissue culture-derived trypomastigotes. (A) Parasitemia was estimated at the peak of infection (day 4). The results represent the mean value of four mice  $\pm$  standard error of the mean. A significant reduction in parasitemia was observed in mice that received 0.005, 0.05, or 0.5 mg of MAb 3C9 when compared to that of control mice. The *P* values were  $<0.0015$ ,  $<0.0002$ , and  $<0.0001$ , respectively (Student's *t* test). There were statistically significant differences between groups of mice that received 0.005, 0.05, or 0.5 mg of MAb 3C9, when they were compared one with the other ( $P < 0.001$ ). (B) Survival rates of mice passively transferred with MlgG or MAb 3C9 are shown. Mice that received 0.5 (closed circles) or 0.05 (closed squares) mg of MAb 3C9 had a significant delay in mortality when compared to that of controls (open circles) ( $P = 0.021$  and  $0.034$ , respectively; log rank test).

with  $2 \times 10^6$  tissue culture-derived trypomastigotes. At day 1 and 2 after infection with trypomastigotes, a very small amount of parasite DNA could be detected in tissues or blood. The amount of parasite DNA increased by day 3, with a peak day 4 after infection. At that time, the highest amounts of parasite DNA could be found in the blood, liver, spleen, heart, and skeletal muscle, strongly suggesting that the hybridization signal corresponded to parasites that have multiplied intracellularly in the animal (Fig. 5).

We found that passive transfer to naive mice of 0.5 mg of MAb 3C9, before challenge with tissue culture-derived trypomastigotes, significantly reduced the number of parasites in their blood, heart, liver, and skeletal muscle when compared to that of animals that received PBS (Fig. 6). In contrast, the amount of parasite DNA in organs of mice that had received the nonprotective MAb 39 was comparable to that present in control animals. It is noteworthy that in the spleens of mice treated with MAb 3C9, the reduction in the amount of parasite

DNA was much less pronounced. To determine whether there was a difference in the number of parasites in this organ, we scored the number of amastigote nests in histological tissue sections. We found that the spleens of mice treated with MAb 3C9 had, on average, 4.25 times fewer nests than the spleens of animals injected with PBS (data not shown). In contrast, the number of nests in the spleens of mice treated with MAb 39 was 1.64 times higher than that in control mice injected with PBS.

To determine whether Fab fragments were also protective, we injected each mouse with 250  $\mu$ g of these fragments or with the same amount of intact immunoglobulin. At that dose, Fab fragments of MAb 3C9 produced only a slight reduction in the parasitemia (Fig. 7A). While all mice injected with 3C9 Fab fragments died, 100% of animals treated with MAb 3C9 survived the challenge (Fig. 7B).

The fact that intact antibodies seem to be important for efficient protection raised the possibility that these MAbs could mediate lysis of parasites in the presence of complement or through ADCC. However, we found that MAb 3C9 was unable to induce complement-mediated lysis or ADCC of tissue cul-

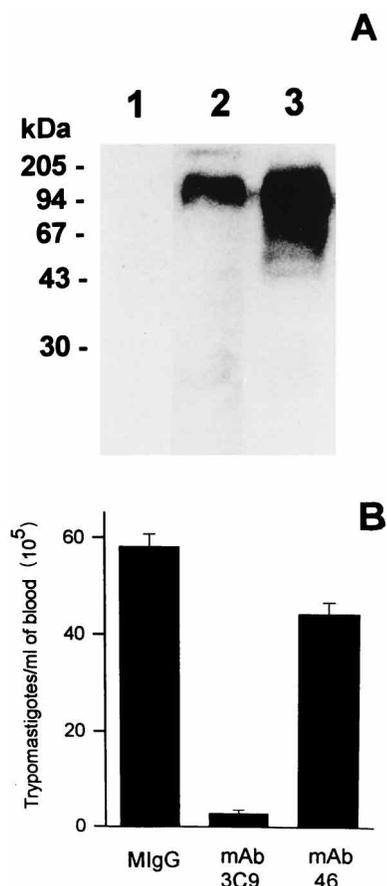


FIG. 4. A second antibody specific for a distinct sialylated epitope on the trypomastigote surface is not protective. (A) Immunoblot of antigens recognized by MAb 46 or MAb 3C9. Bloodstream trypomastigotes ( $3 \times 10^6$ ) lysed with sample buffer were added to each lane of a SDS-PAGE gel. Immunoblot strips were incubated with no antibody (lane 1), MAb 46 (lane 2), or MAb 3C9 (lane 3). (B) Parasitemia of A/Sn mice passively transferred i.v. with 0.5 mg of the indicated purified mouse MAb. After 1 h, the animals were challenged i.v. with  $2 \times 10^6$  tissue culture-derived trypomastigotes. The parasitemia was estimated at the peak of infection (day 4). The bars represent mean values obtained from four mice  $\pm$  standard errors of the mean. MlgG, normal mouse IgG.

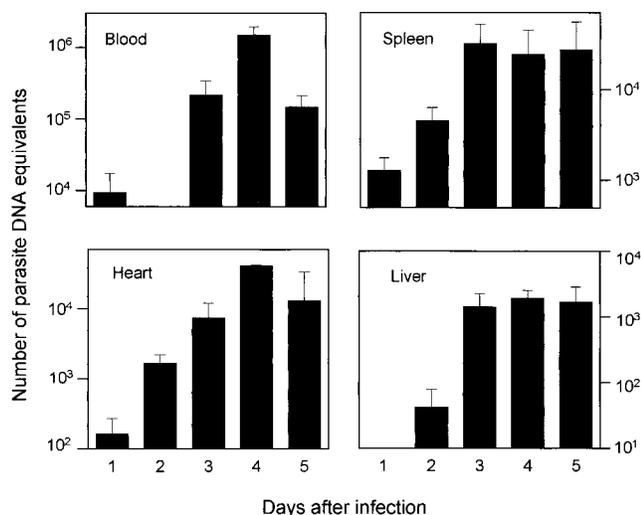


FIG. 5. Kinetics of parasite DNA detection in different organs by use of a DNA probe specific for *T. cruzi*. A/Sn mice were infected i.v. with  $2 \times 10^6$  tissue culture-derived trypomastigotes. At different days after infection, the indicated organs were processed as described in Material and Methods. The number of parasite DNA equivalents per 100  $\mu\text{g}$  of total DNA was estimated by using DNA extracted from known amounts of parasites as a standard. One million parasite DNA equivalents represent 1,285 ng of *T. cruzi* DNA. The bars represent the mean values obtained from three mice  $\pm$  standard errors of the mean.

ture-derived trypomastigotes in vitro as did sera from *T. cruzi*-infected mice used as a positive control (Table 2).

## DISCUSSION

In this work, we have demonstrated that passive transfer of a MAb specific for sialic acid acceptors present on the surface of trypomastigotes reduces the parasitemia and mortality after *T. cruzi* infection in a highly susceptible mouse strain. This MAb abolished mortality of mice infected with tissue culture-derived trypomastigotes and delayed it in mice challenged with blood-stage forms. This discrepancy probably reflects the timing of the acute infection in these two distinct models. While mice infected with  $2 \times 10^6$  culture-derived trypomastigotes had a very short acute phase with peak parasitemia occurring at day 4, animals injected with 500 blood-stage forms had a much longer acute phase with the peak of infection occurring at day 9. Therefore, it is possible that in the latter case, the concentration of MAb 3C9 in the circulation diminished significantly, reducing the protection conferred by the antibody in terms of survival.

Passive transfer of MAb 3C9 inhibited parasite development when relatively small amounts of immunoglobulin were transferred, suggesting that this event is specific. Most important, passive transfer of two other MAbs that recognize other epitopes on the surface of trypomastigotes was unable to mediate protection. Although an unrelated IgG1 MAb was not available for use as a control, the same protection was not produced by passive transfer of 0.5 mg of protein G-purified normal mouse IgG, which contains approximately 0.05 mg of polyclonal IgG1 (13).

The present data show that the parasite load was also dramatically reduced in the blood and in several tissues, with the exception of the spleen, as measured by a DNA probe. In the spleen, a significant decrease could be detected only when we estimated the number of amastigote nests in histological tissue sections. The reason for such a discrepancy is unknown. How-

ever, the presence of parasite DNA could reflect trypomastigotes trapped inside phagocytic cells.

The precise mechanism that mediates protective immunity in vivo by anti-*T. cruzi* trypomastigotes antibodies is unknown. We found that immunity mediated by MAb 3C9 cannot be reproduced by its Fab fragments. Similar results have been reported previously for polyclonal protective antibodies (29). Three main reasons may account for the fact that Fab fragments are not protective. First, the avidity of monovalent fragments may be lower than that of the intact MAb. Second, Fab fragments are not capable of mediating cross-linking. Finally, it is possible that the Fc fragment is important for protection against *T. cruzi* infection. In vitro, protective polyclonal antibodies agglutinate trypomastigotes (14), mediate lysis by complement (15), and mediate cellular toxicity (16, 18). Since MAb 3C9 also agglutinates trypomastigotes but does not lyse them in the presence of complement or cells, it is possible that parasite agglutination plays a role in the protective immunity mediated by this antibody. However, we observed that MAb 46, like MAb 3C9, strongly agglutinates trypomastigotes in vitro at concentrations of 1 to 2  $\mu\text{g}/\text{ml}$  and is not protective in vivo. These findings suggest that parasite agglutination alone may not be sufficient to eliminate trypomastigotes in vivo. A second possibility is that MAb 3C9, being an IgG1, is prefer-

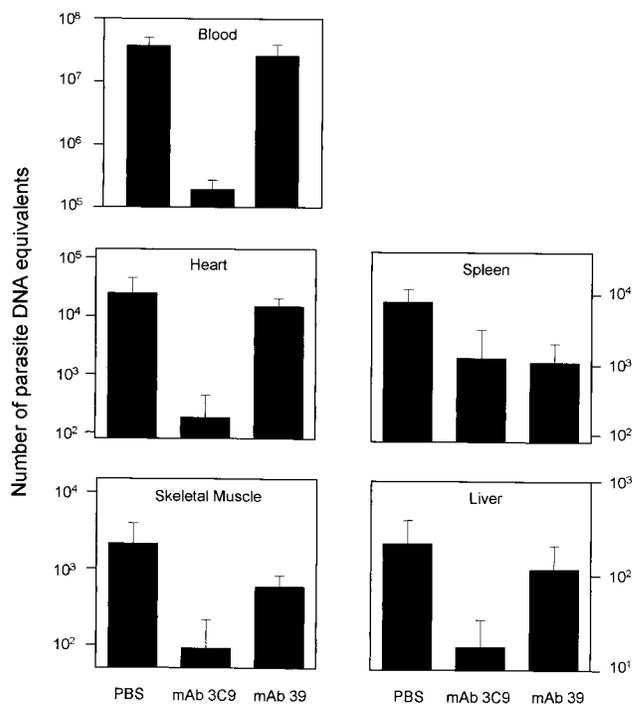


FIG. 6. Passive transfer of MAb 3C9 decreases the number of parasites in different mouse organs after challenge with *T. cruzi*. A/Sn mice were passively immunized i.v. with 0.5 mg of MAb 3C9 or 0.5 mg of MAb 39 or with PBS. After 1 h, the animals were challenged i.v. with  $2 \times 10^6$  tissue culture-derived trypomastigotes. At the peak of infection (day 4), the indicated organs or blood was removed and processed as described in Material and Methods. The number of parasite DNA equivalents are shown per 100  $\mu\text{g}$  of total DNA of each organ or blood. The bars represent the mean values obtained from three mice  $\pm$  standard errors of the mean. The numbers of parasites in the blood, heart tissue, skeletal muscle, and liver of mice injected with MAb 3C9 were significantly lower than the numbers found in mice that received PBS ( $P = 0.0001, 0.01, 0.0001, \text{ and } 0.04$ , respectively; Student's *t* test). In the spleens, the means were not statistically different ( $P = 0.09$ ). No statistically significant reduction in the number of parasites was observed among organs or blood from mice treated with MAb 39 when compared to that of control animals.

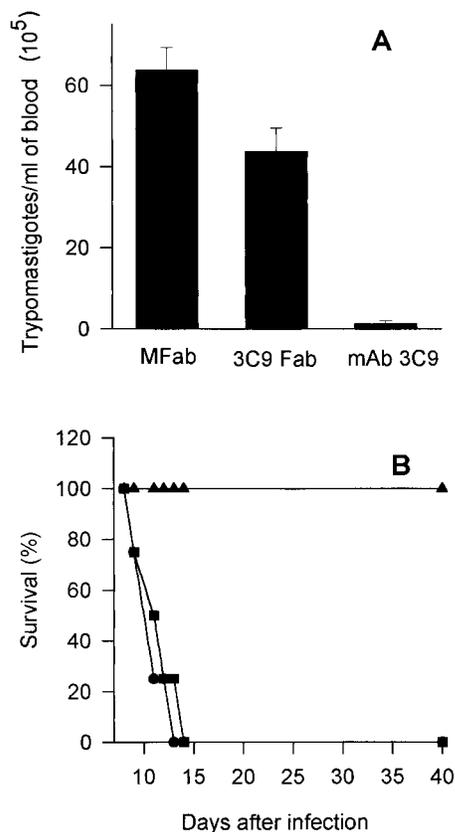


FIG. 7. Fab fragments of MAb 3C9 are not protective. A/Sn mice were passively transferred i.v. with 0.25 mg of the indicated purified mouse Fab (MFab) or MAb 3C9. After 1 h, the animals were challenged with  $2 \times 10^6$  tissue culture-derived trypomastigotes i.v. (A) Parasitemia was estimated at the peak of infection (day 4). The bars represent mean values obtained from four mice  $\pm$  standard errors of the mean. (B) The survival rate after infection is shown. All mice that received intact MAb 3C9 (triangles) survived. The difference in mortality between mice treated with MFab (circles) or 3C9 Fab (squares) was not statistically significant ( $P > 0.05$ ).

entially recognized by high-affinity Fc receptors present in neutrophils and macrophages (20). Because only MAb 3C9 belongs to this subclass, it was not possible to test whether any IgG1 specific for other structures on the surface of trypomastigotes can be protective in vivo. The generation of other trypomastigote-specific IgG1 MAbs and isotype switching of existing MAbs may help to clarify this matter. Finally, MAb 3C9 has been shown to inhibit invasion of nonphagocytic cells in vitro (27). This property may also account for its protective capacity. Nevertheless, it is important to highlight that the precise mechanism(s) used by MAb 3C9, or polyclonal antibodies, to inhibit parasite development in vivo may be extremely complex, involving multiple mechanisms acting simultaneously that remain to be fully elucidated.

The antigens recognized by MAb 3C9 are sialylated O-linked oligosaccharides attached to threonine-rich glycoproteins, present in at least  $2 \times 10^5$  copies per parasite (7). These molecules resemble mucins and cover the entire parasite surface, forming a protective coat, which consequently must be a target for the host immune system. In fact, the same molecules contain oligosaccharides with terminal  $\alpha$ -galactosyl residues recognized by antibodies present in the sera of patients with Chagas' disease (8). Anti- $\alpha$ -Gal antibodies induce strong agglutination and complement-independent killing in vitro, but it

TABLE 2. MAb 3C9 does not lyse trypomastigotes in vitro in the presence of human complement or mouse spleen cells (ADCC)

Antibody	Concn	Comple-ment <sup>a</sup>	Spleen cells <sup>b</sup>	Lysis (%)
MAb 3C9	100 $\mu$ g/ml	+	-	6.0
	10 $\mu$ g/ml	+	-	7.0
Infected mouse serum	25% (vol/vol)	+	-	99.5
Normal mouse serum	25% (vol/vol)	+	-	14.5
MAb 3C9	100 $\mu$ g/ml	-	+	4.6
	10 $\mu$ g/ml	-	+	3.5
Infected mouse serum	25% (vol/vol)	-	+	87.2
Normal mouse serum	25% (vol/vol)	-	+	0.6

<sup>a</sup> In vitro complement-mediated lysis and ADCC were determined as described in Material and Methods. Trypomastigotes used in these assays were derived from tissue culture. The final concentration of human sera used as the complement source was 50%. The results are expressed as an average of duplicate samples. +, presence; -, absence.

<sup>b</sup> Tissue culture-derived trypomastigotes and A/Sn mice spleen cells were used. The final spleen cell/parasite ratio was 40 to 1. Parasites and cells were incubated for 5 h at 37°C. The results are expressed as an average of triplicate samples. +, presence; -, absence.

is unknown whether the same protective mechanism occurs in vivo.

In conclusion, we have shown that a sialylated epitope of a major surface antigen of *T. cruzi* trypomastigotes is the target of a protective MAb. This MAb can be useful to determine the precise chemical structure of the oligosaccharide epitope that is a target for protective immunity. Also, it can be a tool to study the immunological mechanisms of elimination of this protozoan parasite that remain largely unknown.

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