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A Recombinant Protein Based on the *Trypanosoma cruzi* Metacyclic Trypomastigote 82-Kilodalton Antigen That Induces an Effective Immune Response to Acute Infection

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To further investigate the immunological properties of the stage-specific 82-kDa glycoprotein (gp82) of *Trypanosoma cruzi* metacyclic trypomastigotes, previously shown to induce antigen-specific humoral and T-cell responses in mice, we performed a series of experiments with recombinant proteins containing sequences of gp82 fused to glutathione *S*-transferase. Of five fusion proteins tested, only J18b and J18b1, the carboxy-proximal peptides containing amino acids 224 to 516 and 303 to 516, respectively, were recognized by monoclonal antibody 3F6 as well as by various anti-*T. cruzi* antisera and, when administered to mice, were capable of eliciting antibodies directed to the native gp82. The amino-terminal peptide and other carboxy-terminal recombinant proteins lacking the central domain of gp82 (amino acids 224 to 356), which is exposed on the surface of live metacyclic forms, did not display any of these properties. Spleen cells derived from mice immunized with any of the five recombinant proteins proliferated in vitro in the presence of native gp82. J18b was the most stimulatory, whereas J18b3, the peptide containing amino acids 408 to 516, elicited the weakest response. When BALB/c mice immunized with J18b antigen plus $Al(OH)_3$ as adjuvant were challenged with 10^5 metacyclic trypomastigotes, 85% of them resisted acute infection, in comparison with control mice that received glutathione *S*-transferase plus adjuvant. Antibodies induced by J18b protein lacked agglutinating or complement-dependent lytic activity and failed to neutralize parasite infectivity. On the other hand, $CD4^+$ T cells from the spleens of J18b-immunized mice displayed an intense proliferative activity upon stimulation with 1.25 μg of native gp82 per ml, which resulted in increased production of gamma interferon, a cytokine associated with resistance to *T. cruzi* infection.

Attempts by different laboratories to identify potentially immunoprotective or pathology-inducing antigens of *Trypanosoma cruzi*, the etiological agent of Chagas' disease in humans, have revealed several candidate molecules (1, 3, 9, 19, 25); some of these molecules have had their peptide sequences determined and relevant epitopes mapped (15, 24, 26, 28).

We have been studying for several years the immune response induced by insect-stage *T. cruzi* metacyclic trypomastigotes, the developmental forms that can invade a variety of cell types and initiate infection in mammalian hosts. Metacyclic forms express on the surface a set of highly immunogenic molecules (30) that may have immunoprophylactic potential, as indicated by experiments with purified parasite antigens (9) or with anti-metacyclic trypomastigotes monoclonal antibodies (MAbs) capable of neutralizing parasite infectivity in vivo and/or in vitro (2, 17, 32). Among these antigens is an 82-kDa glycoprotein (gp82) which has been shown to elicit both humoral and T-cell responses in mice (31).

Recently, a cDNA clone coding for metacyclic trypomastigote gp82 was characterized, sequenced, and expressed in a bacterial system (4), opening the possibility of analyzing in more detail the immunological properties of this antigen. In the present study, we have used a set of recombinant proteins corresponding to sequences of gp82, aiming at the identification of its immunogenic domains. Our results indicate that major linear B-cell epitopes and several T-cell epitopes of gp82, including those relevant in conferring resistance to acute *T. cruzi* infection, are located in the central region of the molecule, between amino acids 224 and 356.

MATERIALS AND METHODS

Parasites. *T. cruzi* CL (5) and G (29) were maintained cyclically in mice and in liver infusion tryptose medium. To accelerate differentiation into metacyclic trypomastigotes, parasites were also grown in Grace's medium (Life Technologies Inc.) for 7 to 10 days before harvest. Metacyclic forms were purified through passage in a DEAE-cellulose column as described previously (22).

Purification of native gp82. Strain G metacyclic trypomastigotes were used to purify gp82 by affinity chromatography on immobilized antibody 3F6 as described elsewhere (22). The amount of protein in the purified preparation was determined in 96-well microtiter plates by use of the Coomassie Plus protein assay reagent (Pierce), followed by reading the optical density at 620 nm.

Antibodies. The MAb 3F6, directed to gp82, was obtained as described previously (22). Polyclonal antiserum to gp82 was prepared by immunizing mice with four doses of purified antigen (10 μg per mouse) plus $Al(OH)_3$ as adjuvant. Immune sera to metacyclic trypomastigotes were obtained by inoculating mice with four doses of heat-inactivated parasites (10^7 per mouse) at 10-day intervals.

Flow cytometry. Metacyclic trypomastigotes (5×10^6) were incubated for 1 h, on ice, with MAb 3F6 or with unrelated MAb 1C3 against *Leishmania* antigen, which was kindly provided by Clara Lucia Barbieri, Escola Paulista de Medicina. After two washes with phosphate-buffered saline (PBS), the parasites were fixed with 2% paraformaldehyde in PBS for 30 min. The fixative was washed away, and the parasites were incubated with fluorescein-labeled goat anti-mouse immunoglobulin G for 1 h at room temperature. Following two more washes, the number of fluorescent parasites was estimated with a Becton Dickinson FACscan cytometer.

Expression of peptide sequences of gp82 in pGEX3. We used a bacterial expression system (glutathione *S*-transferase [GST] gene fusion system; Pharmacia) to subclone and express subfragments of a cDNA clone (MTS-gp82) encoding the metacyclic trypomastigote gp82 (4) (Fig. 1). The subfragment J18a,

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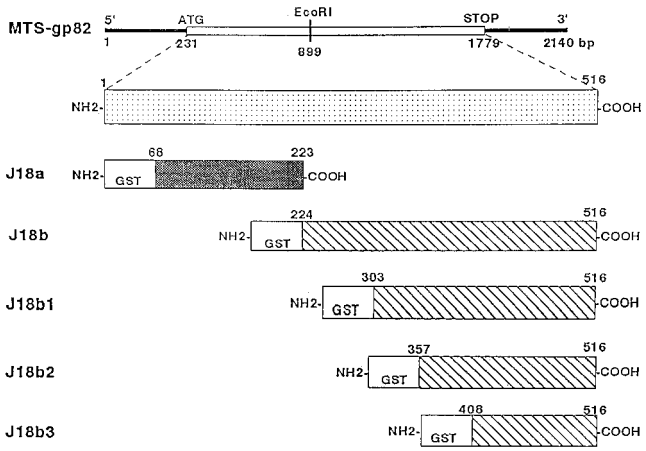

FIG. 1. Schematic representation of a cDNA clone (MTS-gp82), encoding gp82 and the recombinant proteins fused to GST, containing different sequences of the molecule.

encoding 156 amino acids of the N-terminal domain and all N-terminal fragments with deletions, except J18b, were obtained through PCR amplification by use of sense primers derived from different sequences of the coding region of MTS-gp82 and carrying an artificial *Bam*HI restriction site to allow insertion of the fragments in frame with the GST gene of plasmid pGEX3 (20). The 24-mer reverse primer derived from nucleotides 1776 to 1796, used in all constructs, carried the codon for the last amino acid of the cDNA coding region, the stop codon, and an artificial restriction site for *Eco*RI. Amplification products were digested with *Eco*RI and *Bam*HI endonucleases, separated by agarose gel electrophoresis, and cloned directionally into pGEX3. The 1,241-bp *Eco*RI fragment J18b, which encodes 239 amino acids of the C-terminal domain, was inserted into the *Eco*RI site of plasmid pGEX3 in frame with the GST gene (Fig. 1). The sequence arrangement of the recombinant plasmid constructs was ascertained by restriction enzymes and DNA sequencing.

Purification of recombinant proteins. The recombinant proteins were obtained from isopropyl-β-D-thiogalactopyranoside (IPTG)-induced *Escherichia coli* lysates as follows. Bacterial preparations were washed in PBS, sonicated for 10 min (1-min pulse at 30-s intervals), and centrifuged at 12,000 × *g* for 10 min at 4°C. The precipitate was resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, boiled, and subjected to SDS-PAGE. The gels were treated with iced 250 mM KCl to visualize the bands on a black background. By use of molecular size markers ranging from 94 to 14 kDa (Pharmacia) as a reference, a band of high intensity, of the size expected for each recombinant protein, was excised, cut into pieces, and incubated for 16 to 20 h at room temperature in 1 ml of a mixture containing 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 200 mM NaCl, and 0.1% SDS. After centrifugation at 12,000 × *g* for 10 min at 4°C, 1% *n*-octyl β-D-glucopyranoside (NOG) was added, the sample was dialyzed against distilled water, and the final preparation was vacuum dried. The amount of purified proteins was determined by the same procedure used for the native gp82, and their purity was analyzed by SDS-PAGE (Fig. 2A). We observed that the J18b preparation invariably appeared in SDS-polyacrylamide gels as a double band that was recognized by MAb 3F6 (Fig. 2B), suggesting that the lower band is a product of degradation.

Immunization of mice with recombinant antigens. Groups of 6- to 8-week-old female BALB/c mice were immunized, by the intraperitoneal route, with four doses of purified recombinant antigen (5 μg per mouse) plus $Al(OH)_3$ (0.5 mg per mouse) as an adjuvant at 2-week intervals. The same schedule was used for sex- and age-matched controls that received GST plus adjuvant. One week after the last immunizing dose, mice were bled from the retro-orbital plexus, the sera were collected, and 3 days later, some groups of vaccinated animals were challenged with 10^5 metacyclic trypomastigotes. The course of infection was monitored by examining 5-μl blood samples three times a week, under a phase-contrast microscope, up to 31 to 33 days postinoculation.

Competitive binding assay between MAb 3F6 and immune sera to gp82 or the recombinant protein J18b. The competitive binding assay was performed essentially as described previously (9). Briefly, wells of flexible polyvinyl chloride microtiter plates were incubated at 37°C with 50 μl of sonicated metacyclic trypomastigote extract corresponding to 5×10^8 parasites in PBS. After washings and incubation with PBS containing 1% bovine serum albumin for 1 h at room temperature, 50-μl samples of sera of mice immunized with purified gp82 or the recombinant antigen J18b, at various dilutions, were added to separate wells. Following 2 h of incubation at room temperature, the wells were washed and 50 μl (10^5 cpm) of ^{125}I-labeled MAb 3F6 was added. After 1 h of incubation, the wells were washed and dried, and the bound radioactivity was measured with a gamma counter. Controls included wells incubated with nonlabeled MAb 3F6 or unrelated MAb 1C3, directed to a *Leishmania* antigen. Experiments were carried out in triplicate.

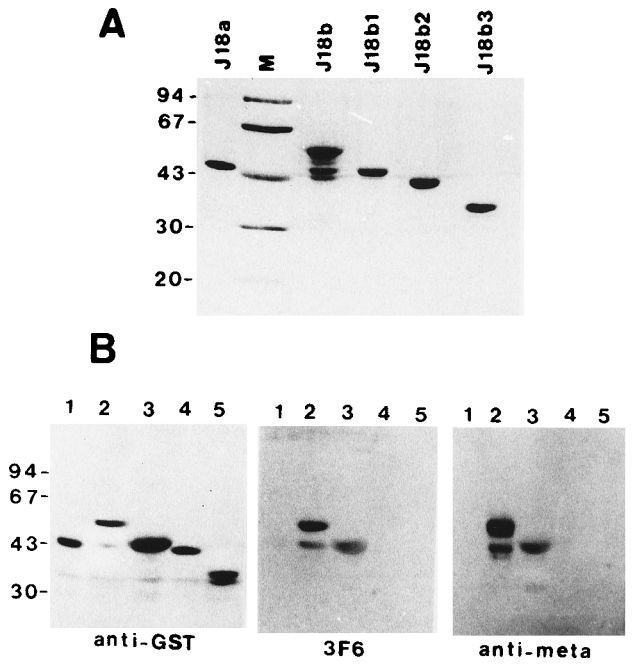

FIG. 2. Analysis of recombinant fragments of *T. cruzi* metacyclic trypomastigote surface antigen gp82. (A) SDS-polyacrylamide gel containing the purified recombinant antigens, stained by Coomassie blue; (B) immunoblot analysis for detection of reactivities of recombinant antigen J18a (lane 1), J18b (lane 2), J18b1 (lane 3), J18b2 (lane 4), and J18b3 (lane 5) with anti-GST antibodies (anti-GST), MAb 3F6 (3F6), or sera of mice immunized with heat-inactivated metacyclic trypomastigotes (anti-meta). Values to the left of the gels are molecular size markers in kilodaltons.

In vitro proliferative response of spleen cells from mice immunized with recombinant antigen. Mice were immunized with four doses of recombinant protein or GST adsorbed in $Al(OH)_3$. One week after the last immunizing dose, the spleens of individual mice were removed and dissociated. Cells were washed twice with RPMI 1640 and adjusted to a concentration of 3×10^6 cells per ml in RPMI 1640 supplemented with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 2 mM L-glutamine, 1% minimum essential medium containing nonessential amino acids, 1 mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.5% normal syngeneic mouse serum. Aliquots of 0.1 ml of the cell suspension were deposited onto 96-well plates to which purified native gp82 was added, in triplicate. The cultures were incubated at 37°C in 5% CO_2 for 3 days and then pulsed with 1 μCi of [5′-^{3}H]thymidine (5 Ci/mol; Amersham) per well for 18 h. At the end of the incubation period, the cells were harvested onto glass fiber, dried, and counted by beta emission spectroscopy. Lymphocyte proliferation was also determined in the presence of anti-CD4 MAb GK1.5 (27) or anti-CD8 MAb H35-89.9 (16), kindly provided by Momtchillo Russo, Universidade de São Paulo, São Paulo, Brazil.

Determination of IFN-γ and nitrite in the supernatant of spleen cell cultures. Spleen cells from mice immunized with the recombinant antigen or GST were plated in 24-well plates and stimulated with native gp82. After 96 h, the supernatant was collected and gamma interferon (IFN-γ) was measured with an enzyme-linked immunosorbent assay (ELISA) kit for quantification of mouse IFN-γ (Genzyme). Nitrite in the culture supernatant was determined by adding 100 μl of Griess reagent (6) to 100 μl of supernatant. The optical density of the resulting chromophore, as well of the reference $NaNO_2$ solution (1 to 100 μM), was read at 540 nm.

Other methods. The standard procedures for SDS-PAGE, immunoblotting, indirect immunofluorescence (IF) assay, agglutination, complement-mediated lysis, and in vitro parasite neutralization have all been detailed elsewhere (14, 22, 32).

RESULTS

Identification of immunoreactive domains of metacyclic trypomastigote gp82. We analyzed by immunoblotting the reac-

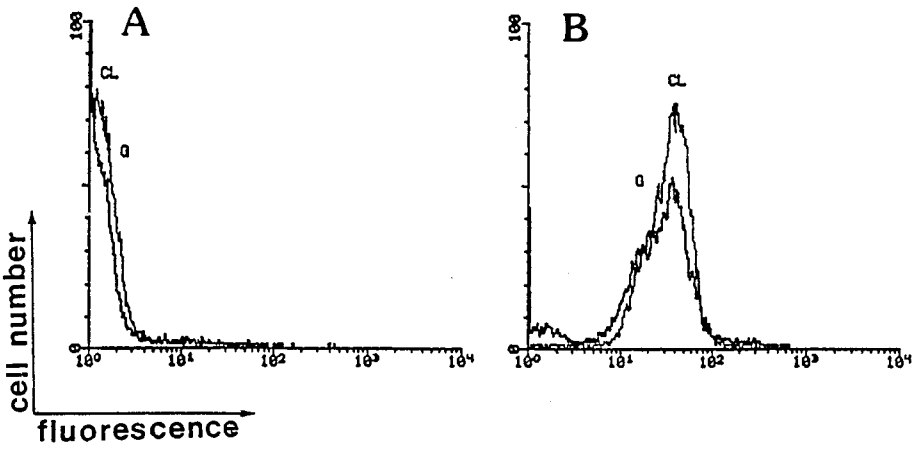

FIG. 3. Expression of 3F6-reactive epitope by *T. cruzi* metacyclic trypomastigotes. Metacyclic forms of strains G and CL were incubated with unrelated MAb 1C3 (A) or with MAb 3F6 (B), reacted with fluorescein-labeled goat anti-mouse immunoglobulin G, and analyzed by FACS.

tivities of the recombinant proteins shown in Fig. 1 with various MAbs and polyclonal antibodies. When nitrocellulose membranes containing purified recombinant antigens were probed with anti-GST antibodies, all fusion proteins displayed similar reactivities (Fig. 2B) together with a few weak bands of lower molecular size, which are likely to be products of protein degradation. Of five gp82 peptides, only J18b and J18b1 were recognized by MAb 3F6 and by sera of mice immunized with heat-inactivated metacyclic forms. The reaction was negative with peptide J18a, which contains the amino-terminal portion of gp82, and with peptides J18b2 and J18b3, representing regions more proximal to the carboxy terminus. Fusion proteins J18b and J18b1, but not the other recombinant peptides, also reacted positively with antibodies elicited by native gp82 and with sera of mice chronically infected with *T. cruzi* (data not shown). These results suggest that regions outside the central domain of gp82 (amino acids 224 and 356) do not contain major linear B-cell epitopes.

Identification of gp82 domain exposed on the surface of live metacyclic trypomastigotes. From our previous studies, we had indications that the 3F6-reactive epitope was exposed on the parasite surface. In fact, we found that this epitope is accessible to antibody 3F6 on the surface of live metacyclic trypomastigotes. When strain G or CL metacyclic forms, processed for flow cytometry analysis as detailed in Materials and Methods, were analyzed by fluorescence-activated cell sorting (FACS), parasites intensely labeled with fluorescein could be detected upon reaction with MAb 3F6 (immunoglobulin G1) but not with unrelated isotype-matched MAb 1C3 (Fig. 3). In the same manner as MAb 3F6, antibodies elicited by native gp82 or by killed metacyclic forms, which react only with recombinant antigens J18b and 18b1 (Fig. 2B), also bound to live parasites. All of these data suggest that the whole gp82 domain containing amino acids 224 to 356 may be exposed on the parasite surface.

Immunogenicity of gp82 recombinant fragments. To determine the abilities of the various recombinant proteins to elicit anti-*T. cruzi* antibodies, groups of mice were immunized with either one of five subunit antigens plus $Al(OH)_3$ as adjuvant, and their sera were used to probe nitrocellulose sheets containing metacyclic trypomastigote extract. As shown in Fig. 4A, gp82 was recognized by anti-J18b and anti-J18b1 antibodies but not by sera of mice immunized with other recombinant proteins, indicating that the region between amino acids 224 and 356 is required for stimulation of B-cell responses. A positive IF reaction, with paraformaldehyde-fixed metacyclic forms, was also observed only with antibodies elicited by peptide J18b or J18b1. Furthermore, anti-J18b antibodies bound to live metacyclic trypomastigotes, as visualized by the IF assay (data not shown).

Determination of T-cell response elicited by gp82 recombinant peptides. We also investigated the abilities of various gp82 subunit antigens to stimulate the T-cell response, which is dependent on the recognition of linear peptide sequences. Spleen cells from recombinant antigen-immunized mice were used to measure the in vitro proliferative activity towards intact gp82. Figure 4B shows proliferation of cells derived from mice immunized with any of the tested antigens. Peptide J18b was the most stimulatory, and J18b3, containing the last 109 amino acids of gp82, elicited the weakest response, slightly higher than that displayed by control spleen cells from GST-immunized mice (Fig. 4B).

Analysis of functional activity of anti-J18b antibodies. Since the J18b recombinant protein elicited antibodies that reacted with gp82 on the surface of live metacyclic trypomastigotes, we analyzed in more detail the properties of these antibodies. Sera of J18b-immunized mice gave IF and ELISA titers of 1,280 and 10,240, respectively, and were unable to agglutinate or lyse parasites in the presence of complement (Table 1), activities that have been associated by some authors with protection against acute *T. cruzi* infection (11, 12). Unlike MAb 3F6, which inhibits penetration of *T. cruzi* into mammalian cells (17), anti-J18b antibodies did not display such activity and also failed to neutralize parasite infectivity in mice: when strain CL metacyclic forms were preincubated for 45 min at room temperature with the sera of GST- or J18b-immunized mice, at a 1:10 dilution, and then injected into naive BALB/c mice (10^5 parasites per mouse), both groups of animals developed comparable parasitemia levels (data not shown).

To determine whether sera of J18b-immunized mice contained antibodies that recognized the same site as MAb 3F6, a competitive binding assay was carried out as described in Materials and Methods. Wells of microtiter plates coated with metacyclic trypomastigote extract were incubated with serially diluted anti-J18b antisera or with control antibodies before addition of ^{125}I-labeled MAb 3F6. As shown in Fig. 5, anti-J18b antibodies only partially inhibited the binding of labeled MAb 3F6, this effect being significantly lower than that of anti-gp82 antibodies. The inhibition was specific since the un-

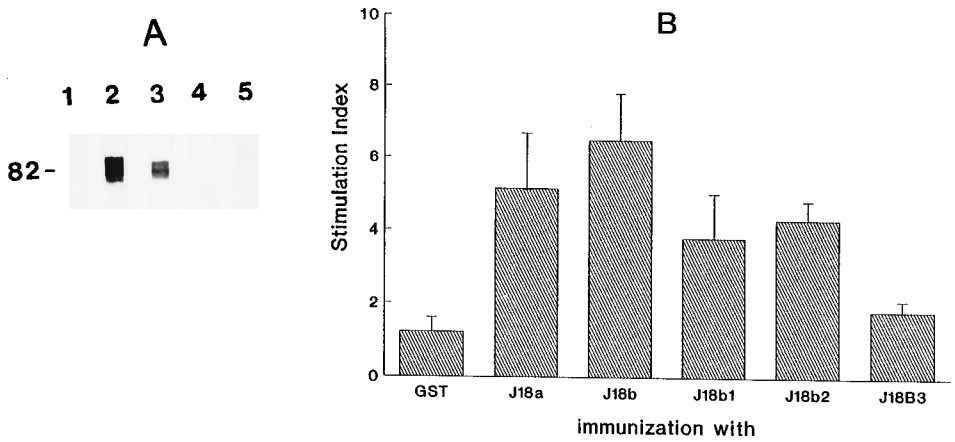

FIG. 4. Antibody and T-cell responses elicited by recombinant fragments of *T. cruzi* metacyclic stage antigen gp82. (A) Metacyclic trypomastigote extracts were subjected to SDS-PAGE, blotted onto nitrocellulose membranes, and then probed with sera of mice immunized with fusion protein J18a (lane 1), J18b (lane 2), J18b1 (lane 3), J18b2 (lane 4), or J18b3 (lane 5). Note that only anti-J18b and anti-J18b1 antisera recognized gp82. (B) The in vitro proliferative response of spleen cells from mice immunized with the indicated recombinant protein, in the presence of 1.25 μg of intact gp82 per ml, was determined by measuring the incorporation of [*methyl*-^{3}H]thymidine as detailed in Materials and Methods. The stimulation index is the counts per minute of T-cell cultures in the presence of gp82 divided by the counts per minute in the absence of antigen. The means ± standard deviations of three experiments performed in triplicate are shown.

related MAb 1C3 did not show any effect, whereas nonlabeled MAb 3F6 prevented binding of labeled antibody.

T-cell proliferation and production of IFN-γ and nitrite induced by immunization with peptide J18b. We further examined the T-cell response elicited by J18b which, among the recombinant proteins tested, showed the highest T-cell stimulatory effect (Fig. 4B). When stimulated in vitro with 1.25 μg of native gp82 per ml, spleen cells from J18b-immunized mice displayed an intense proliferative response, which was abrogated by anti-CD4$^+$ antibodies but not by anti-CD8$^+$ antibodies (Fig. 6). No significant proliferative activity was detected in the presence of unrelated proteins. Proliferation was not further augmented by doses higher than 1.25 μg/ml. Lymph node cells from mice primed subcutaneously with 40 μg of J18b emulsified in Freund's complete adjuvant, and stimulated in vitro with 2.5 μg of native gp82 per ml, displayed lower proliferation rates, with a stimulation index of 4.0 ± 1.2 in four independent experiments.

Also, we measured the concentrations of IFN-γ, a cytokine implicated in resistance to *T. cruzi* (13, 18, 23), and of nitric oxide (as nitrite), which is induced by IFN-γ and whose microbicidal effect against *T. cruzi* has been reported (8). In the supernatants of spleen cell cultures derived from J18b-immunized mice and stimulated in vitro with 1.25 μg of intact gp82 per ml, the levels of IFN-γ were several times higher than those produced by control GST-immunized spleen cells (Fig. 6) or by T cells from J18a- or J18b1-immunized mice (Table 2). Regarding the average concentration of nitrite in J18b-immunized spleen cell preparations, it was significantly higher than that of the control or the spleen cell cultures derived from J18a- or J18b1-immunized mice (Table 2). The concentrations of IFN-γ and nitrite in supernatants of spleen cell cultures of J18b-immunized mice without antigen stimulation, or in gp82-stimulated spleen cells from naive mice, were never higher than 1 ng/ml and 1 μM, respectively. Immunization with a combination of peptides (J18a plus J18b or J18b plus J18b1 plus J18b2) resulted in ~1.4-fold increase in IFN-γ production when compared with immunization with J18b alone.

Resistance of mice immunized with peptide J18b to acute *T. cruzi* infection. The results of experiments with the recombinant protein J18b, which induces high antibody and T-cell responses, led us to test the immunoprotective capacity of this antigen. For that purpose, BALB/c mice immunized with four doses of J18b antigen adsorbed in $Al(OH)_3$ were challenged, by the intraperitoneal route, with 10^5 metacyclic forms of *T. cruzi* CL 10 days after receiving the last immunizing dose. Since the mortality rate upon infection with metacyclic forms is very low, we monitored the course of infection by counting the number of parasites in 5 μl of blood collected 3 days a week starting on day 18 postinoculation. As shown in Fig. 7, immunized mice were resistant to acute *T. cruzi* infection; they either failed to develop parasitemia or displayed very low parasitemia levels in comparison with those of GST-immunized controls. In four independent experiments, in which five to six mice per

TABLE 1. Analysis of anti-*T. cruzi* antibodies induced by recombinant protein J18b

Mice immunized with[a]:	IF titer[b]	ELISA titer	Agglutinating activity[c]	Complement-mediated lysis[d] (%)	Binding to live parasites[e]
GST			−	0	−
J18b	1:1,280	1:10,240	−	6.5	+

[a] Mice were immunized with four doses of fusion protein J18b or GST adsorbed in $Al(OH)_3$ by the schedule described in Materials and Methods.
[b] The IF titer was determined by use of formaldehyde-fixed metacyclic trypomastigotes as antigen.
[c] Agglutination was assayed with different dilutions of serum, from 1:2 to 1:128, and by microscopic examination of parasites after incubation at 37°C for 1 h.
[d] Ten microliters of metacyclic forms (10^8/ml) was incubated with 40 μl of heat-inactivated immune sera. After 10 min at room temperature, 50 μl of normal human serum was added as a source of complement, and incubation proceeded for 30 min at 37°C. The percent lysis was determined by counting the relative numbers of motile versus lysed parasites under a phase-contrast microscope.
[e] Metacyclic trypomastigotes processed as for FACS analysis (see Materials and Methods) were visualized in a fluorescence microscope.

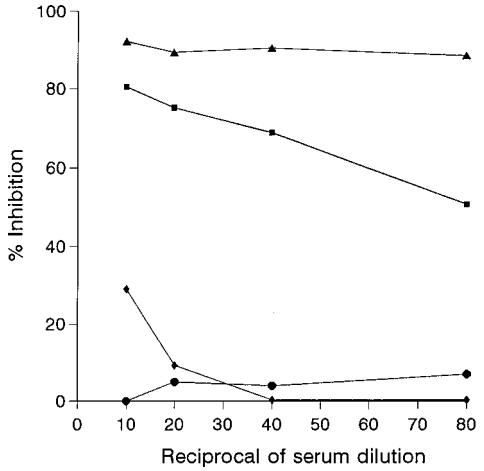

FIG. 5. Competition between MAb 3F6 and immune sera to gp82 or to recombinant antigen J18b. Serial dilutions of sera from mice immunized with four doses of purified gp82 or J18b plus $Al(OH)_3$ were added to microtiter plates coated with sonicated extract of metacyclic trypomastigotes. ^{125}I-labeled MAb was then added. Percent inhibition = 100 − [(cpm of wells with antibody)/(cpm in absence of antibody)] × 100, where cpm is counts per minute. The means of triplicate samples are shown. Standard deviations were never greater than 10% above or below the mean. Note the extensive inhibition of binding of labeled MAb 3F6 by nonlabeled antibody and the lack of inhibition by unrelated MAb 1C3. Symbols: ■, anti-gp82; ◆, anti-J18b; ▲, MAb 3F6; ●, MAb 1C3.

group were challenged, 18 of 21 animals (85.7%) immunized with J18b peptide developed significantly lower parasitemias than those of controls. It is important to note that in J18b1-immunized mice, such a decrease in parasitemia was not observed, suggesting that the gp82 domain, between amino acids 224 and 302, may be relevant in eliciting an effective immune response.

DISCUSSION

In this study, we have further analyzed the immunogenic properties of gp82, the stage-specific 82-kDa antigen of *T. cruzi* metacyclic trypomastigotes, by use of subunit antigens containing peptide sequences of gp82 fused to GST.

Our results indicate that stimulation of the B-cell response by gp82 requires the central domain of the molecule, i.e., that between amino acids 224 and 356. While peptides J18b and J18b1, the carboxy-proximal peptides starting at amino acids 224 and 303, respectively, were capable of inducing antibodies that recognize the native gp82, immunization of mice with fusion proteins corresponding to amino-terminal or other carboxy-terminal sequences of gp82 devoid of the central region (amino acids 224 to 356) did not elicit any detectable antibody response. This finding is compatible with the observation that J18b and J18b1 are also the only peptides that react with MAb 3F6 and with anti-metacyclic trypomastigote antisera. In addition to the metacyclic stage-specific 3F6 epitope, the central region of gp82 may contain epitopes shared by mammalian-stage trypomastigotes. We have found that J18b and J18b1 fusion proteins but not the other recombinant peptides also react positively with sera of mice chronically infected with *T. cruzi*. The presence of such cross-reactivity is not surprising since, as pointed out by Araya et al. (4), the metacyclic trypo-

TABLE 2. Production of IFN-γ and nitrite by spleen cells derived from mice immunized with *T. cruzi* gp82 recombinant proteins[a]

Immunization with:	IFN-γ (ng/ml)	Nitrite (μM)
GST	0.68 ± 0.10	0.75 ± 0.21
J18a	2.10 ± 1.69	0.65 ± 0.08
J18b	7.40 ± 0.98	3.13 ± 0.31
J18b1	1.15 ± 1.05	0.13 ± 0.17

[a] Spleen cells from mice immunized with four doses of purified recombinant antigen or GST plus $Al(OH)_3$, as described in Materials and Methods, were stimulated in vitro with 1.25 μg of native gp82 per ml. Culture supernatants were collected and assayed for the presence of IFN-γ and nitrite. In each case, values obtained in the absence of gp82 have been subtracted. Results are expressed as the mean ± standard deviation of three independent experiments performed in triplicate.

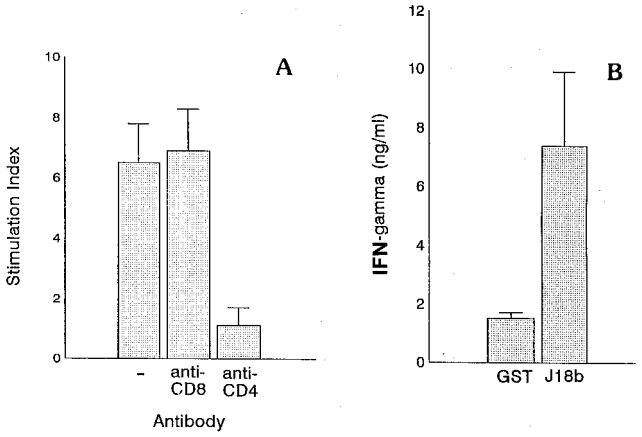

FIG. 6. Analysis of the T-cell response induced by immunization with recombinant protein J18b. (A) The in vitro proliferative response of spleen cells from mice immunized with J18b peptide was measured in the presence of 1.25 μg of gp82 per ml plus anti-CD4 or anti-CD8 antibodies. The stimulation index is the counts per minute of T-cell cultures in the presence of gp82 divided by counts per minute in the absence of antigen. The means ± standard deviations of two experiments and of three experiments, performed in triplicate, are shown. (B) Production of IFN-γ was determined for supernatants of spleen cells of J18b-immunized mice upon stimulation with 1.25 μg of gp82 per ml. Values represent the means ± standard deviations of four experiments carried out in triplicate.

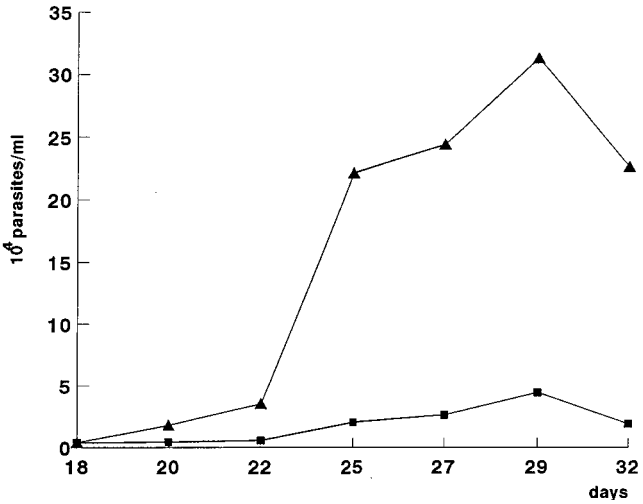

FIG. 7. Time course of *T. cruzi* infection in mice immunized with fusion protein J18b and challenged with metacyclic trypomastigotes. BALB/c mice were immunized with purified J18b antigen plus $Al(OH)_3$ (■) or with GST plus adjuvant (▲), by the protocol described in Materials and Methods and then challenged, by the intraperitoneal route, with 10^5 metacyclic trypomastigotes of strain CL. The values correspond to the mean parasitemia levels of five mice. The average parasitemia level of immunized mice was significantly lower ($P < 0.005$) than that of controls by Student's *t* test.

mastigote gp82 has considerable sequence identity (40 to 56%) with a number of bloodstream trypomastigote surface antigens such as SA-85-1 (10), TSA-1 (7), and Tt34c1 (21).

When our data are compared with those of similar studies performed with the bloodstream trypomastigote stage surface antigen TSA-1 (28), which is a member of the 85-kDa family (7) that displays sequence similarity with metacyclic stage gp82 (4), a number of interesting points emerge. In their study, Wrightsman et al. (28) have reported that the GST-fused recombinant protein corresponding to the amino-proximal portion of TSA-1 is capable of stimulating host-protective immune responses. It should be noted that this TSA-1 amino-terminal portion (amino acids 78 to 607) contains amino acid sequences identical or similar to those of the central domain of gp82 spanning amino acids 224 to 356, which according to our results may be contributing to an effective immune response against acute *T. cruzi* infection. On the other hand, Wrightsman et al. (28) have shown that mice and rabbits immunized with the recombinant TSA-1 developed strong immune responses to the carboxy-proximal but not to the amino-proximal region of TSA-1. On the basis of these findings, they have suggested that responses to immunodominant epitopes within the carboxy-terminal portion of TSA-1, which contains nonapeptide repeats, mask epitopes within the amino-proximal region. Unlike TSA-1, metacyclic stage gp82 lacks repetitive sequences, and its putative immunodominant domain (amino acids 224 to 356) is apparently exposed on the parasite surface.

As regards gp82 T-cell epitopes, they are dispersed along the molecule, as indicated by in vitro proliferative activity of spleen cells from recombinant protein-immunized mice, in the presence of native gp82. Of all subunit antigens tested, the J18b peptide was the most stimulatory. This is in accord with the computer analysis, based on the identification of regions containing amphipathic α-helices, that indicated the presence of more T-cell sites in the sequence spanning amino acids 224 to 302, which is contained in the J18b peptide, in comparison with those in regions more proximal to the amino or carboxy terminus.

Immunization of mice with recombinant J18b antigen (amino acids 224 to 516) conferred resistance to acute *T. cruzi* infection. It is important to note that immunization of mice with peptide J18b1, corresponding to amino acids 303 to 516, was unable to reduce the parasite load to any significant degree, suggesting that the contribution of the gp82 domain, which consists of amino acids 224 to 302, is important for the induction of an effective immune response.

The mechanisms by which acute *T. cruzi* infection is controlled in J18b-immunized mice may be predominantly T cell mediated rather than dependent on the B-cell response. Our results suggest that the contribution of antibodies elicited by the J18b peptide, if any, may be minimal. For instance, they failed to inhibit the penetration of metacyclic forms into cultured mammalian cells or to reduce parasite infectivity in mice. As indicated by the competitive binding assay, only some of the anti-J18b antibodies recognize the same epitopes as MAb 3F6 (Fig. 5), an antibody that, at concentrations of 250 to 500 μg/ml, significantly inhibits *T. cruzi* entry into target cells (17). Thus, it is possible that the lack of cell invasion inhibitory activity of anti-J18b antisera is associated with the low concentrations of 3F6-related antibodies. On the other hand, the resistance conferred by immunization with J18b antigen may well be the result of stimulation of a T-cell response that leads to production of IFN-γ, a cytokine that has been shown by several investigators to be an important factor for protection against *T. cruzi* (13, 18, 23). Spleen T cells of J18b-immunized mice displayed a strong proliferative activity in vitro towards the native gp82 and produced IFN-γ at amounts significantly higher than that of controls (Fig. 6). In this regard, also significant are the increased levels of nitric oxide (Table 2), a potent microbicidal agent implicated in resistance to *T. cruzi* infection (8).

Our study with recombinant proteins based on the metacyclic trypomastigote surface 82-kDa antigen has allowed us to define some sequences of this molecule that are implicated in eliciting B- and T-cell responses and may even confer resistance to acute *T. cruzi* infection. On the basis of these data, we can now construct and test other recombinant antigens and synthetic peptides, towards a fine mapping of gp82 epitopes, including those relevant for immunoprotection.

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