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Expression of a Recombinant Fab Antibody Fragment against Cruzipain, the Major Cysteine Proteinase of *Trypanosoma cruzi*

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Cruzipain, the major proteinase of *Trypanosoma cruzi,* plays an important role in the biology of this parasite. This study reports the development of a recombinant Fab antibody, using RNA isolated from the anti-Ag163B6 hybridoma against cruzipain. This procedure involves the use of cDNAs obtained with the aid of a specific set of primers complementary to the complete light kappa chain (Lκ) and the first two domains of the IgG1 heavy chain (VH/CH1). These products were subsequently cloned in the pComb3 system, from which the gIII gene had been removed, and expressed in *Escherichia coli* cells. The recombinant Fab molecule recognized cruzipain by ELISA, in a fashion similar to the original mAb anti-Ag163B6. Nucleotide sequence analysis of the recombinant molecule, together with its immunological recognition by specific anti-mouse IgG (Fab)2, indicated the immunoglobulin nature of the recombinant product. Moreover, both the mAb anti-Ag163B6 and the soluble Fab fragment described here react similarly with the intact parasite surface, as observed in an indirect immunofluorescence assay. In conclusion, our recombinant Fab anti-Ag 163B6 allows the possible use of this molecule for diagnosis, antigen purification, and eventually treatment of Chagas-afflicted individuals. © 1998 Academic Press

Trypanosoma cruzi is the parasitic protozoan causative agent of Chagas' disease, which affects more than 20 million people on the American Continent (1). The disease has two clinically different stages: the acute stage characterized by the presence of trypomastigotes in blood which is, in most cases, symptomless, while the chronic stage presents low levels of parasitemia. The most severe consequence of the chronic stage is the occurrence of different cardiopathies, such as mild arrhythmia, right or left branch blockages, and/or severe myocardiopathies.

Another endemic illness in Latin America, Leishmaniasis, is caused by infection with parasites of the genus *Leishmania,* also a Trypanosomatidae (2). Both diseases constitute serious public health problems within this geographical region, where it is possible to find individuals infected by both parasites (3, 4).

Conventional serological reactions for the diagnosis of Chagas' disease and leishmaniasis, such as the indirect immunofluorescence assay, indirect hemagglutination, and direct agglutination, use as antigens either whole parasites, or subcellular fractions of *Trypanosoma cruzi* and *Leishmania spp.* When these complex antigen preparations are used, a marked cross-reactivity among sera from patients suffering from these parasitoses is found (5, 6). Since the therapeutic approaches to Chagas' disease and leishmaniasis are completely different, it is important to have a diagnostic tool to differentiate among Chagas' disease, Leishmaniasis or mixed infection (7). Recently, patients carrying a double infection were identified by means of an immunoblotting assay using a *T. cruzi* epimastigotes homogenate and an ELISA with Ag163B6 *T. cruzi* antigen, a glycoprotein identical to cruzipain (8). Studies performed by Martinez *et al.* (9), and by Malchiodi *et al.* (4), showed that most sera from chagasic patients reacted with the purified antigen, whereas leishmaniasis sera showed no reactivity. These characteristics make this antigen a powerful candidate to perform differential diagnoses of both infections. Since the purity of Ag163B6 is crucial in designing new approaches in diagnosis and treatment, monoclonal antibodies anti-

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Ag163B6 (mAb anti-Ag163B6), bound to affinity columns were used to obtain highly purified preparations of this antigen (10).

During growth of hybridomas for large-scale production of mAb, problems in cell-line stability often occur (11). This prompted us to explore new avenues in the production of improved immunoglobulin-derived reagents against cruzipain. The expression of recombinant proteins in *Escherichia coli*, is a well established technology, that allows unlimited gene constructions to obtain recombinant immunoglobulin products. The fast growth of *E. coli* and their simple fermentation requirements, make the large-scale production of antibody fragments relatively convenient.

The present study reports the production of a Fab recombinant antibody fragment, by cloning the variable regions of the mAb anti-Ag163B6 monoclonal antibody into the pComb3 expression vector (12). The performance of the recombinant Fab and the mAb anti-Ag163B6, were compared by both the ELISA and IIF techniques. The recombinant Fab recognized cruzipain by ELISA on cruzipain-coated plates and by IFF on the intact parasite, in a fashion similar to the original monoclonal antibody. Currently, we are attempting to use this development in the design of innovative diagnostic tools, as well as a possible therapeutic molecule.

MATERIALS AND METHODS

Reagents, strains, and media. All restriction enzymes and buffers for cloning and ultra pure agarose, were purchased from GIBCO BRL-Life Technologies. Agarase and T4 ligase were from New England Biolabs Inc. Primers for PCR and sequencing were purchased from GIBCO BRL-Life Technologies, and used according to Huse *et al* (13). Mini preparation plasmid DNA solutions were prepared as described by Sambrook *et al* (14). Escherichia coli strain XLI-Blue [F′*pro AB, lac* I^q *Z*Δ*M15,* Tn *10* (tet^r)] was from Stratagene. LB (10 g bacto tryptone, 5 g bacto yeast extract, 10 g NaCl per liter, pH 7.0) was used for liquid bacteria cultures. Mouse 163B6 (IgG1) was obtained as previously described by Carbonetto *et al* (10). Standard LB agar plates supplemented with 200 μg/ml ampicillin, were utilized as solid media. Isopropyl β-D-thiogalactopyranoside (IPTG) from Sigma Chemical Co., was used as inductor. All other reagents were of analytical grade.

RNA isolation and reverse transcription. Total RNA was extracted as described (14), from 5×10^8 163B6 hybridoma cells, harvested and washed with GKN. The RNA was redissolved in 200 μl of TE (10 mM Tris–HCl, pH 7.4, 1 mM EDTA pH 8.0), 500 μl of ethanol were added and the final solution stored at −70°C until needed. Total copy DNA (cDNA) was obtained by a reverse transcription reaction. RNA (2 μg) was mixed with 2.5 μM random hexamers, 1 mM dNTP (both from Promega Corporation), and buffer (10 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 10 mM Tris HCl, pH 9.0). The mixture was incubated 10 min at 65°C and cooled to 20°C. AMV reverse transcriptase (2.5 units) and 1 unit RNAse inhibitor, (Promega Corp.), were added and incubated 10 min at 22°C, followed by 40 min at 42°C, and finally 5 min at 95°C to stop the reaction.

Construction of a plasmid expressing the Fab fragment (pCHL-$gIII^-$*).* *E. coli* XLI-Blue cells were transformed with pComb 3 phagemid (Fig. 1), and grown overnight in LB medium supplemented with 200 μg/ml ampicillin. Plasmid DNA preparation was performed as described by Sambrook *et al.* (14). Plasmidic DNA was resuspended in 500 μl of deionized water, and extracted with phenol-chloroform and chloroform followed by ethanol precipitation, and concentrated by centrifugation. Vector DNA (1 μg) was resuspended in deionized water and digested 1 h with 10 of units *Xho*I and 9 units *Spe*I, in a final volume of 100 μl at 37°C. The reaction mixture was then extracted with phenol-chloroform and chloroform, followed by ethanol precipitation. The pellet was resuspended in 75 μl of deionized water, and the DNA purified on a preparative 1% low melting point agarose gel (14).

Heavy IgG1 chain Fd PCR DNA amplification was performed with total cDNA obtained as described above, using the following set of primers: upstream 5′-AGGTCCAGCTGCTCGAGTCTGG-3′, and downstream 5′-AGGCTTACTAGTACAATCCCTGGGCACAAT-3′. Five microliters of the cDNA was mixed with 0.03 mg/ml of each primer, 1.3 mM $MgCl_2$, 0.167 mM of each dNTP, 0.5 units *of Thermus aquaticus* DNA polymerase (Promega Corp.), and buffer (10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100) in a final volume of 50 μl. Amplification cycles for the first run of PCR were 95°C for 5 min, 60°C for 90 s, 72°C for 90 s, and 95°C for 90 s, for 30 cycles, followed by a final extension at 72°C for 5 min. All PCRs were carried out in a Perkin–Elmer Model 9600 thermal cycler. The PCR products (500 ng) were digested with 10 units of *Xho*I and 9 units of *Spe*I, and purified on low melting point agarose gel, as described above. Fifteen ng of this DNA were ligated overnight with 20 ng of the prepared *Xho*I/*Spe*I pComb 3 vector, in a final volume of 20 μl together with 0.1 units of T4 DNA-ligase at 16°C. Transformation was carried out by incubating 10μl of ligation mixture 30 min with 100 μl of competent *E. coli* XLI-Blue on ice, followed by 2 min at 42°C, and a further 10 min on ice. Nine hundred microliters of LB were subsequently added, and incubated 60 min at 37°C. The culture was then pelleted by microcentrifugation, and resuspended in 800 μl of LB solution, and an aliquot was withdrawn for plating at 37°C overnight.

Light chain (Lκ) was amplified and cloned as previously described above using the following set of primers: upstream 5′-CCAGTT-CCGAGCTCGTGCTCACCCAGTCTCCA-3′, and downstream 5′-GCGCCGTCTAGAATTAACACTCATTCCTGTTGAA-3′. The amplified products were digested with 40 units of *Sac*I and 80 units of *Xba*I. Forty ng of the purified *Sac*I/*Xba*I PCR fragments were ligated with 20 ng of the purified *Sac*I/*Xba*I pCH plasmid, to recover the pCHL vector. To obtain pCHL-$gIII^-$ (Fig. 2), 1 μg of pCHL construct was digested 2 h with 9 units of *Spe*I and with 10 units of *Nhe*I, in a final volume of 100 μl at 37°C, to remove the gIII gene. The vector was purified on a preparative low melting point agarose gel, and self-ligated. Transformation was performed as described above.

Competent cell preparation. LB (50 ml) was inoculated with 3 ml of fresh overnight culture of *E. coli* XLI-Blue, grown in LB supplemented with 40 μg/ml tetracycline. The culture was grown until OD_{600} was 0.5, then centrifuged 10 min at 5000 rpm in a GS 3 (Sorvall) rotor at 5°C. The pellet was resuspended in 5 ml of TSS buffer (10 g/l Bacto-tryptone, 5 g/liter yeast extract, 5 g/l, NaCl, 100 g/liter polyethyleneglycol-4000, 5% dimethyl sulfoxide, 50 mM $MgCl_2$, pH 6.5) and stored at −70°C.

Preparation of periplasmatic fraction Fab (pfFab). Transformed pCHL-$gIII^-$ *E. coli* cells were grown at 37°C in 1 liter of LB medium containing ampicillin until $OD_{600} = 0.1$ was achieved. IPTG (1 mM), was then added, and the culture continued overnight at 30°C. Control cultures were grown under the same conditions but in the absence of IPTG. Cells were pelleted by 15 min centrifugation at 4000 rpm in a GS3 (Sorvall) rotor at 5°C, resuspended in 50 ml of cold PBS containing 35 μg/ml phenylmethylsulfonyl fluoride, and lysed by sonication. Debris was pelleted by 20 min centrifugation at 10000 rpm in a GS3 (Sorvall) rotor at 4°C. The supernatant (pfFab) was used directly for ELISA and SDS–PAGE-immunoblotting analysis and for indirect immunofluorescence (IIF).

ELISA. Multiwell plates (Nalge Nunc International) were coated with 0.5 μg/well of cruzipain-rich fraction from *T. cruzi* (8). The pfFab preparation was assayed at a onefold dilution, while in a

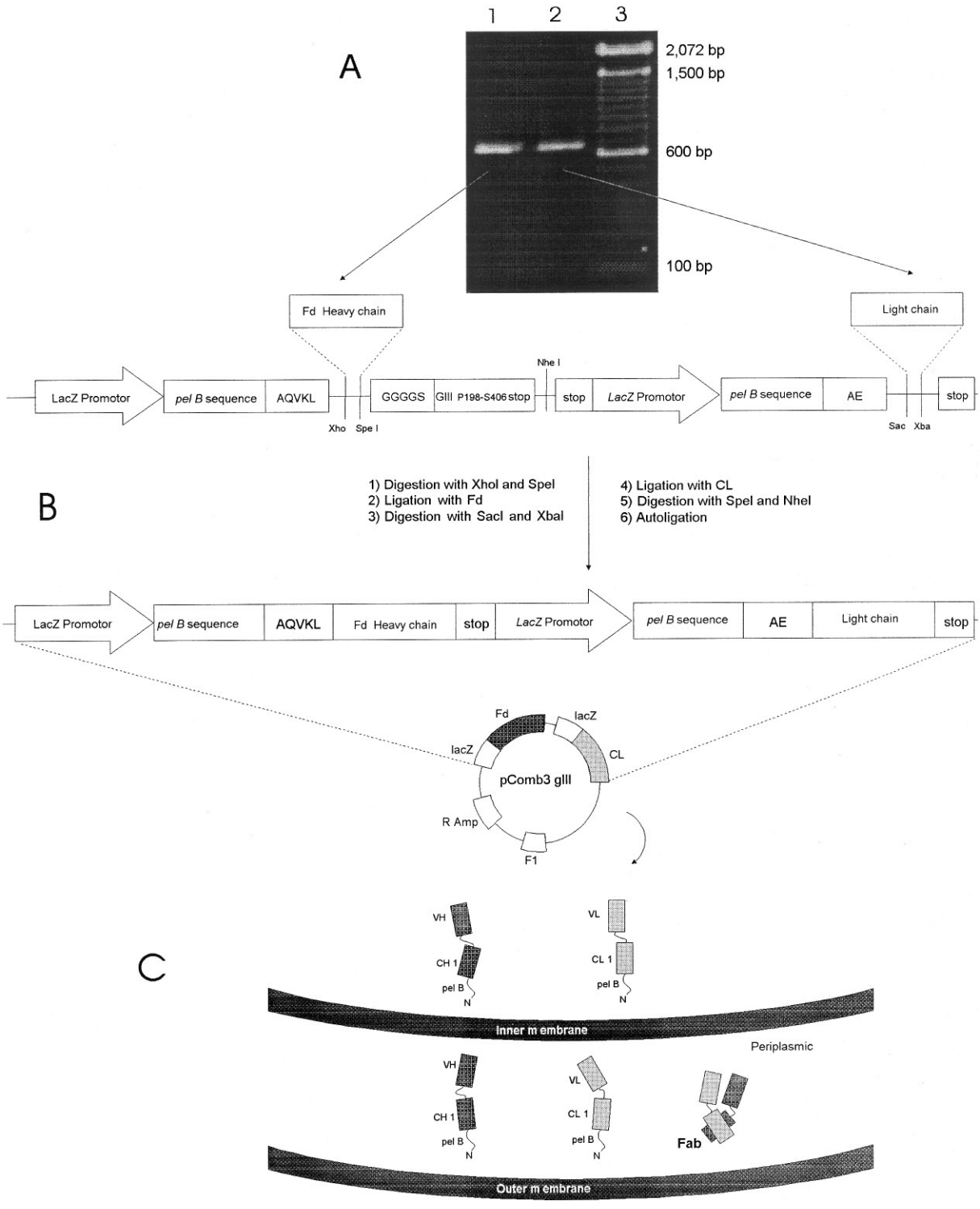

FIG. 1. Representative diagrams that depicts the principal pathway to obtain Fab fragments. (A) PCR amplification of Fd and CL from total RNA of hybridoma 163B6. Amplification was performed with cDNA obtained after reverse transcription with primer specific for amplification of heavy or light chain sequences (Materials and Methods). Lane 1: heavy chain amplification product. Lane 2: light-chain amplification product. A band of about 700 base pairs is observed after successful amplification. Lane 3: molecular size markers (100 bp DNA ladder GIBCO BRL). Fd and CL bands were purified and treated with *Xho*I/*Spe*I and with *Sac*I/*Xba*I, respectively. (B) Fd and CL cDNA PCR products were cloned into the pComb3 plasmid treated with *Xho*I/*Spe*I and *Sac*I/*Xba*I. To obtain soluble periplasm Fab products, the pCHL plasmid was treated with *Spe*I and *Nhe*I to release gIII P198-S406. (C) Fd and CL are directed to the periplasm by pel B signal sequence. Fd and Cl assembly takes place in the oxidizing environment of the periplasm, yielding the heterodimer Fab.

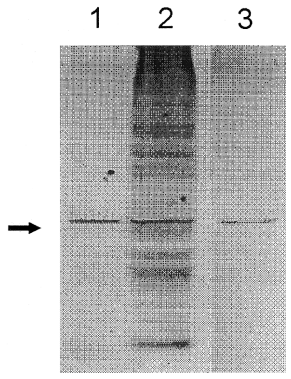

FIG. 2. Western blot analysis of the recombinant Fab anti-Ag163B6 protein. Lanes 1 and 2: *E. coli* total cellular extracts cultured without and with IPTG, respectively. Lane 3: Periplasmic space fraction of *E. coli* cultured with IPTG developed with anti-mouse-Fab. Samples were resolved in 12% polyacrylamide gels, blotted and developed with rabbit anti mouse IgG (Fab′). Arrow indicates the position of Fab anti-Ag163B6.

separate assay, as a positive control, mAb 163B6 was assayed at 1:400 dilution. Both in 1.5% nonfat dried milk in PBS (8.5 mg/ml NaCl, 1.43 mg/ml K_2HPO_4, 0.25 mg/ml KH_2PO_4). Biotinylated-conjugated horse immunoglobulins to IgG mouse immunoglobulins (Vectastain, Vector Laboratories Inc.) were used at a 1:1600 dilution in 1.5% non-fat dried milk in PBS. Avidin–alkaline phosphatase (Sigma Chemical Co.) was used at a 1:500 dilution in 1.5% nonfat dried milk in PBS. Finally, P-nitro phenyl phosphate was used as substrate.

SDS–PAGE and immunoblotting. pfFab preparation, 1 ml of crude bacterial extract containing the recombinant Fab, and crude bacterial control cultures, were grown as described and concentrated by centrifugation, resuspended in 100 μl of 50 mM Tris–HCl, 4% sodium dodecyl sulfate (SDS), 20% glycerol, and 2 mg bromophenol blue, and boiled 3 min. Samples were loaded on a 10% SDS–polyacrylamide gel, run for 1 h, and electrotransferred onto nitrocellulose membrane (Amersham International plc., England). Filters were incubated with 4 μg/ml of rabbit anti mouse IgG $(Fab')_2$ (Pierce, Rockford) in 1.5% nonfat dried milk in PBS. Reactivity was visualized by using an anti-rabbit polyclonal antibody linked to horseradish peroxidase (Amersham International plc, England) at a 1/1000 dilution in PBS containing 4-Cl-naftol and H_2O_2.

Indirect immunofluorescence (IIF) on intact fixed parasites. Formalin-fixed epimastigotes of the RA strain, were used for IIF as described by Alvarez *et al* (15). Monoclonal antibody anti-163B6 ascitic fluid and dilute 1:30 pfFab, were assayed. Rabbit anti-mouse IgG $(Fab')_2$ immunoglobulin coupled to fluorescein isothiocyanate (Sigma Chemical Co.) was used as second antibody. An isotype-matched anti-*Brucella abortus* BI24 (16) was used as a negative control. The slides were observed in a Zeiss standard 14 IFD epifluorescence-equipped microscope.

Sequencing of heavy and light chains from the recombinant immunoglobulin. Plasmid DNA preparation was performed as described above. Double-stranded DNA were sequenced by Sanger method, using a SequiTherm EXCEL DNA Sequencing Kit (Epicentre Technologies), according to the manufacturer's instruction. The corresponding up primers were used for both the heavy and light chains.

RESULTS

In this study, we have generated a cruzipain-binding molecule. Using the cDNA from a hybridoma cell line expressing anti-Ag163B6 (cruzipain) specific IgG, the corresponding Fab fragment was successfully cloned and expressed in *E. coli*, comprising the first two domains of the IgG1 heavy chain (VH/CH1) and the complete light kappa chain (Lκ) (Fig. 1). Both chains are joined by a disulfide bond, increasing heterodimer stability. This reaction takes place in the bacterial oxidative environment, within the periplasmic space. The Fdγ1 (VH/CH1) and Lκ (VL/CL) cDNAs were obtained from RNA by RT-PCR using random hexamers as primers, followed by a PCR amplification with specific oligonucleotides (Fig. 1). No bands were detected when PCR was carried out in the absence of primers. Thus, the bands recovered by RT-PCR were due to amplification of RNA by specific IgG primers, and not the result of DNA contamination or RNA carryover.

The multicopy vector pComb3 was used to express the Fab fragment. Both, the VH/CH1 and Lκ cDNAs, were inserted into pComb3, in phase with leader peptide sequences (pelB-HC and pelB-LC). To obtain soluble Fab fragments, removal of P198-S406 in gene III from the pComb3/Fab DNA, was achieved by cleavage with both *SpeI* and *Nhe*I, followed by direct religation, since the *SpeI* and *NheI* ends are compatible. This construct, denominated pCHL-gIII$^-$, was used to transform competent *E. coli* XL1-Blue cells (Fig. 1). Ampicillin resistance allowed the selection of positive clones, and recombinant bacteria were identified by plasmid analysis.

The identity of the cDNAs that encoded the variable region of the Fab molecule, was confirmed by sequencing analysis. A greater than 90% homology with mouse immunogloblulins was detected.

To express the Fab fragment, recombinant bacteria were grown in the presence of IPTG. Total cellular extract and the periplasmic fraction were resolved by SDS–polyacrylamide gel electrophoresis, transferred

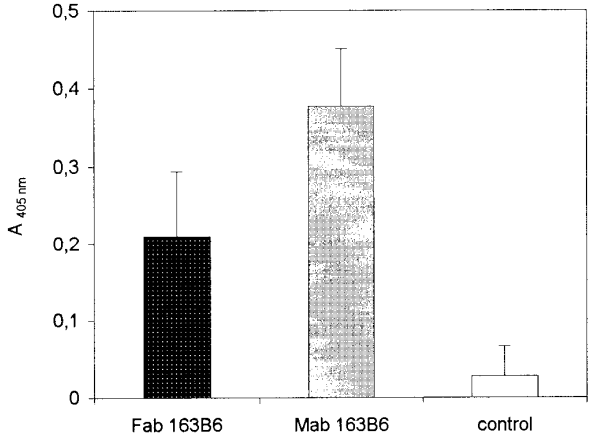

FIG. 3. ELISA reactivity of recombinant Fab anti-Ag163B6 and mAb anti-Ag163B6. The absorbance reading at 405 nm represents binding of the antibodies to cruzipain. A preparation of *E. coli* cells transformed with pComb3 was used as control.

to a filter and probed with anti-mouse Fab. The recombinant protein was visualized as a band of approximately 45 kDa (dimeric version) and as a 22-kDa band (monomeric form) (Fig. 2).

The recombinant Fab molecule recognized cruzipain, as demonstrated by the ELISA and IIF assays. Microwells coated with cruzipain, were incubated with IgG mAb 163B6 monoclonal antibody (the original cognate) and with the newly obtained Fab. Results derived from ELISA (Fig. 3), show that both the monoclonal antibody and the recombinant immunoglobulin fragment bind to cruzipain, while no reaction was observed with the periplasmic fraction from pComb3-transformed bacteria. Accordingly, both the mAb 163B6 and the soluble recombinant Fab anti-Ag163B6, presented the same reactivity. Specific fluorescence was detected on the whole parasite surface (Fig. 4), while no significant fluorescence was detected with an irrelevant monoclonal antibody was used as control.

Neither the mAb 163B6, nor the Fab anti-Ag163B6 recognized cruzipain in a western blotting assay (data not shown).

DISCUSSION

The importance of having purified specific antigens for the diagnosis of parasitic infections has been previously assessed (17). This statement is enhanced in endemic regions, where several antigenically-related parasites coexist.

Malchiodi *et al.* using the immunoaffinity-purified Ag163B6 in ELISA, were able to discriminate between chagasic and leishmaniasic infection (4).

As mentioned above, large-scale monoclonal production has some disadvantages. Our purpose was to produce a recombinant antibody exhibiting the same specificity as mAb 163B6, but easier to produce and more convenient to handle. The procedure involved the use of total mAb 163B6 RNA, followed by RT-PCR to obtain cDNA. The pComb3 system was chosen for cloning and expression. This pBluescript-derived phagemid, contains both the origin of replication of the multicopy plasmid ColE1, and the origin of replication of the filamentous bacteriophage f1. The presence of two *lac* promoters, allows the transcription of both the heavy and light chains. Phagemid pComb3 is also equipped with the carboxyl-terminal half of gene III. This gene was removed to obtain a product that would not be exposed, instead, the pelB-Hc and the pelB-Lc direct recombinant peptides to the periplasmatic space were Fd and Lκ assembly takes place by the formation of disulfide bonds. Subsequently *pelB* is cleaved by the signal peptidase, releasing fragments into the periplasmic space.

Nucleotide identification of the Fab anti-Ag163B6 as a recombinant gene, derived from a total RNA of the

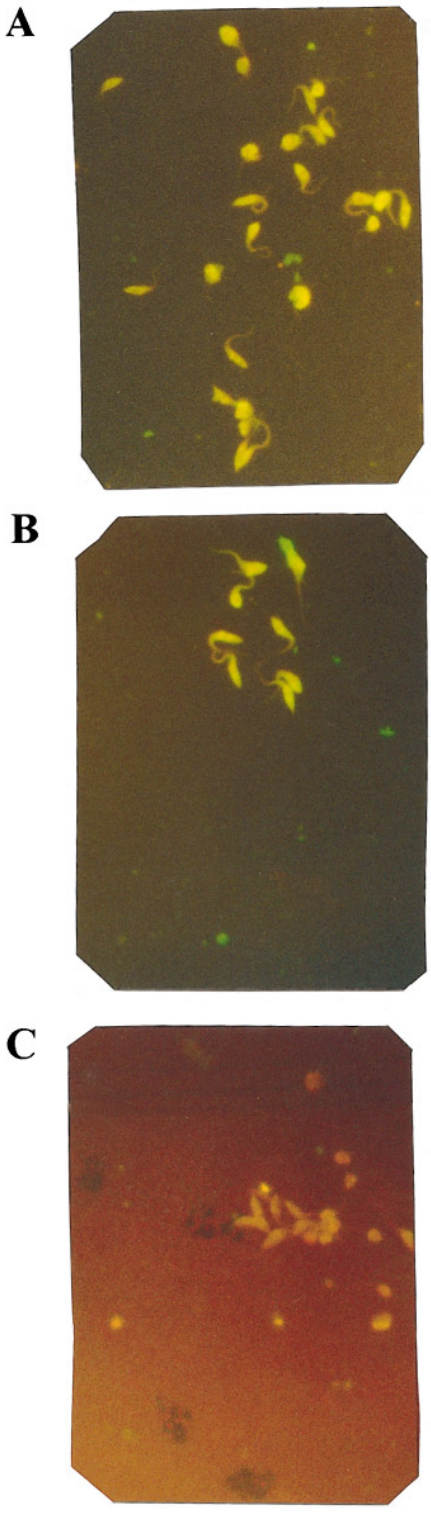

FIG. 4. Indirect immunofluorescence (IIF). Reactivity of mAb anti-Ag163B6 ascitic fluid (A) and Fab 163B6 (B), with formalin-fixed epimastigotes. Rabbit anti-mouse IgG (Fab′)2 immunoglobulins coupled to fluorescein isothiocyanate was used as the second antibody. An isotype-matched anti-Brucella abortus Mab (BI24) (C), was used as negative control. The exposure time for negative control was increased in order to visualize the parasites.

monoclonal antibody anti-cruzipain, showed a great homology with available murine IgG data sequence. In addition, the recombinant product was recognized by an anti-mouse Fab-antibody in a western blotting assay. Moreover, we demonstrated the selective performance of the recombinant Fab anti-Ag163B6, by comparison with the ability exhibited by the mAb anti-Ag163B6, when probed with a cruzipain-enriched extract by ELISA, and on the intact parasite by IIF. The latter allows us to speculate on the potential of using this recombinant immunoglobulin as a therapeutic tool for Chagas' disease.

We have recently reported that antibodies elicited specifically against *T. cruzi*, are implicated in the induction of functional myocardial impairments in chronic Chagasic heart disease (18). Viotti *et al.* suggested that the best solution to alleviate Chagas' disease symptoms, is to eliminate the parasite with an efficient trypanocidal chemotherapeutic protocol (19). In accordance, most of the later approaches to design anti-trypanocidal drugs are focused in their ability to inactivate the proteinase-associated activity of cruzipain (20, 21). It is important to note that the C-terminal domain of cruzipain has been reported to contain a number of post-translational modifications, and is responsible for the immunodominant antigenic character of cruzipain in natural human infections (22, 23). In this sense, the recent elucidation of the crystal structure of cruzipain (24), would allow a more rational development of trypanocidal agents.

Our results, specially those concerning IIF, suggest the possibility of using cruzipain as a specific target against the parasite, by means of the recombinant mAb 163B6 Fab fragment conjugated with trypanocidal drugs. Among the most promising are cystein protease inhibitors such as Z-Phe-Ala-FMK (24) or LVG-CHN2 (25). Studies are being currently conducted to elucidate the possible discriminatory capacity of the recombinant Fab, in recognizing the parasite during its morphogenetic changes throughout its life cycle, in both the insect vector and in the vertebrate host. This is an important issue, since cysteine proteinases are relevant in the life cycle of *T. cruzi*, and it has been suggested that the differentiation steps are the most susceptible to cysteine protease inhibitors (20).

This does not rule out the possible use of recombinant mAb 163B6 Fab fragment for affinity purification purposes as formally proposed.

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