Evidence for the Participation of the Ssp-3 Antigen in the Invasion of Nonphagocytic Mammalian Cells by *Trypanosoma cruzi*

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Summary

Trypomastigotes of *Trypanosoma cruzi* have to invade mammalian cells in order to multiply. They bear on their plasma membrane a sialic acid–containing epitope (Ssp-3) defined by a series of monoclonal antibodies (mAbs). Previous investigations have shown that Fab fragments of these mAbs inhibit the attachment of trypomastigotes to 3T3 fibroblasts. To further define the role of Ssp-3 in invasion, here we use, as targets for infection, L cells and CHO cells stably transfected with cDNA coding for the mouse Fc receptors genes. When the trypomastigotes are incubated with small, nonagglutinating amounts of antibodies to Ssp-3, their attachment to the transfected cells is greatly enhanced, without a parallel increase in invasion. The enhancement in attachment is Fc mediated, since it is abolished by treatment of the transfected cells with mAbs to Fc receptors. In contrast, both attachment to, and invasion of, the transfected cells are increased if the parasites are incubated with polyclonal or monoclonal antibodies against *T. cruzi* surface membrane antigens other than Ssp-3. If, however, antibodies to Ssp-3 are added to the incubation mixtures containing any of the other anti-*T. cruzi* antibodies, the enhancement of invasion (but not of attachment) is reversed. These results suggest that Ssp-3-bearing molecules participate in the process of parasite internalization.

Indirect but compelling evidence from several laboratories indicates that invasion of target cells by trypomastigotes of *Trypanosoma cruzi* is receptor mediated (1–9). Nevertheless, the nature of the parasite ligand and the nature of target cell receptors are unknown. Also unresolved is the question of whether the same or different receptor-ligand pairs are involved in invasion of the various cell types. One obstacle for approaching these problems experimentally has been the lack of methodology to distinguish between the complex series of events that are likely to occur during the invasion process.

We have recently developed a method that permits us to separate unequivocally *T. cruzi* adhesion from penetration, and that demonstrates that the attachment of trypomastigotes to fibroblasts is receptor mediated (10). Furthermore, we have found that adhesion probably involves the sialilated, trypomastigote-specific Ssp-3 epitope (11), since a Fab fragment of a mAb directed to Ssp-3 inhibits attachment to, and invasion of, the target cells in a dose-dependent manner (10).

Here we study further the function of Ssp-3. We ask whether the encounter between the Ssp-3 epitope and its receptor is necessary to trigger the events leading to internalization of the parasite. The alternative possibility is that this encounter merely serves to bring the parasite into close contact with the membrane of the target cells, and that the ensuing penetration is an active phenomenon dependent on parasite energy, and not contingent on specific signalling events. In other words, does the parasite enter cells irrespective of where it initially binds?

The experiments to be described were performed in an effort to distinguish between these two alternatives. We reasoned that if the Ssp-3-receptor interaction serves only a binding function, then it could be replaced by other unrelated ligand-receptor pairs. We have used as targets nonphagocytic cells (CHO and L cells) transfected with cDNA coding for the murine Fc receptors FcγRIII and FcγRII (12). The transfected cells were then incubated with trypomastigotes coated with monoclonal and polyclonal antibodies directed to different surface molecules of the parasite. This experimental model seemed appropriate to study this problem because in the nonphagocytic CHO and L cells, the transfected Fc receptors do not mediate internalization of antibody-coated inert particles (13).

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Materials and Methods

Parasites and Cell Lines. *T. cruzi* trypomastigotes, Y strain (14), were grown in cultures of LLC-MK2 cells (CCL-7; American Type Culture Collection [ATCC], Rockville, MD). Usually, 75-cm^2 flasks, with subconfluent cultures of LLC-MK2 cells, were infected with 5×10^6 trypomastigotes. The LLC-MK2 cells were grown in low glucose DME with penicillin and streptomycin (Gibco Laboratories, Grand Island, NY), containing 10% FCS at 37°C, 5% CO_2. Free parasites were removed 24 h later, and the cultures were maintained in 10% FCS-DME. After day 5 postinfection, the culture supernatants contained trypomastigotes, intermediate forms, and amastigotes. Isolation of trypomastigotes from this mixture was performed as described (10).

BALB/3T3 fibroblasts, clone A31 (CCL-163; ATCC), were grown in 10% FCS-DME. Chinese hamster ovary (CHO) cells K1 (CCl 61; ATCC) and mouse L-M(TK$^-$) fibroblasts (CCL 1.3; ATCC) were grown in the α modification of Eagle's medium (αMEM) (Gibco Laboratories), supplemented with proline, nonessential amino acids, deoxyribonucleotides, penicillin, and streptomycin, and 10% gamma globulin–free FCS (Gibco Laboratories). CHO cells were stably transfected with murine FcγRIII-2 plus γ chain of FcεRI (CHO FcRIII α + γ) (15), or with the same FcγRIII-2 truncated at the cytoplasmic domain by conversion of the glutamine 210 codon to a stop codon (CHO Q210). This mutant is expressed on the cell surface without the expression of the γ chain (our unpublished observations). Fibroblast Ltk$^-$ was stably transfected with murine FcγRII (16). Transfected cells were maintained in the above medium containing 0.077 mg/ml of geneticin G-148 (Gibco Laboratories).

Antibodies and Other Reagents. The mAbs 3C9, anti-Ssp3 (IgG1), and 2H1 anti-Ssp2 (IgG2a) (11) were purified from ascitic fluids by ammonium sulfate precipitation, followed by DEAE-cellulose and protein A–Sepharose chromatography. The mAb 46 (IgG2a), and mAbs 27, 50, and 87 (IgG1), were generated as described in reference 17, and purified from ascitic fluids by elution from protein A–Sepharose columns. The mAb H1A10 (anti-Tc85) (3) was a gift of Dr. J. Alves (Instituto de Quimica, Universidade de Sao Paulo, Brazil) and was used after ammonium sulfate precipitation of culture supernatants. The mAb 2.4G2 (18) was used as ascites at a 1/1,000 dilution. The anti-*T. cruzi* polyclonal antiserum was prepared by immunizing rabbits with live trypomastigotes treated with 8-methoxypsoralen (19), and the IgG purified after elution from protein A–Sepharose column. Fab fragments were prepared using immobilized papain (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions, and purified by passage through a column of protein A–Sepharose. No remaining intact IgG was detected in the preparations, as determined by coomassie blue staining of SDS-PAGE gels that had been loaded with 50 μg of the Fab preparation.

Attachment and Invasion Experiments. The assays to detect the attachment of trypomastigotes to glutaraldehyde-fixed 3T3 fibroblasts were performed in Terasaki plates as described in reference 10. For the inhibition assays, the Fabs in PBS were preincubated 10 min at room temperature with the parasites before addition to live or glutaraldehyde-fixed cells.

CHO and L cells, transfected or nontransfected with Fc receptors, were each plated onto 12-mm glass coverslips in 24-well plates in αMEM containing 10% gamma globulin–free FCS. When using Fc receptor–transfected cells, the medium also contained geneticin G-418. After 24–48 h, the medium was removed and the cells were pretreated 2 h with αMEM-10% without geneticin-G418. Then, 10^7 trypomastigotes in 0.3 ml of αMEM containing 10% gamma globulin–free FCS preincubated with antibodies were added to each well. The following antibodies were used at subagglutinating concentrations: mAb 46, 2 μg/ml; mAb 3C9, 5 μg/ml; and polyclonal antibody, 5 μg/ml. The other mAbs are nonagglutinating, and were used at 20 μg/ml. After 30 min of incubation of trypomastigotes with cells, at 37°C, the medium was aspirated, each well rapidly washed with 1 ml of Hanks bicarbonate, and the cells fixed for 30 min with freshly prepared 4% paraformaldehyde in PBS at 4°C. Wells were washed with PBS, and treated with 10% FCS in PBS for 30 min before addition of 1/50 dilution in 10% FCS-PBS of the anti-*T. cruzi* rabbit antiserum. After three washes in PBS, cells were further incubated with a fluorescein-labeled anti–rabbit IgG and with 0.01 mM of Hoechst dye 33258 (Sigma Chemical Co., St. Louis, MO) for 30 min. The total number of trypomastigotes per at least 100 cells were counted by visualizing the typical Hoechst dye staining of the nuclei and kinetoplast of the parasites under an epifluorescence microscope. Extracellular trypomastigotes were stained by immunofluorescence, and invasion was determined by the difference between total and extracellular parasites (20). The data shown are means and standard deviations of four determinations.

Immunoprecipitation and Western Blots. Trypomastigotes were lysed in 1% NP-40, 50 mM Tris-HCl, pH 7.4, 1 mM PMSF, 0.1 mM EDTA, 5 μg/ml leupeptin, pepstatin, and antipain. The lysates were centrifuged 5 min at 10,000 *g*, the supernatants treated with a nonrelevant antibody and protein A–Sepharose, centrifuged, and then incubated for 1 h with 20 μg of the purified antibodies. The immunocomplexes were collected by incubation with 50 μl of a 50% suspension of protein A–Sepharose. The samples were processed as described (11), and loaded onto 7.5% SDS-PAGE gels.

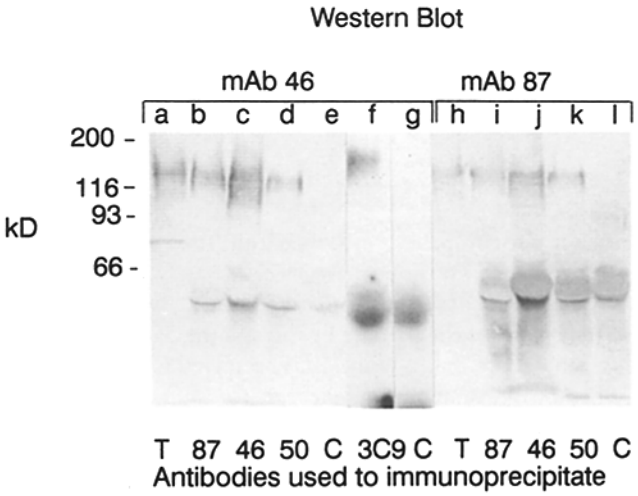

Figure 1. Western blotting demonstrating that mAb 46 recognizes the molecules immunoprecipitated by mAb anti-Ssp-3 and, vice versa, that the antigens immunoprecipitated by mAb 46 are recognized by mAbs anti-Ssp3. 2×10^8 trypomastigotes were lysed and immunoprecipitated with mAb anti-Ssp-3 (87, 46, 50, 3C9), or with an unrelated antibody (C), and loaded into SDS-PAGE gels as indicated in the bottom of each lane. The total parasite lysate (T, lanes *a* and *h*) was also applied into the gel. After transfer to nitrocellulose membranes, the blots were incubated with mAb 46 (lanes *a–g*), or mAb 87 (lanes *h–l*), and the bound antibodies revealed, respectively, with a biotinylated anti–mouse IgG and streptavidin-peroxidase-biotin complex, or with an anti–mouse IgG coupled to alkaline phosphatase. Lanes *f* and *g* contained antigens immunoprecipitated with mAb 3C9, and were revealed by incubation with ^{125}I–protein A followed by autoradiography.

After electrophoresis, the gels were blotted onto nitrocellulose paper. The paper was blocked with 1% BSA in 0.15 M NaCl, 10 mM Tris-HCl, pH 7.4 (TBS), and treated with 20 μg/ml of mAbs in the same buffer. Bound antibodies were detected as described (10) using an anti-mouse IgG conjugated with alkaline phosphatase, or with an anti-mouse IgG linked to biotin, followed by a mixture of biotinylated peroxidase and streptavidin.

Results and Discussion

The Ssp-3 epitope is defined by the mAb 3C9 (11). We have previously shown that Fab fragments of other anti-Ssp-3 mAbs (87 and 50) inhibit the attachment of *T. cruzi* trypomastigotes to glutaraldehyde-fixed 3T3 fibroblasts (10). In the experiments to be described, however, we have instead used mAb46, which also recognizes the Ssp-3 epitope by the following criteria: (*a*) the molecules immunoprecipitated from *T. cruzi* extracts by mAbs 50, 87, and 3C9 are recognized by mAb 46 in Western blotting. Conversely, the molecules immunoprecipitated by mAb 46 are recognized by mAb 87 (Fig. 1). (*b*) The epitope recognized by mAbs 46, 50, 87, and 3C9 is sialic acid dependent (18).

Our choice of mAb 46 was based on the results of preliminary experiments in which we compared the ability of the various mAbs, at subagglutinating concentrations, to enhance the binding of trypomastigotes to the Fc receptors of transfected cells. At these low concentrations, none of the anti-Ssp-3 antibodies inhibited parasite attachment, but rather enhanced the binding of the trypanosomes to target cells bearing Fc receptors. As shown elsewhere, inhibition of *T. cruzi* attachment required much higher concentrations of Fab fragments of these mAbs (10), presumably because most of the Ssp-3 epitopes have to be covered to prevent parasite adhesion to specific cellular receptors.

T. cruzi trypomastigotes were pretreated either with polyclonal IgG from a rabbit anti-*T. cruzi* antiserum, or with mouse mAbs that recognize trypomastigote surface antigens, and then incubated for 1 h with CHO cells transfected with mouse FcγRIII (21). We also used cells transfected with a truncated

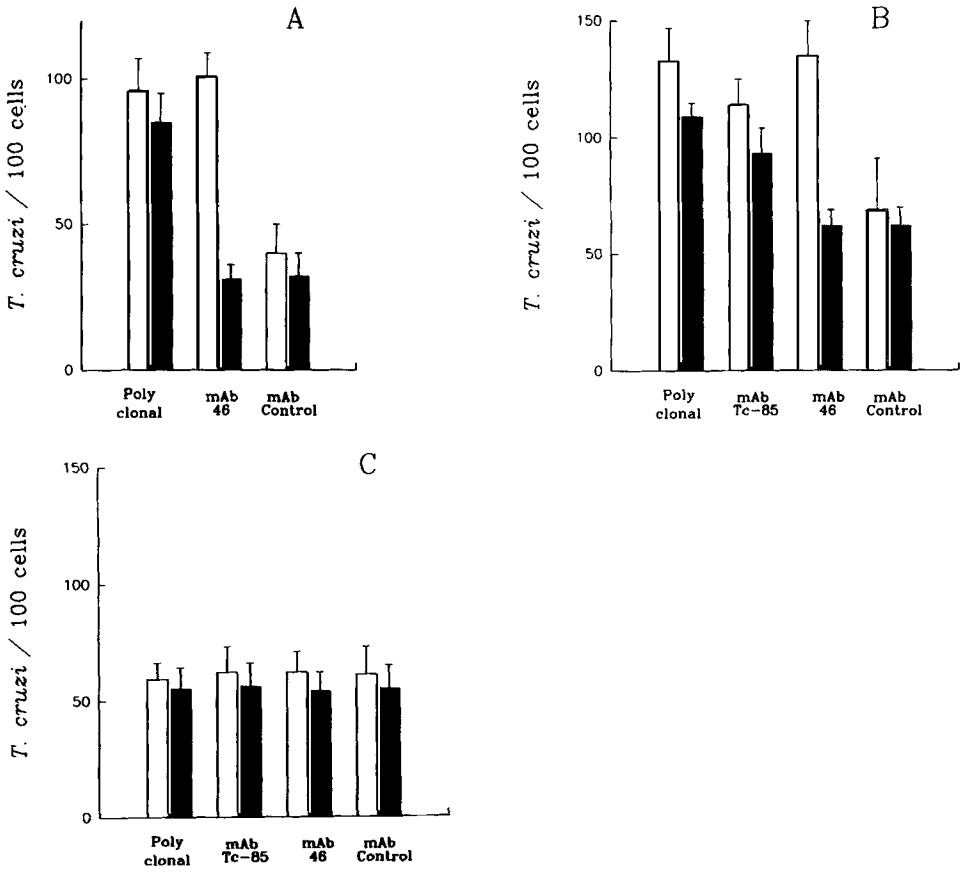

Figure 2. Effect of anti-*T. cruzi* antibodies on the attachment and invasion of Fc receptor-transfected cells. Trypomastigotes pretreated with different IgGs were added to CHO cells transfected with a truncated form of FcγRIII (CHO-Q210) (*A*), CHO cells transfected with FcγRIII (α + γ) (*B*), and nontransfected CHO cells (*C*). After 1 h at 37°C, parasites were removed, and the number of trypomastigotes associated per 100 cells was counted. The open bars represent the total number of trypomastigotes associated with cells determined by counting the trypomastigotes stained with Hoechst dye. The filled bars indicate intracellular parasites, estimated by subtracting the total number of parasites from the number of extracellular parasites, revealed by immunofluorescence staining of nonpermeabilized cells. The bars are means ± SDs of four independent determinations. The difference of the mean values for intracellular parasites pretreated with the polyclonal and mAb 46 in *A* and *B* is significant, $p < 0.001$.

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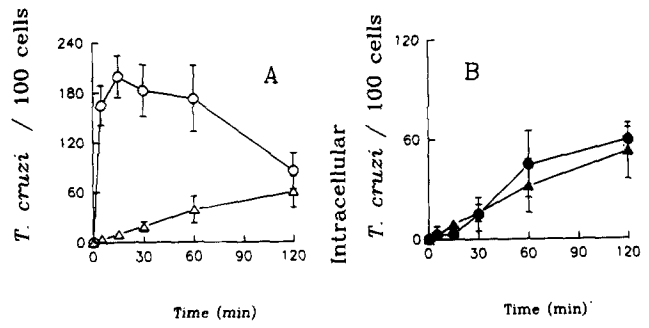

Figure 3. Kinetics of attachment and invasion of CHO cells, and of CHO cells transfected with FcγRIII (α + γ) by trypomastigotes coated with anti-Ssp-3 IgG. Trypomastigotes were pretreated with mAb 46 and incubated with CHO cells (*triangles*), or CHO cells transfected with FcγRIII (α + γ) (*circles*). At different incubation times the supernatants were removed, the cells fixed with 4% paraformaldehyde, and the number of trypomastigotes associated with the cells was determined as in Fig. 2. (*A*) The total number of trypomastigotes associated with the cells; (*B*) the number of intracellular parasites.

form of FcγRIII, lacking the cytoplasmic domain which transduces the interiorization signals in phagocytic cells (15).

As shown in Fig. 2, in the presence of anti-*T. cruzi* antibodies, there are more parasites associated with the transfected cells, as compared with the parental cells. This enhancement is mediated by the transfected Fc receptors, since it is not observed when anti-*T. cruzi* Fab fragments are used to precoat the trypomastigotes, and is completely reversed by pretreatment of the transfected CHO cells with mAb 2.4G2, an antibody directed to the mouse FcγRIII (13) (not shown).

Notably, however, while most trypomastigotes coated with mAb 46 anti-Ssp-3 remain outside the transfected CHO cells, most parasites incubated with the polyclonal antibodies, or with mAbs against non-Ssp-3 epitopes, are found inside the CHO cells (Fig. 2). Kinetic experiments show that in the presence of mAb 46, the attachment of trypomastigotes to FcRIII-bearing cells is faster than to nontransfected cells (Fig. 3 *A*). In agreement with the prior findings, there is no increase in penetration despite the rapid binding (Fig. 3 *B*), while the polyclonal antibody enhances in a parallel fashion the speed of attachment and invasion (Fig. 4).

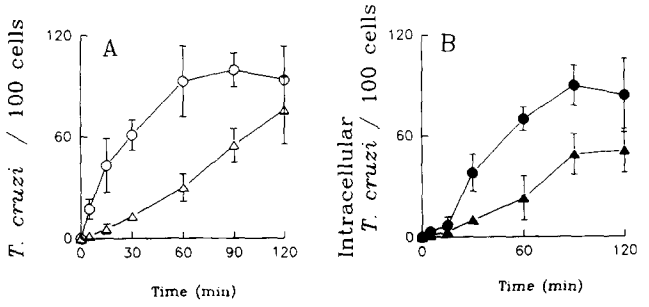

Figure 4. Kinetics of attachment and invasion of CHO cells, and of CHO cells transfected with FcγRIII (α + γ) by trypomastigotes coated with polyclonal anti-*T. cruzi* IgGs. Trypomastigotes were pretreated with the rabbit polyclonal anti-*T. cruzi* IgG and incubated with CHO cells (*triangles*), or CHO cells transfected with FcγRIII (α + γ) (*circles*). At different incubation times the cell supernatants were removed, the cells fixed with 4% paraformaldehyde, and the number of trypomastigotes associated with the cells determined as in Fig. 2. (*A*) The total number of trypomastigotes associated with the cells; (*B*) the number of parasites that had entered the cells.

Identical results were obtained when the target cells were L cells (instead of CHO cells) transfected with mouse FcγRII. In Fig. 5, all trypomastigotes are labeled with the Hoechst dye, but only the extracellular parasites are labeled by indirect immunofluorescence (see Materials and Methods). As illustrated, very few parasites pretreated with a polyclonal antibody are fluorescent, indicating that they have entered the cells. In contrast, most parasites pretreated with mAb 46 are fluorescent, indicating that they remain extracellular.

To further substantiate the finding that the anti-Ssp-3 antibody inhibits invasion, we precoated the parasites with mAb 46 simultaneously with the polyclonal antiserum. As shown in Fig. 6, the presence of mAb 46 reverses the enhancement in internalization mediated by the polyclonal IgG. This rabbit antiserum reacts strongly with many antigens in desialylated extracts of *T. cruzi*, but does not immunoprecipitate the Ssp-3-bearing molecules. By Western blotting, it reacts very weakly with antigens immunoprecipitated by mAb 46 (not shown).

One interpretation of the present results is that the anti-Ssp-3-coated parasites attached to target cells via Fc receptors invade less efficiently because Ssp-3 participates in some essential step of the internalization process. It may be of relevance that Ssp-3 is assembled by the transfer of sialic acid from exogenous glycoconjugates, to acceptor molecules on the parasite surface. The enzyme that catalyzes this reaction is a *trans*-sialidase associated with the surface membrane of trypomastigotes (18). During trypomastigote invasion, the *trans*-sialidase can utilize the target cell glycoconjugates as donors of sialic acid, and in theory it should be able to perform the opposite reaction, that is, transfer sialic back onto the host's cells. One intriguing possibility is that consecutive sialic acid transfers involving the Ssp-3 epitope, as well as the recognition of the host's donor surface molecules by the enzyme, are essential steps in the invasion process. Antibodies to Ssp-3 might prevent internalization by inhibiting these transfers. In fact, others have suggested that surface-associated glycosyl transferases might participate in cell surface recognition processes (22), but no enzymes with the characteristics of the *T. cruzi trans*-sialidase have yet been found on the membrane of eucaryotic cells.

Nevertheless, it could be argued that bridging the trypomastigotes to the surface of the target cells with an excess of antibody might immobilize the parasites, and prevent their interaction with a cellular receptor essential for internalization. This seems unlikely since the mAb 46–sensitized trypomastigotes are attached through one of their ends to the surface membrane of the target cells, and remain fully motile during the time of incubation. The polar accumulation of Ssp-3 is not unexpected: although the Ssp-3-bearing molecules are homogeneously distributed on the parasite surface, they cap when crosslinked by mAb 46, and accumulate in one of its extremities (S. Schenkman, unpublished observation). That the parasites are not trapped is also shown by

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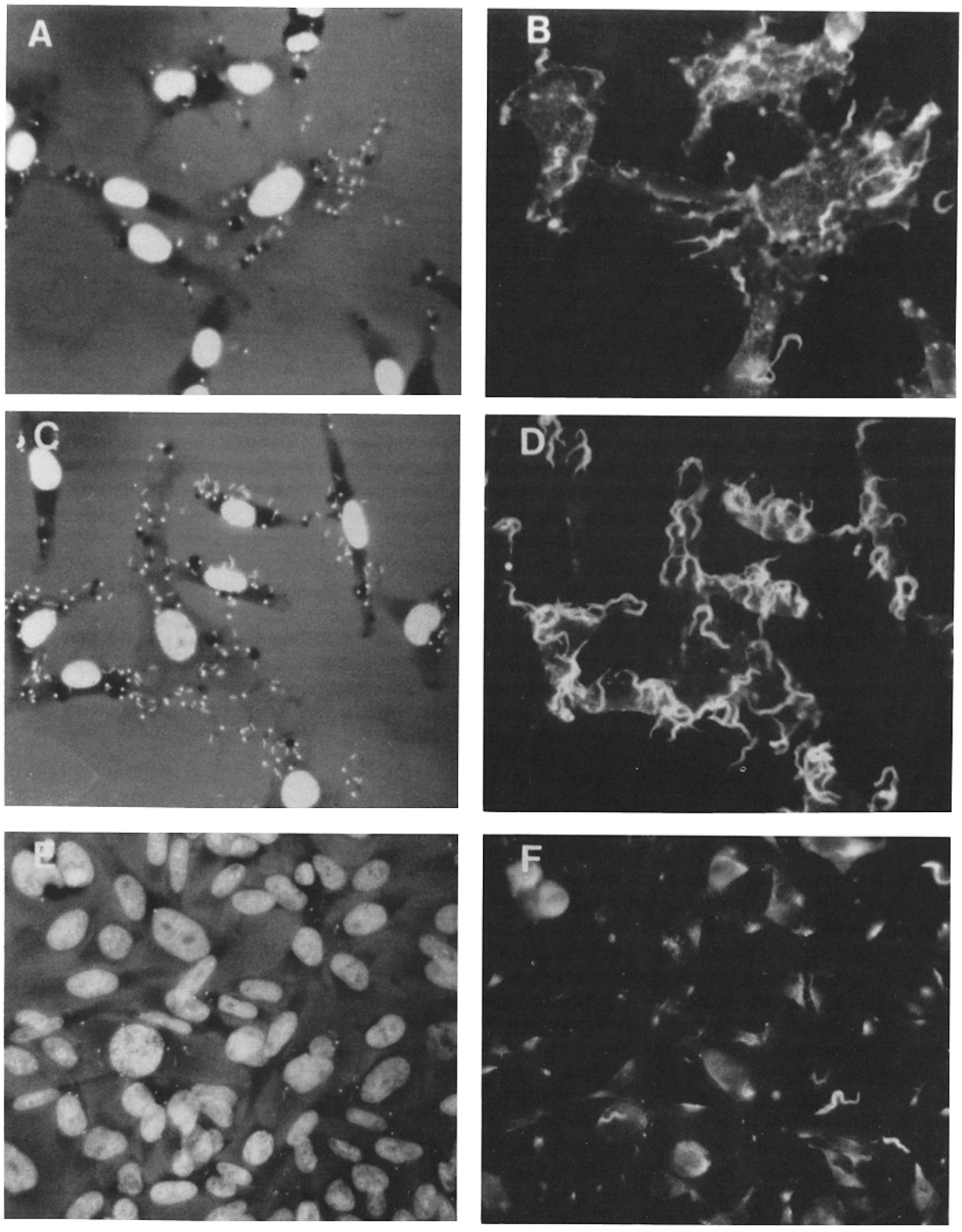

Figure 5. Trypomastigotes treated with anti-Ssp-3 attach to L cells transfected with FcγRII, but are not internalized. Trypomastigotes pretreated with a rabbit IgG anti-*T. cruzi* (*A* and *B*), mAb 46 (*C* and *D*), or with an unrelated mAb (*E* and *F*) were incubated at 37°C with L cells transfected with FcγRII. After a 1-h incubation, unbound trypomastigotes were removed. The cells were fixed with paraformaldehyde, stained with Hoechst dye, and processed for immunofluorescence. The slides were examined microscopically using the appropriate filters for Hoechst dye fluorescence (*A*, *C*, and *E*) or for FITC-immunofluorescence (*B*, *D*, and *F*).

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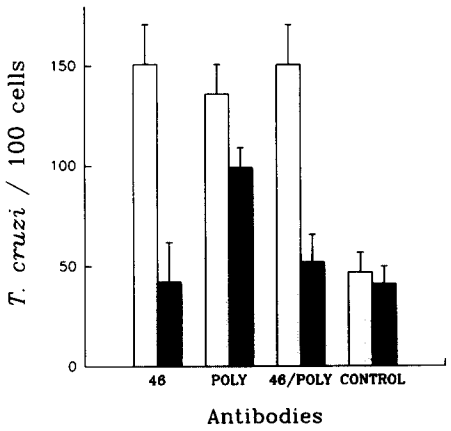

Figure 6. Anti-Ssp-3 reverses the enhancement in internalization mediated by polyclonal antibodies to trypomastigotes. Trypomastigotes were incubated 15 min either with mAb 46, polyclonal antibody, a control nonrelevant antibody, or with a mixture of mAb 46 and the polyclonal antibody. The parasites were then added to CHO transfected with FcγRIII (α + γ), and incubated 1 h at 37°C. The total number of trypomastigotes (*open bars*) and intracellular trypomastigotes (*filled bars*) were determined as in the previous figures.

the fact that after prolonged incubation, they can disengage from the cell surface as indicated in Fig. 3 *A*.

In conclusion, our findings provide further evidence of the participation of Ssp-3 in the penetration of *T. cruzi* trypomastigotes into nonphagocytic cells. Nevertheless, penetration of *T. cruzi* into mammalian cells is most likely a complex process involving several recognition events. In addition, the present results illustrate a new application of a strategy previously used to study the mechanisms that hinder phagosome-lysosome fusion in cells infected by *Toxoplasma gondii* (23). Target cells transfected with Fc receptors can be used to separate the attachment and penetration phases of cell invasion by microorganisms, thus providing an additional experimental tool to dissect these complex molecular interactions.

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