Vol. 182, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1992 Pages 6-13

ATTACHMENT OF Trypanosoma cruzi TO HOST CELLS : A MONOCLONAL ANTIBODY RECOGNIZES A TRYPOMASTIGOTE STAGE-SPECIFIC EPITOPE ON THE gp 83 REQUIRED FOR PARASITE ATTACHMENT

Fernando Villalta*[1], Maria F. Lima+, Antonio Ruiz-Ruano*, and Lin Zhou*

*Division of Biomedical Sciences and +Department of Microbiology , Meharry Medical College, 1005 D.B. Todd Boulevard, Nashville, Tennessee 37208

Received November 22, 1991

A set of monoclonal antibodies against the purified surface gp 83 of T. cruzi trypomastigotes was produced and the ability of these monoclonals to inhibit the attachment of trypomastigotes to heart myoblasts was investigated. Western blots of solubilized trypomastigotes, epimastigotes or amastigotes probed with this set of monoclonal antibodies show that the gp 83 is present in invasive trypomastigotes, but not in non-invasive epimastigotes or amastigotes. One monoclonal antibody (Mab 4A4) from this set inhibits the attachment of trypomastigotes to heart myoblasts, whereas the others (MAbs 2H6, 4B9, 2D11) do not. These results show that the Mab 4A4 recognizes an epitope on the gp 83 of invasive trypomastigotes required for parasite binding to host cells.

Trypanosoma cruzi, the protozoan which causes Chagas' disease and affects millions of people in South and Central America (1,2), must attach to mammalian cells before it can invade them. The disease is acquired by infection with invasive trypomastigotes which are transmitted by insect vectors, or by blood infected with trypomastigotes during blood transfusion (3). Recent cases of blood-transfused Chagas' disease have been reported in the United States (4,5). Trypomastigotes attach to and penetrate mammalian host cell membranes to multiply intracellularly as amastigotes and disseminate in the body. An understanding of how T. cruzi attaches to mammalian host cells is critical to the development of molecular means of intervention.

Recently we have identified a glycoprotein of molecular weight 83 kDa (gp 83) on the surface of T. cruzi trypomastigotes that binds to heart myoblasts in a ligand-receptor interaction manner (6-7). Addition of the purified gp 83 to heart myoblast monolayers inhibits the attachment and internalization of trypomastigotes into host cells (8). The gp 83 is more expressed on the surface of highly infective than weakly infective trypomastigote clones, suggesting that the regulation of its expression in the parasite modulates cellular invasiveness (7). In this paper we report novel findings showing that the gp 83 is trypomas-

[1]To whom correspondence should be addressed.

0006-291X/92 $1.50

tigote- stage- specific and that from a set of monoclonal antibodies specific against the purified gp 83 one, the Mab 4A4, recognizes an epitope that is required for parasite attachment to host cells.

MATERIAL AND METHODS

Trypanosoma cruzi. The highly infective trypomastigote clone MMC 20A of the Tulahuen strain of T. cruzi was used in this work (7). Pure culture trypomastigotes were obtained from the supernatant of infected rat heart myoblast monolayers (6,7). Pure amastigotes were produced under conditions previously reported (9) and epimastigotes as described (10). Parasites were washed by centrifugation with Hank's balanced salt solution and resuspended at the concentrations described below.

Purification of the gp 83 from T. cruzi trypomastigotes. The surface gp 83 was purified from trypomastigote membrane fractions by combining preparative isoelectric focusing and anion-exchange chromatography on a Mono-Q column (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ.) using a Fast Pressure Liquid Chromatography system (Pharmacia LKB Biotechnology, Inc.) as described in detail (8). Nonreducing SDS-PAGE (11) of the lyophilized purified gp 83 molecule stained with silver showed only the trypomastigote 83 kDa band. Staining the gels with silver-Commassie blue indicated its glycoprotein nature. In addition, treatment of the purified gp 83 with N-glycanase (Genzyme, Cambridge, MA) resulted in a electrophoretic mobility shift, indicating that the purified protein is glycosilated. The lyophilized purified gp 83 was resuspended in PBS, pH 7.2 and used as the antigen for monoclonal antibody production.

Production of hybridomas. We used the method described in detail by Kohler and Milstein (12) to produce monoclonal antibodies against the purified gp 83. Briefly, Balb/c mice were first i.p. immunized with 50 μg of the purified gp 83 resuspended in PBS mixed with complete Freund's adjuvant. After seven days, mice received a second i.p. injection of the gp 83 (50 μg) resuspended in PBS mixed with incomplete Freund's adjuvant. Fourteen days later, mice received 50 μg of the purified gp 83 resuspended in PBS intravenously. Spleen cells of immunized Balb/c mice were obtained four days after the last booster and fused with the plasmacytoma cell line P3X63-Ag8.653 in the presence of polyethylene glycol as described (12). Cells were resuspended in MEM/HAT media supplemented with 20% Fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μg/ml), and gentamycin (50 μg/ml). The cell suspension was distributed in aliquots in 96-well plates (Limbro Chemical Co., Hamden, CT). Supernatants were tested for the presence of antibodies by ELISA (13) using the purified antigen, indirect immunofluorescence using pure trypomastigotes as antigen and by western blots of solubilized trypomastigotes. Supernatants were also tested for the ability to block the attachment and internalization of trypomastigotes to heart myoblasts as described below. Selected hybridomas were cloned two times by limiting dilution on 96-well plates adding 0.1 cells/ well and then screened as described above. Isotyping of monoclonal antibodies was performed by ELISA using the isotyping kit from Amersham (Arlington Heights, IL) following the recommendations described by the manufacturer.

Purification of monoclonal antibodies. Selected hybridomas were grown to 2 liters in MEM supplemented with 10% FBS and antibiotics as described above at 37°C in 5% CO_2 with a water saturated atmosphere. Culture supernatants were precipitated with 50% ammonium sulfate. The selected monoclonal antibodies were isotyped as IgM. A fraction enriched for IgM was obtained by DEAE chromatography using a pH gradient as described in detail (13). IgM was further purified by immunoaffinity chromatography, linking purified goat IgG anti-mouse IgM (Cappel, Durham, NC) to Affi-gel Hz using an immunoaffinity kit from Biorad (Richmond, CA) following the manufacturer recommendations. The purified antibody was found to be pure when analyzed by SDS-PAGE under reducing conditions. Protein concentration of the purified monoclonal antibody was determined using a Pierce Micro-BCA assay.

Western blots. The presence of monoclonal antibodies recognizing the gp 83 in hybridoma culture supernatants was tested by western blots using solubilized trypomastigotes as described in detail (14). Purified monoclonal antibodies recognizing the gp 83 were similarly tested by western blots. Briefly, washed trypomastigotes (1x 10^9 organisms) were solubilized with 0.8% (3-[(3-Cholamidopropyl)dimethyl-ammonio 1-propanesulfonate), CHAPS, (6-8) in PBS in the presence of protease inhibitors (1 mM phenyl-methylsulfonylfluoride, 1mM N-p-tosyl-L-lysine-chloro-methylketone, and 2.8 μg/ ml aprotinin (6-8). Two hundred μg protein of solubilized trypomastigotes were separated by SDS-PAGE (11) under non reducing conditions using a Mini-Protean II Electrophoresis Cell (Biorad) and blotted onto nitrocellulose membranes. When required the same protein concentration (200 μg) of epimastigotes or amastigotes were separated similarly by SDS-PAGE. Biotinylated molecular weight standards (Biorad) were also included. Nitrocellulose membranes strips were incubated with monoclonal antibodies using a Mini-Protean II immunomultiscreen apparatus (Biorad). Control lanes received supernatants containing irrelevant monoclonal antibodies or purified irrelevant monoclonal antibodies of the appropriate class. When required individual nitrocellulose membrane strips were incubated with monoclonal antibodies. Since the set of selected monoclonal antibodies recognizing the gp 83 was found to be of the IgM class by isotyping analysis, blots were incubated with biotinylated goat IgG anti-mouse IgM (μ chain specific) (Cappel) and then developed with Avidin-alkaline phosphatase as described (8).

Immunofluorescence. We analyzed the binding of monoclonal antibodies to the surface of T. cruzi by indirect immunofluorescence as described (10,15). Briefly, live parasites resuspended in PBS supplemented with 1% Bovine Serum Albumin (BSA) (PBS+BSA) were incubated with monoclonal antibodies diluted in PBS+BSA for 2 hours at 4°C. Parasites were washed with cold PBS and incubated with fluorescein-labeled goat IgG anti-mouse IgM diluted in PBS+BSA for 1 hour at 4°C. Controls received an irrelevant IgM monoclonal antibody. Parasites were washed at 4°C, fixed with 1% formaldehyde and the fluorescence was observed using a Nikon microscope.

Inhibition of the attachment of trypomastigotes to host cells. The ability of monoclonal antibodies raised against the purified gp 83 from trypomastigotes to inhibit the attachment of trypomastigotes to heart myoblasts was evaluated using a microassay as described (7). Forty μl of hybridoma supernatants regulated at the same absorbance units or different concentrations of purified monoclonal antibodies diluted in DMEM supplemented with 1% BSA (DMEM+BSA) were incubated with 40 μl of trypomastigotes (1 x 10^7 organisms/ml) resuspended in DMEM+BSA for 1 hour at 4°C. The concentration of monoclonal antibodies selected for these assays did not agglutinate parasites. Controls were carried out by incubating the same concentration of parasites with DMEM +BSA. Then, twenty μl of antibody-pretreated parasites or mock-pretreated parasites were added to heart myoblast micromonolayers in triplicate and incubated at 37°C for 2 hours. Under these conditions the parasite attaches to host cells and minimal internalization occurs (7). After washing off the non-bound trypomastigotes with DMEM, the monolayers were fixed and stained with Giemsa as described (16,17). The number of trypomastigotes attached per 100 cells and the percentage of myoblasts containing attached parasites were microscopically determined by screening no less than 200 cells per monolayer. Binding experiments were also performed at 4°C incubating antibody-treated or untreated trypomastigotes with myoblast monolayers for 4 hours. Parasite internalization assays were also performed as described in detail (7). Treated or untreated parasites were added to myoblast micromonolayers and incubated for 2 hours at 37°C. After washing off the non-bound trypomastigotes, co-cultures were incubated for an additional two hours. After this time extracellular parasites that remained bound to myoblasts were lysed by a 2 min hypotonic pulse (7), the monolayers were fixed and the number of internalized parasites were microscopically determined as described (7).

Presentation of results. Results presented in this paper typically represent four independent experiments with the same design. Differences were considered to be significant if $p \leq 0.05$ as determined by the Student's t test.

RESULTS

We have produced a set of monoclonal antibodies against the purified gp 83 of T. cruzi. Four selected monoclonal antibodies named 4A4, 2H6, 4D11 and 2B9 that bound to the cell surface of trypomastigotes by indirect immunofluorescence recognized this molecule in invasive trypomastigotes by western blots (Figure 1). All of these monoclonal antibodies were of the IgM class as indicated by isotyping assays. An irrelevant monoclonal antibody of the IgM class, named IMab, did not recognize any proteins in trypomastigotes (Figure 1). Monoclonal antibodies 4A4, 2H6, 4D11 and 2B9 are highly specific since they recognized the gp 83, but they did not cross react with any other epitopes on other trypomastigote proteins (Figure 1). Indirect immunofluorescence studies indicate that Mab 4A4, Mab 2H6, Mab 4D11 and Mab 2B9 bind to the cell surface of trypomastigotes, whereas IMab does not. Western blot of solubilized trypomastigotes, epimastigotes or amastigotes probed with the set of monoclonal antibodies 4A4, 2H6, 4D11 and 2B9 recognized the 83 kDa molecule in invasive trypomastigotes, but not in non-invasive epimastigotes or amastigotes (Figure 2). From the set of monoclonal antibodies recognizing the gp 83, only the Mab 4A4 inhibits the attachment of trypomastigotes to heart myoblasts. Trypomastigotes were pre-treated with Mab 4A4, Mab 2H6, Mab 4D11, Mab 2B9 and IMab and then exposed to heart myoblast monolayers. Only pre-treatment of trypomastigotes with Mab 4A4 inhibited the attachment of trypomastigotes to heart myoblasts (Figure 3). Pre-treatment of trypomastigotes with Mab 2H6, Mab 4D11, Mab 2B9 and

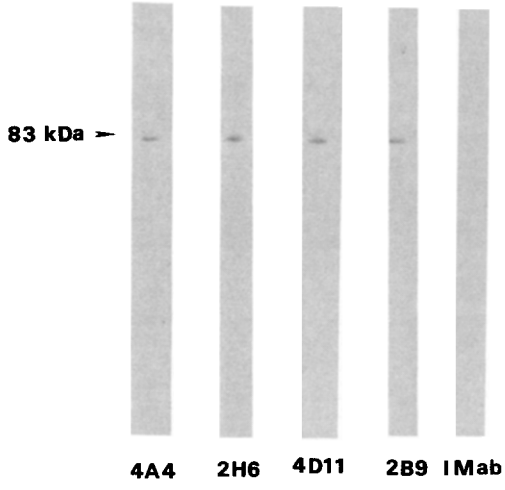

Figure 1. Western blots of solubilized T. cruzi trypomastigotes probed with a monoclonal antibody set produced against the purified gp 83 from trypomastigotes indicate that this set of monoclonal antibodies recognize only the gp 83 in trypomastigotes and there is no cross reaction with any other molecule of trypomastigotes. Solubilized trypomastigotes (200 μg) were separated by SDS-PAGE in 10% polyacrylamide slab gels under non reducing conditions, blotted onto nitrocellulose membranes and nitrocellulose strips were probed with supernatants of hybridoma clones Mab 4A4, Mab 2H6, Mab 4D11 and Mab 2B9 of the IgM class as described in Material and Methods. An irrelevant monoclonal antibody of the IgM class (IMab) was used as a control. Blots were incubated with biotinylated goat IgG anti-mouse IgM (μ chain) and developed with Avidin-alkaline phosphatase (Biorad). Biotinylated molecular weight standards were included. Arrow points to the gp 83.

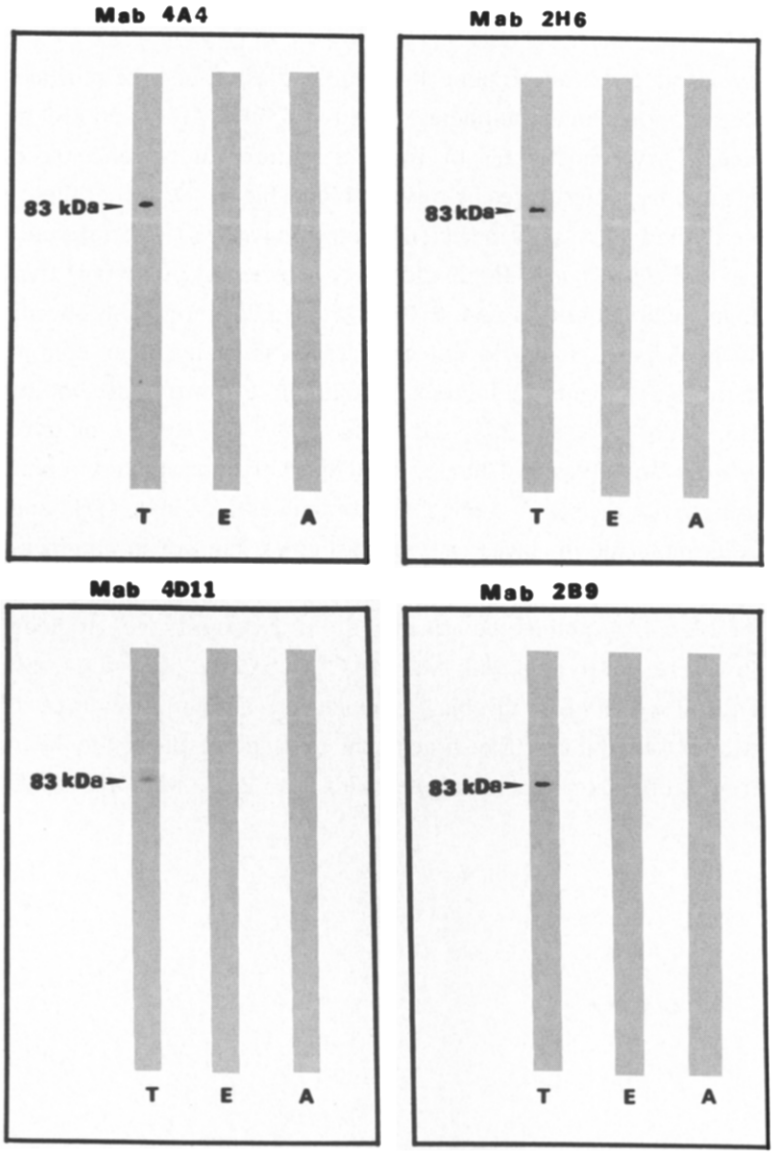

Figure 2. A set of monoclonal antibodies against the purified surface gp 83 of trypomastigotes recognizes the 83 kDa molecule in invasive trypomastigotes, but not in non-invasive epimastigotes or intracellular amastigotes. The same protein concentration (200 μg) of solubilized trypomastigotes, epimastigotes or amastigotes was separated by SDS-PAGE in 10% polyacrylamide slab gels under non-reducing conditions, blotted onto nitrocellulose membranes, nitrocellulose strips probed with a set of monoclonal antibodies (4A4, 2H6, 4D11 and 2B9) and developed with biotinylated goat IgG anti-mouse IgM (μ chain) and Avidin-alkaline phosphatase as described in Material and Methods. Biotinylated molecular weight standards were included. Arrow points to the gp 83. T, trypomastigotes; E, epimastigotes; and A, amastigotes.

IMab did not affect the attachment of trypomastigotes to host cells (Figure 3). The purified Mab 4A4 inhibits the binding of trypomastigotes to heart myoblasts monolayers in a concentration dependent manner (Figure 4). Inhibition of the attachment of trypomastigotes to myoblasts by Mab 4A4 requires low nanogram concentrations of monoclonal antibody

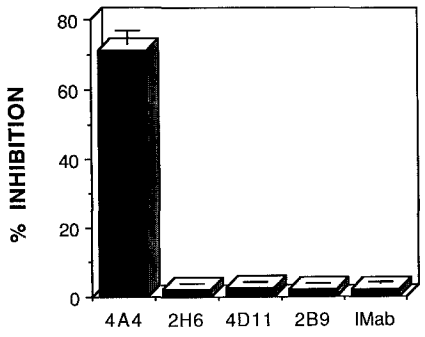

Figure 3. Effect of monoclonal antibodies produced against the purified gp 83 of trypomastigotes on the attachment of trypomastigotes to heart myoblast monolayers. 1 x 10^7 trypomastigotes were treated with the same concentration of monoclonal antibodies Mab 4A4, Mab 2H6, Mab 4D11, Mab 2B9 and IMab for 1 hour at 4°C and then exposed to heart myoblasts. Trypomastigote binding to heart myoblasts was evaluated as described in detail in Material and Methods. IMab represents an irrelevant monoclonal antibody. This is a representative experiment of four performed in triplicate showing the same results. Each column represents the mean of triplicate determinations and the bars one standard deviation.

(Figure 4). Our results indicate that Mab 4A4 blocks the attachment of trypomastigotes to heart myoblasts; it also prevents the internalization of trypomastigotes into heart myoblasts as indicated by parasite internalization assays.

DISCUSSION

These results show that a highly specific monoclonal antibody (4A4) that recognizes an epitope on the trypomastigote stage-specific gp 83 is required for T. cruzi trypomastigote attachment to host cells. Our results also show that the gp 83 of T. cruzi is develop-

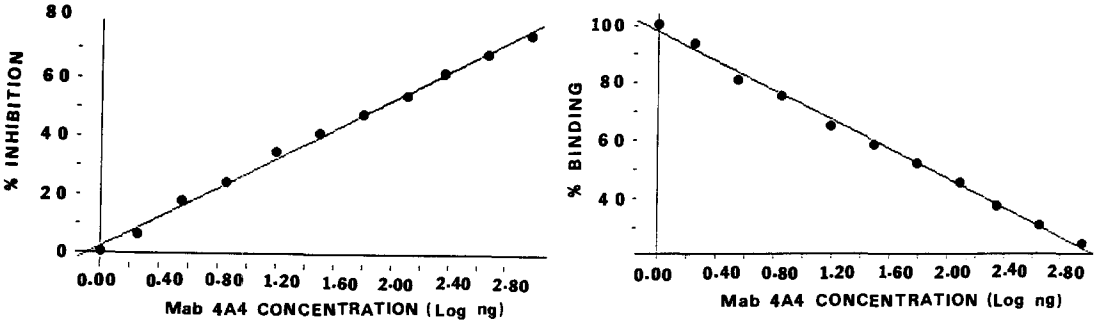

Figure 4. Inhibition of the binding of T. cruzi trypomastigotes to heart myoblasts by the purified Mab 4A4. Different concentrations of the purified Mab 4A4 diluted in DMEM+BSA were incubated with 1 x 10^7 trypomastigotes for 1 hour at 4°C and then exposed to heart myoblast monolayers as described in Material and Methods. Control cultures received the same number of trypomastigotes incubated only with DMEM+BSA. Unbound parasites were removed and parasite binding was determined as described in Material and Methods. Binding data were analyzed by the ENZFITTER computer program (18). This is a representative experiment of four performed with the same results.

mentally regulated in the cell cycle of T. cruzi, since this molecule is expressed in invasive trypomastigotes but not in non-invasive epimastigotes or intracellular amastigotes.

We have produced a highly specific monoclonal antibody (4A4) against the purified gp 83 of trypomastigotes which neutralizes the attachment of T. cruzi trypomastigotes to heart myoblasts. This monoclonal antibody 4A4 recognizes an epitope on the gp 83 necessary for parasite attachment to host cells, since from a set of different monoclonal antibodies recognizing the purified gp 83 only Mab 4A4 inhibits the attachment of trypomastigotes to host cells. The other members of the set of monoclonal antibodies 2H6, 2B9 and 4D11 which recognize the gp 83 but do not inhibit the attachment of trypomastigotes to heart myoblasts recognize other epitopes on the gp 83 that are not required for parasite attachment to host cells. These findings are consistent with a previous report showing that addition of the purified gp 83 of T. cruzi trypomastigotes to heart myoblast monolayers abolishes the attachment of trypomastigotes to heart myoblasts by competing with host cell receptors for the parasite (8). The novel findings reported in this paper show that an epitope on the trypomastigote stage- specific gp 83, recognized by the Mab 4A4, binds to heart myoblasts and mediates the first critical step by which this human pathogen attaches to its major host cell target.

The gp 83 of T. cruzi trypomastigotes binds to heart myoblasts in a ligand receptor-interaction manner (6). This molecule, which is expressed on the surface of invasive trypomastigotes but not on non-invasive epimastigotes, is highly expressed in highly invasive trypomastigote clones and is weakly expressed in weakly infective trypomastigote clones (7), implying that its expression regulates parasite cellular invasiveness (7). Therefore, the molecular elucidation of the binding of trypomastigote adhesion molecules, eg. gp 83, to host receptors is paramount to understanding the attachment of T. cruzi to host cells.

Mab 4A4 does not cross react with any other epitopes in other trypomastigote proteins, as indicated by the high monospecificity of Mab 4A4 for the gp 83 adhesion molecule shown in Figure 1. In addition, the epitope recognized by the monoclonal antibody 4A4 does not cross react with any molecule present in other developmental stages of the parasite, such as the non-invasive epimastigotes or intracellular amastigotes (Figure 2). Highly purified Mab 4A4 strongly inhibits the attachment of trypomastigotes and consequently prevents trypomastigote internalization into heart myoblasts. This inhibition is linear and concentration dependent in the range of 3 to 600 ng of Mab 4A4, indicating that this inhibition is specific and that the epitope recognized by Mab 4A4 participates in the process by which the human protozoan T. cruzi binds to heart myoblasts to gain an intracellular location in the mammalian host.

Knowledge of the mechanisms by which this invasive protozoan binds to host cell receptors of mammalian cells may facilitate molecular intervention to prevent T. cruzi infection.

ACKNOWLEDGMENTS: This work was supported by grants AI 25637 from the National Institutes of Health, DAN-5053-G-00-0030-00 from the US Agency for International Development and RII-9005826 from the National Science Foundation. A.R.R. is a postdoctoral fellow supported by grant 5G12RR03032 from the National Institutes of Health. The Hybridoma Facility at MMC is supported by grant RII-8804-780 from the National Science Foundation.

REFERENCES

1. Brener, Z. (1973) Annu. Rev. Microbiol. 27, 347-382.
2. Kirchoff, L.V., and Wilson, M. E. (1991). Curr. Opin. Infec. Dis. 4, 273-281.
3. Gutteridge, W.E. (1985) Brit. Med. Bull. 41, 162-168.
4. Grant, I.H., Gold, J.W.M., Wittner, M., Tanowitz, H.B., Nathan, C., Mayer, K., Reich, L., Wollner, N., Steinherz, L., Ghavimi, F., O'Reilly, R.J., and Armstrong, D. (1989) Ann. Int. Med. 111, 849-851.
5. Nickerson, P. Orr, P., Shroeder, M.L., Sekla, L., and Johnston, J.B. (1989) Ann. Int. Med. 111, 851-853.
6. Lima, M.F., and Villalta, F. (1988) Biochem. Biophys. Res. Commun. 155, 256-262.
7. Lima, M.F., and Villalta, F. (1989) Mol. Biochem. Parasitol. 33, 159-170.
8. Villalta, F., Lima, M.F., and Zhou, L. (1990) Biochem. Biophys. Res. Commun. 172, 925-931.
9. Villalta, F. and Kierszenbaum, F. (1982) J. Protozool. 29, 570-576.
10. Villalta, F. and Kierszenbaum, F. (1983) J. Protozool. 30, 329-331.
11. Laemmli, V.K. (1970) Nature 227, 680-685.
12. Kohler, G., and Milstein, C. (1975) Nature 256, 495-497.
13. Coligan, J.E., Kruisbeek, A.M., Margulies, D.H., Shevach, E.M., and Strober, W. (1991). Current Protocols in Immunology. (J.E. Coligan, Ed.), Vol I, p.2.7.10. John Wiley & Sons, New York.
14. Lima, M.F., and Villalta, F. (1990) Mol. Biochem. Parasitol. 38, 245-252.
15. Kierszenbaum, F., Villalta, F, and Tai, P.C. (1986) J. Immunol. 136, 662-666.
16. Villalta, F., and Kierszenbaum, F.(1983) Biochem. Biophys. Acta. 736, 39-44.
17. Villalta, F., and Kierszenbaum, F. (1985) Biochem. Biophys. Acta. 845, 216-222.
18. Leatherbarrow, R.J. (1987) ENZFITTER. p. 13. Elsevier Science Publishers, Amsterdam.