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MEMORANDUM

TO: PPC/CDIE, Maury Brown

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SUBJECT: Publication of Research Finding from Meharry Medical College

The attached reprint by Drs. F. Villalta, M.F. Lima and L. Zhou was published in Biochemical and Biophysical Research Communications. The basis for these research findings is a project funded by S&T/RUR under A.I.D.'s Small Research Program for Historically Black Colleges and Universities (**Trypanosoma Cruzi Binding to Mammalian Cells** - DAN-5053-G-SS-8052-00).

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Attachment:
Purification of Trypanosoma Cruzi Surface Proteins Involved in Adhesion to Host Cells

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PURIFICATION OF *Trypanosoma cruzi* SURFACE PROTEINS INVOLVED IN ADHESION TO HOST CELLS

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We have identified four surface 83 kDa proteins of pI values 6.3, 6.4, 6.5 and 6.6 in *T. cruzi* trypomastigotes which specifically bind to rat heart myoblasts. These proteins were purified by isoelectric focusing and anion-exchange chromatography in an FPLC system. These 83 kDa proteins inhibit the attachment of trypomastigotes to myoblasts in a concentration-dependent manner, indicating that these trypomastigote proteins mediate the attachment of trypomastigotes to heart myoblasts.

Trypanosoma cruzi, the protozoan which causes Chagas' disease and affects millions of people in South and Central America (1), 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. Recently two cases of blood-transfused Chagas' disease have been reported in the United States (2,3). Trypomastigotes attach to and penetrate mammalian host cell membranes to multiply intracellularly and disseminate in the body. An understanding of how the parasite attaches to mammalian host cells is critical to the development of effective means to prevent the infection.

Previous work from several laboratories has shown that free sugars (4,5), removal of sugar residues from trypomastigotes (6-9), inhibition of parasite glycoprotein biosynthesis (10) and parasite glycoprotein processing (11) can affect the attachment of the parasite to mammalian cells. It has been shown that the parasite can infect some cells in a polarized manner (12) and it has also been suggested that neuraminidase may be involved in the infection (13).

We recently identified an 83 kDa band present on the cell surface of invasive trypomastigotes which binds to heart myoblasts and appears to mediate the attachment of trypomastigote to host cells (14,15). This 83 kDa component, which is present in invasive trypomastigotes but not in noninvasive epimastigotes, is more expressed in highly than

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weakly infective trypomastigote clones (15). In this paper we report that this trypomastigote 83 kDa which binds to heart myoblasts is composed of four proteins with different isoelectric points; we purify these proteins and show that they inhibit the attachment of the parasite to heart myoblasts.

MATERIAL AND METHODS

The highly infective trypomastigote clone MMC 20A of the Tulahuen strain of T. cruzi (15) was used in this work. In some experiments other highly invasive trypomastigote clones (15) were used. Pure culture trypomastigotes were obtained from the supernatant of infected rat heart myoblast monolayers (14,15). Parasites where washed by centrifugation with Hank's balanced salt solution and resuspended at the appropriate concentrations as described below.

Pure trypomastigotes (2 x 10^{10} organisms) were radioiodinated with 5 mCi of Na[^{125}I] (specific activity, 17 Ci/mg I, ICN Radiochemicals, Irvine, CA) in the presence of iodobeads (Pierce Chemical Co., Rockford, IL) as previously described (14-15). Trypomastigote surface proteins were also biotinylated with N-hydroxisuccinimide (NHS)-biotin (Biorad, Richmond, CA) using living parasites as described in detail (14,15). In some experiments trypomastigotes were metabolically labeled with ^{35}S-Methionine or double labeled with ^{35}S-Methionine first and then biotinylated as described above (14-15). Trypomastigotes were metabolically labeled by incubating 1x 10^{10} parasites per ml in methionine deficient MEM (Sigma Chemical Co, St. Louis, MO) and supplemented with 1 mCi of ^{35}S-Methionine (specific activity, 1250 Ci/mmol, ICN Biochemicals) for 5 hours at 37°C.

Labeled or unlabeled trypomastigotes were washed four times with HBSS at 4°C. Live parasites or their cell membranes, obtained as described (16), were solubilized with 0.8% (3-[(3-Cholamidopropyl)dimethyl-ammonio] 1-propanesulfonate), (CHAPS), (14-15) in PBS in the presence of protease inhibitors (1mM phenyl-methylsulfonylfluoride, 1 mM N-p-tosyl-L-lysine-chloro-methyl ketone, and 2.8 ug/ml aprotinin (14,15).

The identification by two-dimensional gel electrophoresis (2-D) of T. cruzi surface proteins with ability to bind to myoblasts was performed by a binding assay that we have recently described (14,15). Biotinylation or radioiodination of the cell surface of trypomastigotes does not affect the capacity of the parasite to attach to and enter rat heart myoblasts (15). Membrane fractions obtained from pure labeled trypomastigotes (16) were solubilized with 0.8% CHAPS in PBS supplemented with protease inhibitors described above (14,15). After removing the detergent and protease inhibitors by dialysis against PBS at 4°C, 1 mg of parasite proteins was incubated with heart myoblast monolayers at 4°C to avoid ligand internalization as described (14,15). Unbound parasite material was removed by washing the cell monolayers with cold PBS. Myoblasts containing the bound parasite proteins were solubilized with CHAPS in the presence of protease inhibitors (14,15) and processed as described (15). Samples of 120 ug of protein were then subjected to 2-D analysis.

Two dimensional analysis of trypomastigote membrane labeled proteins was performed under equilibrium conditions as described by O'Farrel (17) using a MiniProtean Cell or a Protean Cell II (Bio-rad). Ampholines (Bio-rad) in different pH ranges were combined to obtain pH gradients. After isoelectric focusing (16 h at 400 V and 2 h at 800V) the pH gradient was measured on the first-dimension gel with a surface electrode. The second-dimension slab gels were prepared with 10% polyacrylamide. High and low molecular weight biotinylated standards (Bio-rad) or ^{14}C labeled molecular weight standards (NEN , Boston, MA) were included to determine the relative molecular weight of parasite proteins. Autoradiograms or nitrocellulose membranes were scanned with an Ultroscan XL Laser Densitometer (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ.).

Proteins from trypomastigote membrane preparations were initially separated by a preparative scale isoelectric focusing within a pH of 3 to 10 ampholines (Bio-rad) gradient using a isoelectric focusing cell (Rotofor, Bio-rad) at 4°C. Labeled or unlabeled trypomastigote membrane fractions (5.1 mg protein) solubilized with 0.8 % CHAPS and protease in-

hibitors as described above were mixed with 4.75 ml ampholines and brought to a total volume of 55 ml with Milli-Q deionized water. Thus the final ampholine concentration was 2%. This mixture was loaded into the horizontal electrofocusing cell and focused for 4 to 6 h at 4°C at constant 12 W. After focusing, 20 fractions were collected. Fractions were analyzed by SDS-PAGE (18) and stained with Silver (Bio-rad), autoradiographed when membrane proteins were radioiodinated or when trypomastigotes were metabolically labeled with ^{35}S-Methionine. Biotinylated fractions were also analyzed by western blots and stained with avidin-alkaline phosphatase (16) when biotinylated surface proteins were separated. Fractions were tested for their ability to inhibit the attachment of trypomastigote to heart myoblast monolayers as described below. Fractions that inhibited the attachment of trypomastigotes to heart myoblasts were pooled and further separated by anion-exchange chromatography in a Fast Pressure Liquid Chromatography (FPLC) system.

Selected trypomastigote membrane protein fractions obtained from the Rotofor (10-12), that were able to inhibit the attachment of trypomastigotes to myoblasts, were further separated by anion-exchange chromatography. Trypomastigote proteins (0.42 mg protein) in 20 mM Tris HCl buffer pH 8.0 supplemented with 0.4 % CHAPS were applied at a flow rate of 1.0 ml/min to a Mono Q column (Pharmacia LKB Biotechnology, Inc.) equilibrated with the same buffer and attached to a FPLC system (Pharmacia LKB Biotechnology, Inc.) which was operated with FPLC manager software (Pharmacia LKB Biotechnology, Inc.). Elution was performed at 4°C with a NaCl gradient from 1 to 1000 mM in the same buffer and fractions of 0.5ml were collected. Fractions were automatically read at A_{280} and radioactivity was determined in a gamma counter. Fractions were dialyzed against water, lyophilized, resuspended in 50 ul of PBS and tested for their capacity to inhibit the attachment to and internalization of trypomastigotes by heart myoblasts as described below. The purity of lyophilized chromatographic fractions (5 ul) was analyzed by gel electrophoresis as described above.

The effect of trypomastigote membrane proteins obtained from chromatographic fractions on the attachment to and internalization of highly invasive trypomastigotes by rat heart myoblasts was carried out using a microassay previously described in detail (15). Chromatographic fractions were dialyzed against water at 4°C, lyophilized and resuspended in PBS. Protein concentration in the fractions was determined using a Pierce Micro-BCA assay. Fractions were tested before the inhibition assay for their toxicity and cell release from monolayers and not to affect the viability of the cells. Only fractions which did not affect cell viability and were unable to release myoblasts from monolayers were tested for the inhibition of the binding to and internalization of trypomastigotes into heart myoblasts monolayers (15). Parasites were resuspended in DMEM (GIBCO, Grand Island, NY) containing 1% bovine serum albumin (Sigma Chemical Co, St.Louis, MO) at the concentration of 2x 10^7 organisms/ ml. Ten ul of this parasite suspension and 10 ul of parasite proteins diluted in PBS at increasing concentrations were added to microwells containing heart monolayers. Control cultures received PBS instead of trypomastigote proteins. Cultures were incubated at 37°C for two hours (15). After washing off the non-bound organisms with PBS, the monolayers were fixed with 2.5 % glutharaldehyde in PBS and stained with Giemsa. The parasite internalization assay was performed as described in detail (15). The number of trypomastigotes attached or internalized per 200 cells and the percentage of myoblasts containing attached or internalized parasites were evaluated microscopically by screening no less than 200 cells per well (15) Inhibition and binding data were analyzed by the ENZFITTER computer program as described (19).

All sets of results presented in this paper are typically representative of three independent repeat experiments.

RESULTS

To identify the surface trypomastigote proteins that bind to heart myoblasts we incubated detergent-free labeled parasite surface proteins with live myoblast monolayers. The interaction was carried out at 4°C to avoid ligand internalization. As can be seen in Fig. 1, 2-D analysis indicates that four 83 kDa surface proteins of pI values 6.3, 6.4, 6.5 and 6.6 from virulent T. cruzi trypomastigotes bind to heart myoblasts. These same proteins were

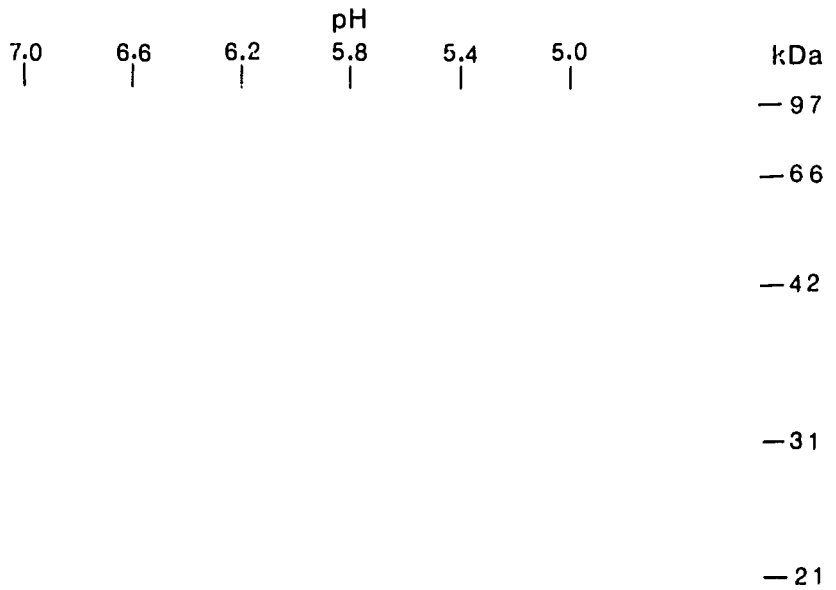
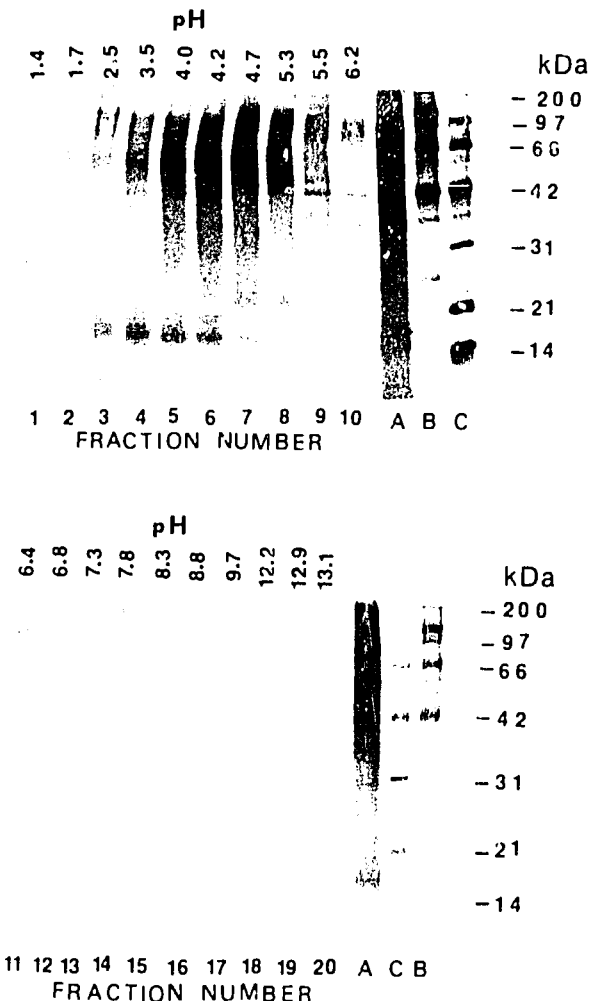


Figure 1. Identification of 83 kDa surface proteins of T. cruzi trypomastigotes that bind to rat heart myoblasts by two-dimensional gel electrophoresis. Biotinylated trypomastigote surface proteins that bound to rat heart myoblasts were obtained as described in Material and Methods and analyzed by two dimensional gel electrophoresis consisting of isoelectric focusing over a pH range of 3-10 in the first dimension and 10% SDS-PAGE in the second dimension. Nitrocellulose blots containing transferred biotinylated proteins were stained with avidin-alkaline phosphatase (Bio-rad) and developed as described in Material and Methods. Biotinylated molecular weight standards (Bio-rad) are indicated in kiloDaltons.

identified when trypomastigote surface proteins were biotinylated, radioiodinated or when the parasite proteins were metabolically labeled with ^{35}S-Methionine. The specificity of the binding of labeled 83 kDa trypomastigote proteins to heart myoblasts was indicated by the concentration-dependent inhibitory effect of unlabeled solubilized trypomastigote membrane proteins (results not shown). Labeled or unlabeled surface proteins of membrane fractions were initially separated by preparative isoelectric focusing (Rotofor). Fig. 2 shows a typical profile separation of trypomastigote membrane proteins by preparative scale isoelectric focusing. 83 kDa proteins can be seen in fractions 10, 11 and 12 with pH 6.2, 6.4 and 6.8, respectively. We found that these fractions inhibited the attachment to and internalization of highly invasive trypomastigote clones into heart myoblasts up to 70%. We pooled these three fractions and pursued further separation on a Mono Q anion-exchange column using an FPLC system. Fig. 3 shows the chromatographic profile separation of rotofor fractions (10 to 12) on a Mono Q column. A peak (the second in the chromatogram) was eluted with 0.25 M NaCl in Tris-HCl buffer, pH 8.0. This peak blocked up to 98% the attachment of trypomastigotes to heart myoblasts (Fig. 3). Western blot analysis of this peak (fractions 18 and 19) shows an 83 kDa band (Insert of Fig. 3). This 83 kDa band was the only band observed when these fractions were lyophilized and analyzed by SDS-PAGE and stained with silver stain. 2-



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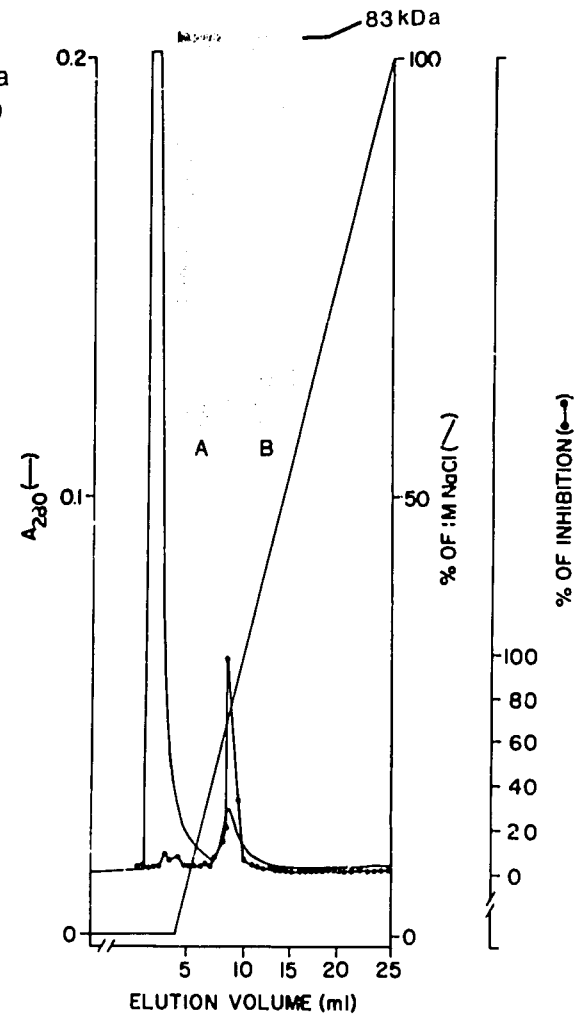

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Figure 2. Separation of biotinylated T. cruzi trypomastigote surface proteins by preparative scale isoelectrofocusing (Rotofor). A solubilized membrane fraction (5.1 mg protein) obtained from biotinylated trypomastigotes was applied to an isoelectric focusing cell (Rotofor) as described in Material and Methods. Twenty fractions (1-20) were harvested and their pH were determined. Fractions were analyzed by SDS-PAGE, blotted onto nitrocellulose and stained with avidin-alkaline phosphatase (Bio-rad). Fractions 10, 11 and 12 are enriched for the 83 kDa proteins. A, represents a western blot of the crude biotinylated trypomastigote membrane preparation (5 ug protein) that was applied to the Rotofor. B and C, are western blots of biotinylated high and low molecular weight standards (Bio-rad).

Figure 3. Elution profile of biotinylated trypomastigote surface proteins (Rotofor fractions 10-12) on Mono Q anion-exchange column using an FPLC system. Rotofor fractions 10-12 were pooled (0.42 mg protein) and applied to a Mono Q column in 20 mM Tris, 0.4% CHAPS, pH 8.0 with a flow rate of 1.0 ml/min. Fractions of 0.5 ml were collected. Elution was performed in a NaCl programmed gradient from 1 to 1000 mM (/) in 20 mM Tris, 0.4% CHAPS, pH 8.0 at 4°C. Fractions were automatically read at A_{280} (—) and tested for their capacity to inhibit the attachment to and internalization of T. cruzi trypomastigotes into heart myoblasts (●-●) as described in Material and Methods. Insert shows a western blot of two lyophilized fractions from the top of the second chromatographic peak eluted with 0.25M NaCl in Tris-HCl buffer, pH 8.0 and stained with avidin-alkaline phosphatase showing the purified 83 kDa (arrow). A, fraction 18 (elution volume 9 ml). B, fraction 19 (elution volume 9.5 ml).

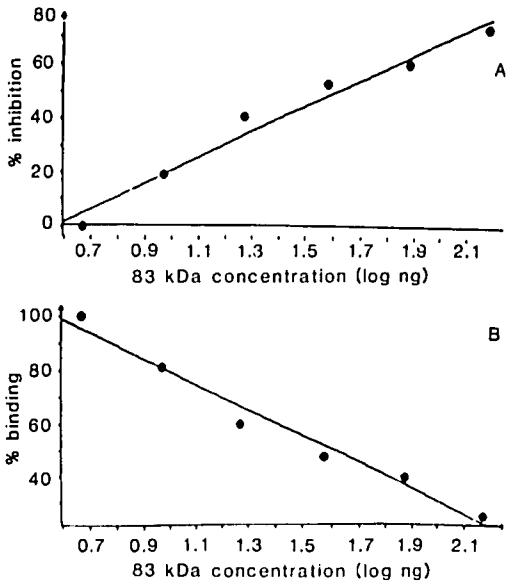

Figure 4. Inhibition of the binding of T. cruzi trypomastigotes to rat heart myoblasts by a Mono Q chromatographic peak containing the surface trypomastigote 83 kDa proteins. Fractions 18 and 19 of the second chromatographic peak (Fig. 3), presenting strong capacity to inhibit the attachment of the parasite to myoblasts, were dialyzed against water at 4°C, lyophilized and resuspended in PBS, pH. 7.2. and tested at different concentrations for their ability to compete with the binding of highly virulent T. cruzi trypomastigotes to rat heart myoblasts as described in detail in Material and Methods. Control cultures received PBS, pH 7.2 instead of parasite proteins. Binding data were analyzed by the ENZFITTER computer program (19).

D analysis of fractions 18 and 19 containing the 83 kDa yielded p.I. values similar to the 83 kDa bound to myoblasts (Fig. 1). Similar results were ob ained when labeled or unlabeled T. cruzi membrane proteins were used to purify the 83 kDa by chromatofocusing and anion-exchange chromatography using an FPLC system. By these procedures the 83 kDa was purified over 60-fold. These two fractions containing the 83 kDa were pooled and found to compete in a concentration dependent manner with the attachment of virulent trypomastigotes to heart myoblasts. Fig. 4 (A and B) shows that the purified 83 kDa is able to inhibit the binding of T. cruzi trypomastigotes to heart myoblasts in a concentration dependent manner at nanogram concentrations.

DISCUSSION

These results show that T. cruzi trypomastigotes attach to rat heart myoblasts through four 83 kDa proteins of pI values 6.3, 6.4., 6.5 and 6.6 which are present on the cell surface of virulent highly invasive trypomastigotes. In addition, these results show that the 83 kDa trypomastigote adhesive proteins can be readily purified by two steps involving preparative chromatofocusing and anion-exchange chromatography. The synthesis of these invasive proteins appears to be fast in trypomastigotes, since 2-D analysis of autoradiograms of 5 hour-metabolically labeled trypomastigotes predominantly shows these proteins. Interest-

ingly these proteins are not present on the surface of noninvasive epimastigotes (Villalta, F. and Lima, M.F., unpublished observations), suggesting that they are developmentally regulated in the cell cycle of the parasite.

One step-separation of membrane proteins through the rotofor isoelectric cell yielded three fractions enriched for the 83 kDa proteins which were able to compete with trypomastigote attachment to and internalization by host cells, facilitating their further separation. Chromatographic separation of these fractions on a Mono Q column using a FPLC system yielded a second peak containing the 83 kDa which strongly inhibited the attachment of the parasite to heart myoblasts. This inhibition is linear and concentration dependent in the range of 1 to 200 ng protein, suggesting that it is specific and that these 83 kDa proteins participate in the process by which T. cruzi binds to heart myoblasts to gain intracellular location and establish the infection in the mammalian host.

The mechanisms by which these 83 kDa proteins participate in this complex interaction between T. cruzi and its host cells may facilitate molecular intervention to prevent T. cruzi infection.

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REFERENCES

1. Brener, Z. (1973) Annu. Rev. Microbiol. 27,347-382.
2. 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.
3. Nickerson, P., Orr, P., Shroeder, M.L., Sekla, L. and Johnston, J.B. (1989) Ann. Int. Med. 111,851.
4. Crane, M.St. J., and Dvorak, W. (1982) Mol. Biochem. Parasitol. 5, 333- 341.
5. de Araujo Jorge, T.C., and de Souza, W. (1984) Acta Trop. 41, 17-28.
6. Villalta, F., and Kierszenbaum, F. (1983) Biochim. Biophys. Acta 736, 39-44.
7. Villalta, F., and Kierszenbaum, F. (1984) Biochem. Biophys. Res. Commun. 199, 228-235.
8. Villalta, F., and Kierszenbaum, F. (1985) Biochim. Biophys. Acta 845, 216-222.
9. Villalta, F, and Kierszenbaum, F.(1987) Mol. Biochem. Parasitol. 22, 109-114.
10. Piras, R., Piras,M.M., Henriquez, D., and Negri, S. (1982) Mol. Biochem. Parasitol. 6, 83-92.
11. Villalta, F., and Kierszenbaum, F. (1985) Mol. Biochem. Parasitol. 16, 1-4.
12. Schenkman, S., Andrews, N.W., Nussenzweig, V., and Robbins, E.S. (1988) Cell 55,157-165.
13. Pereira, M.E.A. (1990) In Modern Parasite Biology. (D.J. Wyler, Ed.), pp. 64-78. Freeman and Company, New York, NY.
14. Lima, M.F., and Villalta, F. (1988) Biochem. Biophys. Res. Commun. 155,256-262.
15. Lima, M.F., and Villalta, F. (1989) Mol. Biochem. Parasitol. 33, 159-170.
16. Lima, M. F., and Villalta, F. (1990) Mol. Biochem. Parasitol. 38, 245-252.
17. O'Farrel, P.H.. (1975) J. Biol. Chem. 250,4007-4021.
18. Laemmli, V.K. (1970) Nature 227,680-685.
19. Leatherbarrow, R.J. (1987) ENZFITTER. p.13. Elsevier Science Publishers, Amsterdam.