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Serodiagnosis of New World Leishmaniasis by Using a Genus-specific Antigen in Enzyme Linked Immunosorbent Assays

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A genus-specific monoclonal antibody (83L-5G9), generated against promastigotes of Leishmania braziliensis panamensis (WRAIR-470), has been used as a ligand in immunochromatography columns for the recovery and purification of the genus-specific antigen. When the purified polypeptide, actually a doublet comprised of a 58 kd and a 31 kd moiety, was used as the antigen in enzyme linked immunosorbent assays (ELISA), it was reactive with 36 of 85 sera from Leishmania patients and with 1 of 49 sera from confirmed cases of Chagas' disease. An additional 25 sera from an unexposed population and five specimens from individuals seropositive for Toxoplasma gondii were nonreactive.

INTRODUCTION

Trypanosoma cruzi and the *Leishmania*, *L. braziliensis ssp.* and *L. mexicana ssp.*, possess several common surface antigens that are crossreactive serologically (Anthony et al., 1980; Badaro et al., 1983; Carmargo and Rebonato, 1969). Moreover, the diseases caused by these parasites, American Trypanosomiasis (Chagas' disease) and the New World Leishmaniases, are indigenous to many of the same villages of South and Central America. Consequently, accurate accounts of the prevalence of human infection, as defined by serum antibody titers, are contingent upon using genus-specific antigens in the serodiagnostic assays. The advent of hybridoma–monoclonal antibody technology has made it possible to identify both genus-specific and species-specific antigens among the parasites of the family Trypanosomatidae (Anthony et al., 1985; McMahon-Pratt and David, 1981; Handman and Hocking, 1982). Moreover, the use of these antibodies as ligands in immunoaffinity chromatography now permits the purification of the reactive antigens, which are prerequisites for the development of the genus-specific and species-specific assays for the serodiagnosis of human infections (Sharma et al., 1984; Kasper et al., 1981). As part of our continued efforts to dissect the complex antigenic profiles of the Trypanosomatidae, we have generated several *Leishmania*-specific monoclonal antibodies. We now report on the use of one

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such antibody for the recovery of a *Leishmania*-specific surface antigen that, when used in an enzyme linked immunosorbent assay (ELISA), is highly reactive with sera from human Leishmaniases but only minimally reactive with >95% of the sera from individuals with Chagas' disease.

MATERIALS AND METHODS

Parasites

Promastigotes of *Leishmania braziliensis panamensis* (WRAIR-470) and epimastigotes of *T. cruzi* (Tulahuen strain) were maintained at 26°C in medium 199 containing 12 mM HEPES, 20 mM L-glutamine, 20% heat inactivated fetal bovine serum, penicillin 50 U/ml, streptomycin 50 U/ml, and gentamycin 50 μg/ml. Parasites were collected in their stationary phase and washed three times with 0.01 M phosphate buffered saline (PBS), pH 7.2, by centrifugation at 1500 rpm. Methods for the extraction of the parasite antigens in CHAPS(3-[(3-cholamidopropyl)-dimethylammonio] 1 propane sulfonate) were outlined in a previous report (Williams et al., 1986b).

Sera

The serum collection was comprised of 85 specimens of individuals (Panamanians) from whom *Leishmania* parasites had been isolated but whom had not yet begun chemotherapy. The Chagasic sera were from 49 persons (Bolivians) whose disease had been confirmed by xenodiagnosis and/or clinical impression. The sera from five cases of Toxoplasmosis were identified by indirect immunofluorescent microscopy using a *T. gondii* substrate (Electo-Nucleonics, Columbia, MD) and, as with the disease-free specimens, were obtained from persons (Marylanders) without any history of exposure to the Trypanosomatids.

Monoclonal Antibodies

Immunization schedules, procedures for cell fusion, and methods of screening hybridomas for production of monoclonal antibodies (Fusion L) have been described in earlier publications (Williams et al., 1986a; Constantine and Anthony, 1983). One hundred thousand hybridoma cells from clone 83L-5G9 were introduced into the peritoneal cavity of a Balb/c mouse that had been primed with Pristane (Oi and Herzenberg, 1980). After 2 weeks, the resultant ascitic fluid was collected and the immunoglobulin was purified on an Affi-gel Blue column (Bio-Rad Laboratories, Richmond, CA) according to procedures outlined by Bruck et al. (1982). The immunoglobulin fraction was dialyzed overnight at 4°C against 0.05 M Tris-HCl buffer, pH 7.2, and lyophilized. The protein content was determined by the Bio-Rad protein assay.

Affinity Chromatography

Thirty milligrams of the 83L-5G9 monoclonal antibody, reconstituted in 0.1 M sodium bicarbonate, were coupled to 2 ml of the activated agarose affinity support, Affi-gel 10 (Bio-Rad Laboratories, Richmond, CA), at 4°C for 18 hr. All remaining active ester sites were then blocked by the addition of 200 μl of 0.1 M glycine ethyl ester. The gel was packed into a 0.7 × 15 cm column and after a single wash with 0.05 M glycine, pH 7.2, unbound protein was eluted with 0.05 M glycine-HCl, ph 2.5. When the transmittance of the effluent, at 280 nm, reached 100%, the column was equili-

brated with the pH 7.2 glycine buffer. The CHAPS extract of the WRAIR 470 promastigotes (1 mg/ml) was applied to the column in a volume equal to the void volume. When the first evidence of protein, <100% transmittance, appeared in the effluent, the outlet was closed and the antigen–antibody binding was allowed to proceed for 2 hr at 4°C. Unbound proteins were then eliminated by elution in the 0.05 M, pH 7.2, glycine buffer containing 10 mM CHAPS. When transmittance again reached 100%, the bound 83L-5G9 antigen was displaced by a final elution with the pH 2.5 glycine buffer, and fractions were collected in 1-ml volumns.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The purity of the 83L-5G9 leishmanial antigen was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1979), using a 12.5% slab gel (1.5 mm × 80 mm × 60 mm), subsequently stained with 0.25% Coomassie Brillant Blue. The molecular weight of the antigen was determined using Bio-Rad molecular weight standards.

Immunoblotting

Proteins, resolved by SDS-PAGE, were electrophoretically transferred to a sheet of nitrocellulose. Precise procedures have been outlined by Towbin et al. (1979). After electroblotting for 2 hr at 1 amp, the sheet was treated with 5% bovine serum albumin (BSA) for 30 min. The sheet was next placed in medium supporting the 83L-5G9 hybridoma for 18 hr at 4°C. After extensive washing in PBS, pH 7.2, containing 0.05% Tween 20, the sheet was submerged for 2 hr at room temperature, in a 1 : 1000 dilution goat anti-mouse immunoglobulin serum labeled with ^{125}I. PBS containing 3% BSA served as the diluent. After another series of washes in large volumes of PBS–Tween 20, the sheet was air-dried and exposed to Kodak X-Omat AR film for 5 days at −70°C on an intensifying screen.

Enzyme Linked Immunosorbent Assay

Three micrograms of the purified 83L-5G9 antigen, in carbonate-bicarbonate buffer, pH 9.6, were added to each well of a flat bottom, polyvinyl microtitration plate (Flow Laboratories, Alexandria, VA). After an overnight incubation at 4°C, the wells were emptied by inversion and 100 μl of human serum, diluted 1 : 20 in PBS–TWeen, was added. Incubation continued for 2 hr at room temperature. Unreactive serum was eliminated by three washes in PBS–Tween 20, and 100 μl of a 1 : 500 dilution of goat anti-human immunoglobulin serum labeled with alkaline phoshastase was added. Incubation continued for 2 hr at room temperature and the washing cycle was repeated. One hundred microliters of p-nitrophenyl phosphate in diethanolamine buffer pH 9.8, was used as the enzyme substrate. Hydrolysis was terminated after 15 min at room temperature by the addition of 50 μl of 3 M NaOH and the resultant color change was measured, spectrophotometrically, at 405 nm in the Titertek Multiscan.

RESULTS

Reactivity of monoclonal antibody 83L-5G9 (IgG_1) with a dominant antigen on the surface membrane of promastigotes and amastigotes of *L. b. panamensis* (WRAIR isolate 470) is illustrated by the immunofluorescent micrographs, Figures 1A and 1B. The genus-specificity of this monoclonal, as assessed by reactivity with whole promastigotes in enzyme-linked immunosorbent assay (ELISA) (Table 1), revealed

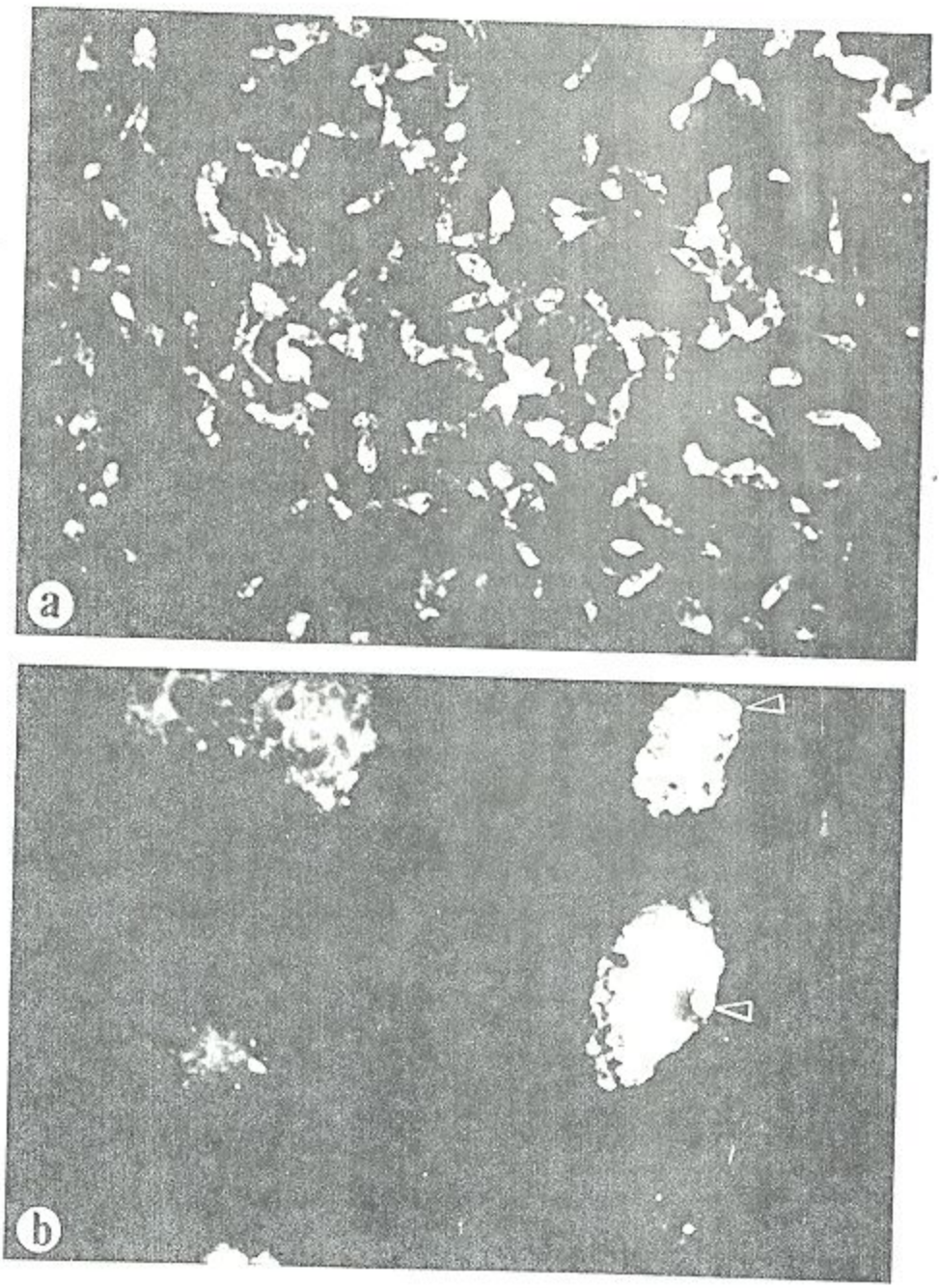

FIGURE 1. Immunofluorescent micrographs depicting reactivity of monoclonal antibody 83L-5G9 with the surface membrane of (A) promastigotes and (B) intracellular amastigotes of *L. b. panamensis*.

that the antigen is expressed on the membrane of the *L. b. panamensis*, *L. m. mexicana*, *L. m. amazonensis*, *L. b. peruviana*, and *L.d. chagasi* isolates, but not of the isolates of *L. b. braziliensis*, *T. cruzi*, and *Endotrypanum schaudinni*. Sequential analyses of the crude CHAPS extract of *L. b. panamensis* promastigotes by SDS-PAGE, immunoblot, and immunoautoradiography verified that the surface epitope

TABLE 1. Reactivity of Monoclonal Antibody 83L-5G9 with New World Trypanosomatids

Isolate (405 nm)	Absorbance
L. b. panamensis (WRAIR-470)[a]	0.345
L. m. mexicana (WRAIR-222B)	0.413
L. m. amazonensis (WRAIR-303)	0.340
L. b. peruviana (WRAIR-140)	0.445
L. d. chagasi (WRAIR-484)	0.418
L. b. braziliensis (WRAIR-508)[b]	0.000
Endotrypanum schaudinni (GML-465)	0.000
Trypanosoma cruzi (Tulahuen)	0.000

[a]Homologous reaction.
[b]Mucocutaneous disease.

recognized by 83L-5G9 is displayed on both a 58 kd and a 31 kd polypeptide (Figure 2). Elution of the extract through the 83L-5G9 affinity column and subsequent displacement of bound protein at pH 2.5, resulted in recovery of a small but compact fraction (Figure 3). An immunoblot of this fraction, using 83L-5G9 monoclonal antibody as the probe, established the purification of both reactive polypeptides (Lane A, Fig. 2).

As anticipated, absorbance values of Chagasic sera were equivalent to those of Leishmania sera whenever air-dried promastigotes of *L. b. panamensis* were used as the ELISA antigen (Figure 4). On the other hand, when the 83L-5G9 antigen was used as the antigen (Figure 5), the difference between the distributions of ELISA determinations for the two sets of sera was statistically significant, $p < 0.005$. Only

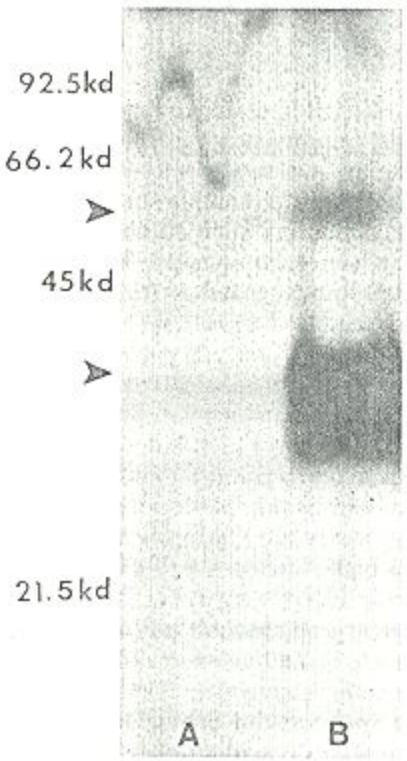

FIGURE 2. Immunoautoradiograph demonstrating reactivity of monoclonal antibody 83L-5G9 with (Lane A) affinity purified L-5G9 antigen and (Lane B) crude CHAPS extract of *L. b. panamensis* promastigotes. The arrowheads indicate the 58 kd and 31 kd components of the antigen.

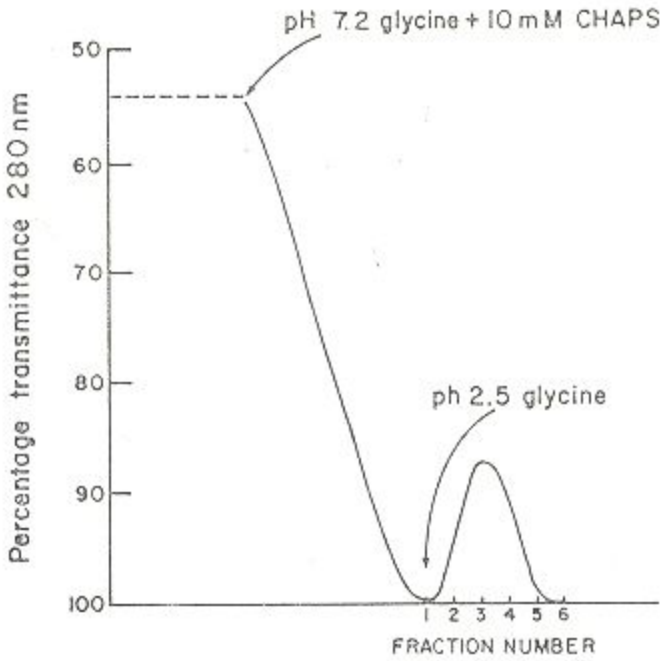

FIGURE 3. Elution profile of the purified L-5G9 antigen. Dotted line represents 2-hr incubation period during which time the outlet was closed.

one of 49 sera from *T. cruzi* infections produced an absorbance value of >0.200, whereas 36 of 85 sera for Leishmanial infections had values greater than 0.200.

DISCUSSION

Although significant progress has been made in increasing the sensitivity of serologic assays for the diagnosis of Leishmaniasis, improvements in specificity have lagged far behind. Badaro et al. (1986) were able to develop a species-specific ELISA for *L. donovani chagasi* by using an unfractionated, saline soluble supernatant of promastigotes as the antigen, but as a rule, assays that use such crude parasite extracts to demonstrate a specific antibody response have been severely limited by a high frequency of false positive results. These false positive assays are attributable, for the most part, to serum antibodies that are produced in response to a large array of antigens that are common to all members of the Family Trypanosomatidae (Badaro et al., 1983; Carmargo and Rebonato, 1969; Anthony et al., 1980). In fact, the cross-reactivity of antitrypanosomal antibodies with Leishmania antigens has been recognized for many years (Menolasino and Hartman, 1954). More recent studies have shown that some of this crossreactivity is due to a composite of antigens displaying epitopes that have been highly conserved throughout phylogeny (Dwyer, 1980) and expressed on vertebrate cells (Anthony et al., 1985). The antibody response to these shared epitopes, in a broad spectrum of both infectious and autoimmune diseases (Boreham and Facer, 1974; Sadigurski et al., 1982; Snary et al., 1983) likewise contributes to the lack of specificity inherent to serodiagnostic assays for Leishmaniasis.

Prior to the advent of hybridoma–monoclonal antibody technology, it was virtually impossible to detect subtle qualitative differences in the antigenic profiles of closely related species. However, the exquisite specificity of monoclonal antibodies now

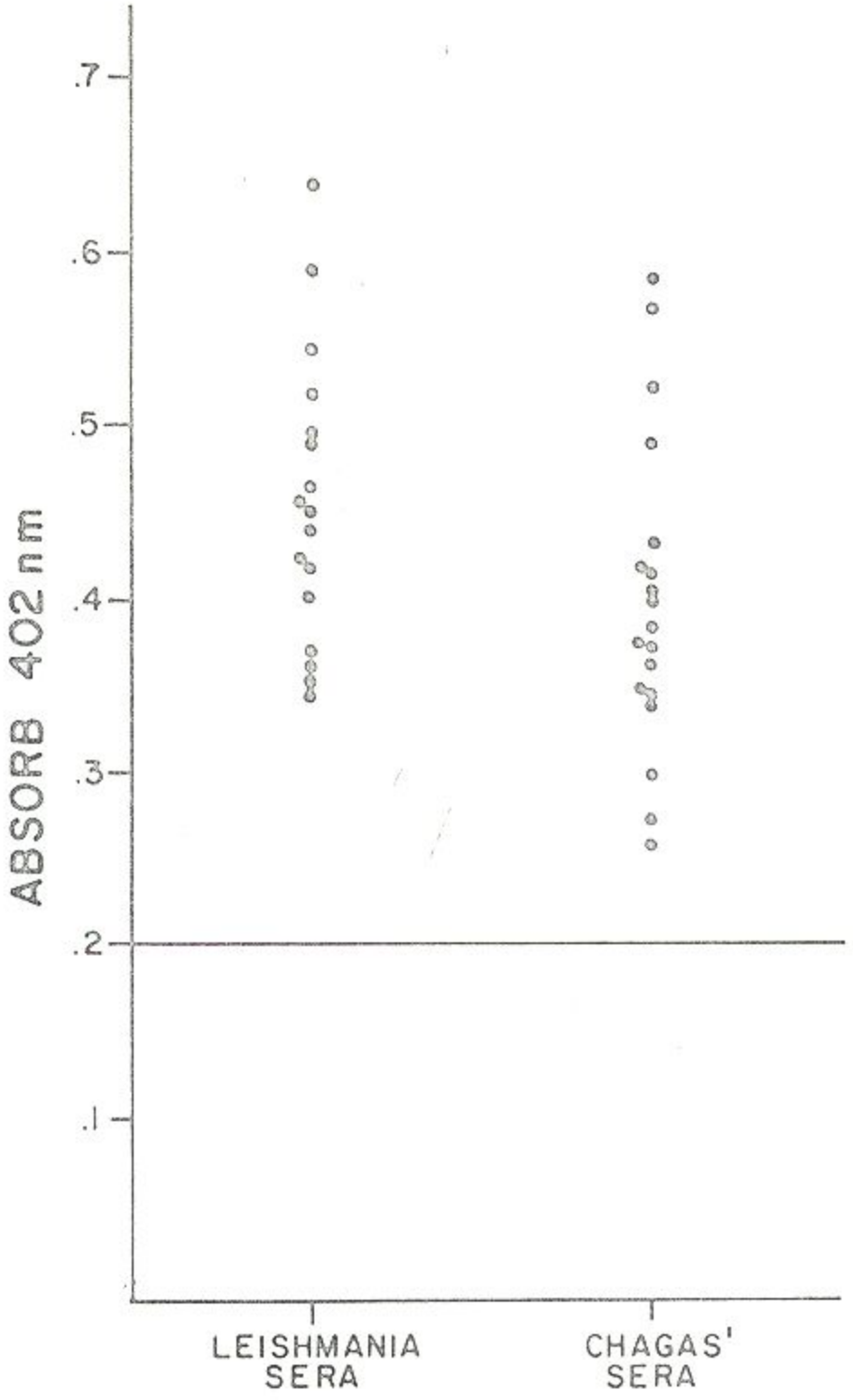

FIGURE 4. Reactivity of Leishmania and Chagasic sera with air-dried promastigotes of L. b. *panamensis*.

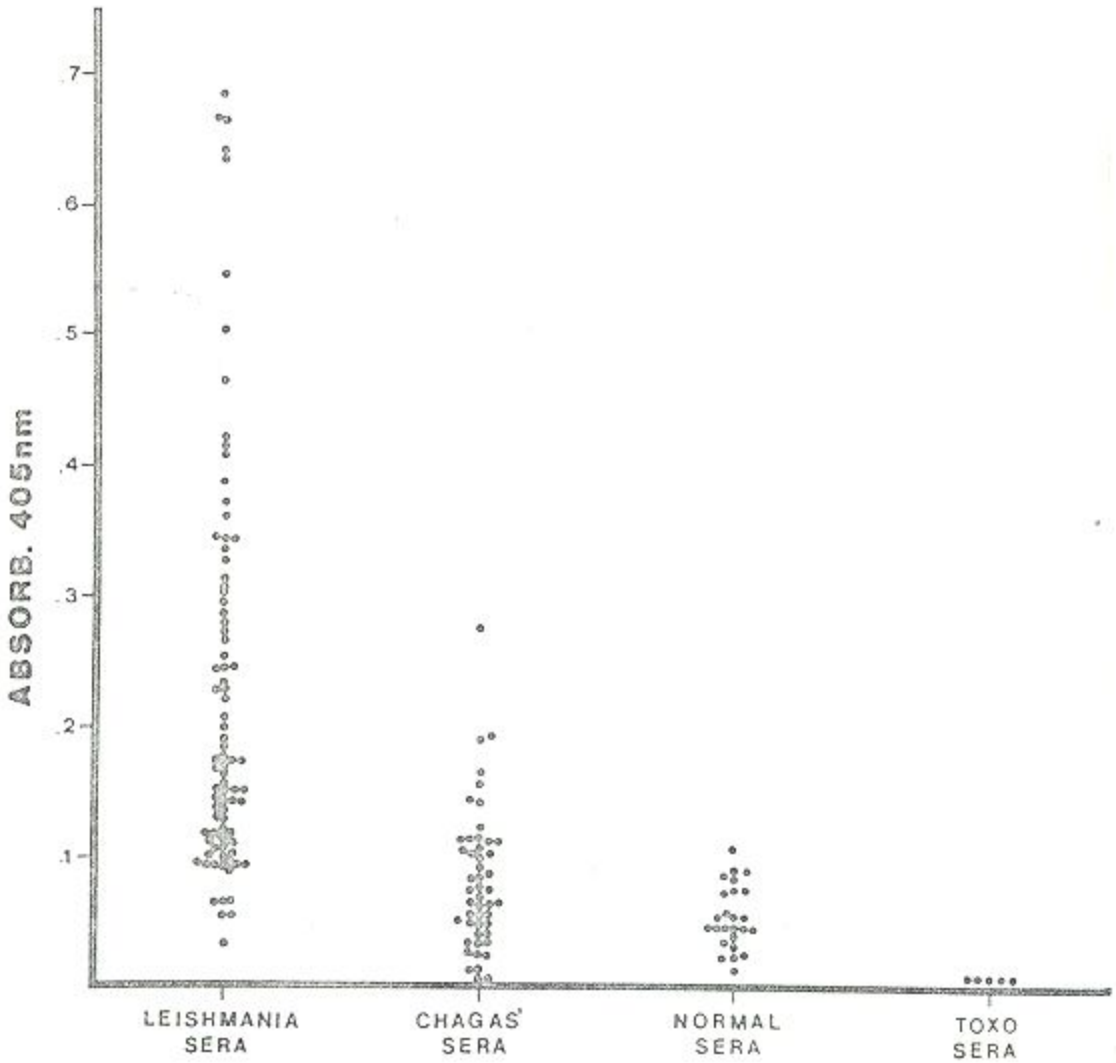

FIGURE 5. Reactivity of 85 Leishmania sera, 49 Chagasic sera, 25 normal sera, and five sera from patients seropositive for *T. gondii*.

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enables development of competitive ELISA for the demonstration of a species-specific antibody response (Lemesre et al., 1986) and, perhaps most importantly, permits recovery and purification of the species-specific antigens from complex extracts (Kasper et al., 1981; Williams, et al., 1986b). The evidence that such purified antigens can be used to demonstrate a species-specific antibody response in American trypanosomiasis (Scharfstein et al., 1983), prompted the current effort.

Although the epitope recognized by 83L-5G9 was common to isolates representative of the three species of New World Leishmania, the *L. b. braziliensis* (WRAIR-508) isolate from a mucocutaneous case of disease was nonreactive (Table 1). This very apparent difference in the antigenic composition of isolates that cause cutaneous versus mucocutaneous disease had been noted in previous reports (Anthony et al., 1985; Williams et al., 1986b). Noteworthy, of course, is the obvious lack of reactivity of the 83L-5G9 monoclonal antibody with epimastigotes of *T. cruzi*, probably the

most common cause of false positive serologic assays in persons living in villages where transmission of both Chagas' disease and Leishmaniasis is common (Lemesre, 1986). The capability of this monoclonal to distinguish between the two genera was the impetus for attempting to recover the polypeptide expressing the Leishmania-specific determinant.

The ELISA data (Figure 5) clearly establish that the L-5G9 antigen is only weakly reactive with antibodies in Chagasic serum. We would like to believe that the one false positive specimen was from an individual who also had Leishmaniasis but, unfortunately, his medical history did not include an examination for scars.

Because all of the Leishmania sera represent cases confirmed by isolation of parasites from lesion aspirates and/or biopsies, we must address the problem of the high frequency of false negative assays when using the L-5G9 antigen. Furthermore, all of these false negative sera were highly reactive, by ELISA, with air-dried promastigotes (unpublished data). Although we cannot relate differences in reactivity to the postinfection time of serum collection, we do suspect that the antibody response to the L-5G9 antigen may be transient. This suspicion will be examined when adequate panels of serial specimens representative of individual cases become available.

In spite of the fact that five of the six isolates examined were reactive with the monoclonal, we must also consider the possibility that the L-5G9 antigen is not expressed on all strains that produce cutaneous disease. Strain-specific antigens have been described (Handman and Hocking, 1982; Anthony et al., 1985). Of course, the antigen could be expressed but, because of an cryptic or inward orientation on the membrane (Kirchhoff et al., 1984; Williams et al., 1986), it does not elicit a demonstrable antibody response. Therefore, it may be necessary to use panels of purified antigens to eliminate false negative assays.

In summary, genus-specific monoclonal antibodies can be used as ligands in immunoaffinity chromatography columns for the recovery of Leishmania antigens required for the development of genus-specific serodiagnostic assays. Although a positive reaction virtually confirms a Leishmania infection, false negative assays are common. We contend, however, that these false negatives can be eliminated by using combinations of antigens in the assasy system.

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REFERENCES

Anthony RL, Christensen HA, Johnson CM (1980) Micro enzyme linked immunosorbent assay (ELISA) for the serodiagnosis of New World Leishmaniasis. *Am J Trop Med Hyg* 29:190.

Anthony RL, Williams KM, Sacci JB, Rubin DC (1985) Subcellular and taxonomic specificity of monoclonal antibodies to New World *Leishmania*. *Am J Trop Med Hyg* 34:1094.

Badaro R, Reed SG, Carvalho EM (1983) Immunofluorescent antibody test in American visceral leishmaniasis: Sensitivity and specificity of different morphological forms of two *Leishmania* species. *Am J Trop Med Hyg* 32:480.

Badaro R, Reed SG, Barral A, Orge G, Jones TC (1986) Evaluation of the micro enzyme-linked immunosorbent assay (ELISA) for antibodies in American visceral leishmaniasis: Antigen selection for detection on infection-specific responses. *Am J Trop Med Hyg* 35:72.

Boreham PFL, Facer CA (1974) Autoimmunity in Trypanosome infections. II. Anti-fibrin/fibrinogen (anti-F) autoantibody in *Trypanosoma* (*Trypanozoon*) *brucei* infections in the rabbit. *Intl J Parasitol* 4:601.

Bruck C, Portelle D, Glineur C, Bollen A (1982) One-step purification of mouse monoclonal antibodies from ascitic fluid by DEAE Affi-gel Blue chromatography. *J Immunol Meth* 53:313.

Carmargo ME, Rebonato C (1969) Cross-reactivity in immunofluorescence test for *Trypanosoma* and *Leishmania* antibodies. A simple inhibition procedure to ensure specific-results. *Am J Trop Med Hyg* 18:50.

Constantine NT, Anthony RL (1983) Antigenic differentiation of the Kinetoplastids *Leishmania braziliensis* and *Trypanosoma cruzi* by means of monoclonal antibodies. *J Protozool* 30:346.

Dwyer DM (1980) Isolation and partial characterization of surface membranes from *Leishmania donovani* promastigotes. *J Protozool* 27:176.

Handman E, Hocking RE (1982) Stage-specific, strain-specific and cross reactive antigens of *Leishmania* species identified by monoclonal antibodies. *Infect Immun* 37:28.

Kasper LH, Bradley MS, Pfefferkorn ER (1981) Identification of stage-specific sporozoite antigens of *Toxoplasma gondii* by monoclonal antibodies. *J Immunol* 132:443.

Kirchhoff LV, Hieny S, Shiver GM, Sanry D, and Scher A (1984) Cryptic epitope explains the failure of a monoclonal antibody to bind to certain isolates of *Trypanosoma cruzi*. *J Immunol* 133:2731.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680.

Lemesre JL, Afchain A, Orozco O, Loyens M, Breniere FS, Desjeux P, Carlier Y, Martin U, Nogueira-Queiroz JA, LeRay D, Capron A (1986) Specific and sensitive immunological diagnosis of Chagas' disease by competitive antibody enzyme immunoassay using *Trypanosoma cruzi*-specific monoclonal antibody. *Am J Trop Med Hyg* 35:86.

McMahon-Pratt D, David JR (1981) Monoclonal antibodies that distinguish between New World Species of *Leishmania*. *Nature* 291:581.

Menolasino NJ, Hartman E (1954) Immunology and serology of some parasitic protozoan flagellates. II. The Hemoflagellate Protozoa, *Leishmania donovani* and *Trypanosoma cruzi*. *J. Protozool* 1:111.

Oi VT, Herzenberg LA (1980) Immunoglobulin producing hybrid cell lines. In Selective Methods in Cellular Immunology. Eds. BB Mischell and AM Shiigi. San Francisco: W.H. Freeman pp. 355–372.

Sadigurski M, Acosta AM, Santos-Buch CA (1982) Muscle sarcoplasmic reticulum antigen shared by *Trypanosoma cruzi* clone. *Am J Trop Med Hyg* 31:934.

Sharma SD, Araujo FG, Remington JS (1984) Toxoplasma antigen isolated by affinity chromatography with monoclonal antibody protects mice against lethal infections with *Toxoplasma gondii*. *J Immunol* 133:2818.

Scharfstein J, Rodrigues MM, Alves CA, deSouza W, Previato JO, Mendonca-Previato L (1983) *Trypanosoma cruzi*: Description of a highly purified surface antigen defined by human antibodies. *J Immunol* 131:972.

Snary D, Flint JE, Wood JN, Scott MT, Chapman MD, Dodd J, Jessell TM, Miles MA (1983) A monoclonal antibody with specificity for *Trypanosoma cruzi*, central and peripheral neurones and glia. *J Exp Immunol* 54:616.

Towbin H, Straehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedures and some applications. *Proc Natl Acad Sci USA* 76:4350.

Williams KM, Sacci JB, Anthony RL (1986a) Flow cytometric analyses of the effects exerted by monoclonal antibodies on binding and uptake of *Leishmania mexicana mexicana* promastigotes by murine peritoneal macrophages. *Infect Immun* 52:36.

Williams KM, Sacci JB, Anthony RL (1986b) Characterization and quantitation of membrane antigens of New World Leishmania species using monoclonal antibodies in western blot and flow microfluorometric assays. *J Protozool* (in press).