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Leishmania: Trypanosomes; Monoclonal antibodies; Specific antigens; Serodiagnosis; Immunopathology.

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

Monoclonal antibodies, generated against the New World Leishmania have been used to identify taxons, dissect complex antigenic profiles, study host cell-parasite interactions, detect parasites in tissues of the host, develop species-specific serodiagnostic assays and to study the immunopathology of the disease.

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SUMMARY:

During this final year of contract no. DAMD 17-83-C-3031, efforts were directed at completing our major objectives. Our accomplishments included the generation of genus-specific monoclonal antibodies, development of flow cytometric assays for quantitating surface antigen expression among the various species and subspecies of New World Leishmania, identification of monoclonal antibodies with the potential of detecting intracellular parasites in infected tissues of the host, assessement of the effects of selected monoclonal antibodies on host cell- parasite interactions, and development of ELISA for the detection of genus-specific antibody response in humans. Results of these studies have been used to prepare a series of theseven manuscripts which makeup the body of this annual report.

1. Anthony, R. L., K. M. Williams, J. B. Sacci and D. C. Rubin. 1985. Subcellular and taxonomic specificity of monoclonal antibodies to New World Leishmania . Am. J. Trop. Med. Hyg., 34: 1085-1094.

2. Williams, K. M., J. B. Sacci, and R. L. Anthony. 1986. Characterization and quantitation on membrane antigens of New World Leishmania species using monoclonal antibodies in western blot and flow microfluorometric assays. J. Protozool. (in press).

3. Williams, K. M., J. B. Sacci, and R. L. Anthony. 1986. Flow cytometric analysis of the effects exerted by monoclonal antibodies on binding and uptake of *Leishmania mexicana mexicana* promastigotes by murine peritoneal macrophages. Infect. Immun. (in press).

4. Williams, K. M., J. B. Sacci, and R. L. Anthony. 1986. Identification and recovery of Leishmania antigen displayed on the surface membrane of mouse peritoneal macrophages infected *in vitro*. J. Immunol. (in press).

5. Anthony, R. L., K. M. Williams and J. B. Sacci. 1986. Identification, recovery and immungenecity of an antigen common to *Trypanosoma cruzi* and the vertebrate cell matrix. Infect. Immun. (submitted for publication).

6. Williams, K. M., J. B. Sacci, and R. L. Anthony. 1986. Rapid identification of *Leishmania* amastigotes using strain- and species-specific monoclonal antibodies. Am. J. Trop. Med. (submitted for publication).

7. Sacci, J. B., H. A. Christensen, A. Vasquez, and R. L. Anthony. 1986. Serodiagnosis of New World *Leishmania* by using a genus-specific antigen in enzyme linked immunosorbent assays. (in preparation).

FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

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Am. J. Trop. Med. Hyg., 34(6), 1985, pp. 1085–1094

SUBCELLULAR AND TAXONOMIC SPECIFICITY OF MONOCLONAL ANTIBODIES TO NEW WORLD *LEISHMANIA*

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Abstract. Murine monoclonal antibodies to flagellar, surface membrane and cytoplasmic antigens of New World *Leishmania* were assessed for their taxonomic specificity in enzyme-linked immunosorbent assays with three genera of the family Trypanosomatidae and three species and seven subspecies of the genus *Leishmania.* Antibodies exhibiting exclusive reactivity with either the flagellum, flagellar pocket, kinetoplast, or nucleus lacked specificity at all phylogenetic levels and, in fact, recognized epitopes common to cultured mammalian cells. Monoclonals to intracellular antigens were capable of distinguishing *Leishmania* from *Trypanosoma* and *Endotrypanum.* Antibodies reactive at the surface membrane could separate six isolates of *L. braziliensis* from three isolates of *L. mexicana* but the differences in antigen expression were frequently quantitative rather than qualitative. Antigenic variability within species and/or subspecies often exceeded that which was observed between species and/or subspecies. At least one monoclonal antibody was specific for a surface antigen peculiar to a subpopulation of promastigotes of an *L. braziliensis panamensis* isolate.

It is generally accepted that different species and subspecies of *Leishmania* are responsible for the broad spectrum of cutaneous, mucocutaneous and visceral diseases which occur in the New World. Consequently, a complete identification of the infectious parasite in cases of human disease is regarded to be of fundamental importance in deciding regimens of treatment, management of the patient and prognosis.[1] Morphologic, physiologic, biochemical and genetic differences between species have been reported,[2–6] and most recently, variations in the antigenic composite of the surface membrane among subspecies and stages, as defined by reactivity with murine monoclonal antibodies in radioimmune binding assays, have been demonstrated.[7–9]

As part of our continued efforts to recover species-specific leishmanial antigens for use as serodiagnostic reagents in field studies, we have generated several hundred monoclonal antibodies to surface and intracellular antigens of six isolates from the New World. We now report on the subcellular specificity of these antibodies, as assessed by their sites of reactivity in indirect immunofluorescent antibody assays, and their taxonomic specificity, as measured by their reactivity with 14 New World isolates in enzyme-linked immunosorbent assays.

Accepted 23 May 1985.

MATERIALS AND METHODS

Parasites

Promastigotes, obtained from the Walter Reed Army Institute of Research (WRAIR) and the Gorgas Memorial Laboratory (GML), were maintained at 26°C in medium 199 containing 10 mM Hepes, 20% fetal calf serum and antibiotics. Isolate designations, countries of origin and references to previous published data are listed in Table 1.

Monoclonal antibody production

Eight-week-old female BALB/c mice were inoculated subcutaneously into the axilla, with 15 μg (0.1 ml) of stationary phase promastigotes which had been rendered noninfectious by repeated freezing and thawing and by sonic disruption. The inoculation was repeated biweekly until a serum sample, taken via the tail vein, exhibited an indirect immunofluorescent antibody (IFA) titer of >1:800 or an absorbance of >0.500 at 405 nm by an enzyme-linked immunosorbent assay (ELISA). One final inoculation was given three days prior to recovery of splenocytes. Exceptions to this method included the use of isolated pellicular membranes[14] as the immunogen (fusion 83U) and immunization of

TABLE 1
Parasite isolates

Designation	Country of origin	Taxon
WR 140	Peru	*L. b. peruviana*[13]
WR 222	Panama	*L. m. mexicana*[13]
WR 303	Brazil	*L. m. amazonensis*[3-7,8,12,13]
WR 359	Panama	*L. b. braziliensis*[10]
WR 390	Brazil	*L. b. guyanensis*[*8,11,18]
WR 470	Panama	*L. b. panamensis*
WR 484	Brazil	*L. d. chagasi*
WR 508	Brazil	*L. b. braziliensis***
GML 1	Panama	*L. b. panamensis*
GML 3	Panama	*L. m. aristidesi*†
GML 18	Peru	*L. b. braziliensis***
GML 111	Brazil	*L. m. amazonensis*†
GML 465	Panama	*Endotrypanum schaudinni*‡
Tulahuen	Chile	*Trypanosoma cruzi*

* Same as WHO International Reference Strain MHOM/BR/75/M4147
** Mucocutaneous disease.
† Rodent isolate
‡ Sloth isolate

mice by exposure to sand flies infected with isolate WR-303 (fusions 84C and 84F).

Splenocytes (10^8) were fused with 10^7 P3X63-Ag8 plasmacytoma cells in the presence of 30% polyethylene glycol. Precise methods for preparation of the fusion partners and detailed recipes for making selection and growth medium have been outlined by Kennett[15] and Oi and Herzenberg.[16] Fifteen days post-fusion, medium supporting healthy hybridomas was assayed by IFA for anti-leishmanial antibodies. Positive hybridomas were expanded and subsequently cloned in semi-solid agarose.

Indirect immunofluorescence

Promastigotes, washed in phosphate buffered saline (PBS) (pH 7.2) and standardized to a concentration of 1×10^5/ml, were air dried, in 25 μl volumes, on eight-well microscope slides. Each well was then covered with 25 μl of medium from a cloned hybridoma for 30 min at room temperature. The unreactive protein was removed by three washes in PBS and 25 μl of goat anti-mouse immunoglobulin serum labeled with fluorescein isothiocyanate (Litton-Bionetics, Charleston, South Carolina) and diluted 1:50 in 0.25%. Evans Blue was added. Incubation was continued for 30 min at room temperature, unreactive conjugate was removed by three washes in PBS and a coverslip was mounted with buffered glycerol. Sites of reactivity were visualized using a ×50 water immersion lens mounted on a Leitz Ortholux immunofluorescent microscope. Medium supporting unfused P3X63-Ag8 cells served as the negative control.

Viable and/or formalin-fixed promastigotes were used as the IFA substrates for confirmation of antibody reactivity with surface antigens.

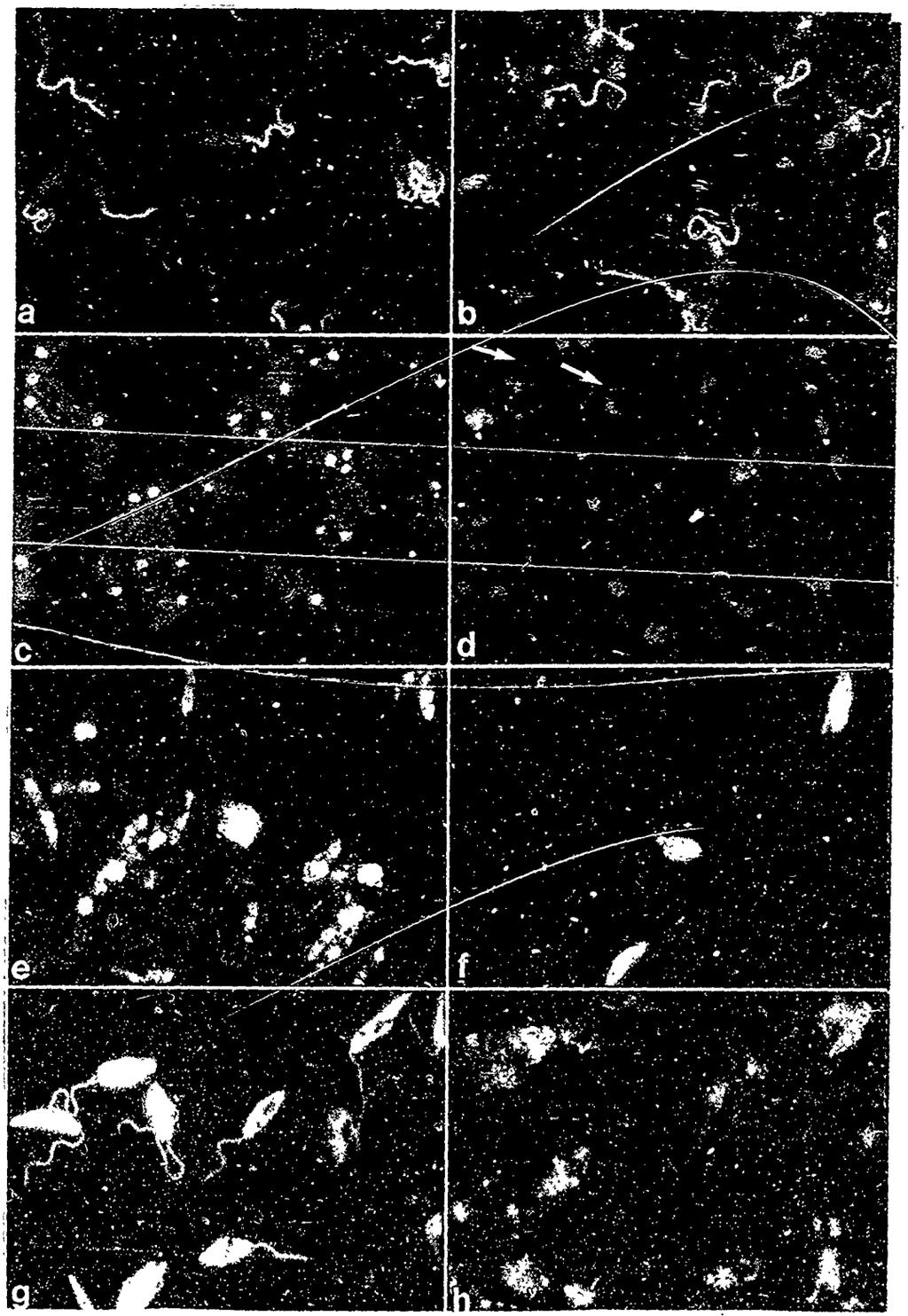
Enzyme-linked immunosorbent assay (ELISA)

The 96 flat-bottom wells of polyvinyl microtitration plates (Flow Laboratories, Alexandria, Virginia) were coated with 25 μg poly-l-lysine (Sigma Chemical Company, Saint Louis, Missouri) for 30 min at room temperature. The wells were emptied and 10^4 promastigotes, in 100 μl PBS, were added to each well. Each of the eight rows, consisting of 12 wells, received promastigotes representative of a different isolate. After centrifugation at 2, 500 rpm for 5 min at room temperature, the plates were inverted to remove the liquid phase and permitted to air dry overnight.

One hundred microliters of the monoclonal antibody (medium from a cloned hybridoma) was added to eight wells, each of which contained a different isolate. Thus, 12 monoclonal antibodies could be assayed against eight different isolates on a single plate. Medium supporting unfused P3X63-Ag8 cells served as the negative control. After 2 hr at room temperature, the unreactive protein was removed by three washes in PBS containing 0.025% Tween 20, and 100 μl of goat anti-mouse immunoglobulin serum labeled with alkaline phosphatase (Kirkegaard and Perry, Gaithersburg, Maryland) and diluted 1:500 in PBS-Tween 20 was added. Incubation continued for 2 hr at room temperature. Unreactive

→

FIGURE 1. Immunofluorescent micrographs of monoclonal antibodies × air dried, unfixed, stationary phase promastigotes. Monoclonals. a = anti-flagellum × *L. m. mexicana*, b = anti-flagellum + pocket × *L. m. mexicana*, c = anti-kinetoplast × *L. b. panamensis*, d = anti-nucleus × *L. m. amazonensis*, e = anti-cytoplasmic granules × *L. b. panamensis*, f = anti-smooth surface × *L. m. mexicana*; g = anti-flagellum + surface × *L. b guyanensis*; h = anti-granular surface × *L. b. guyanensis*. ×500.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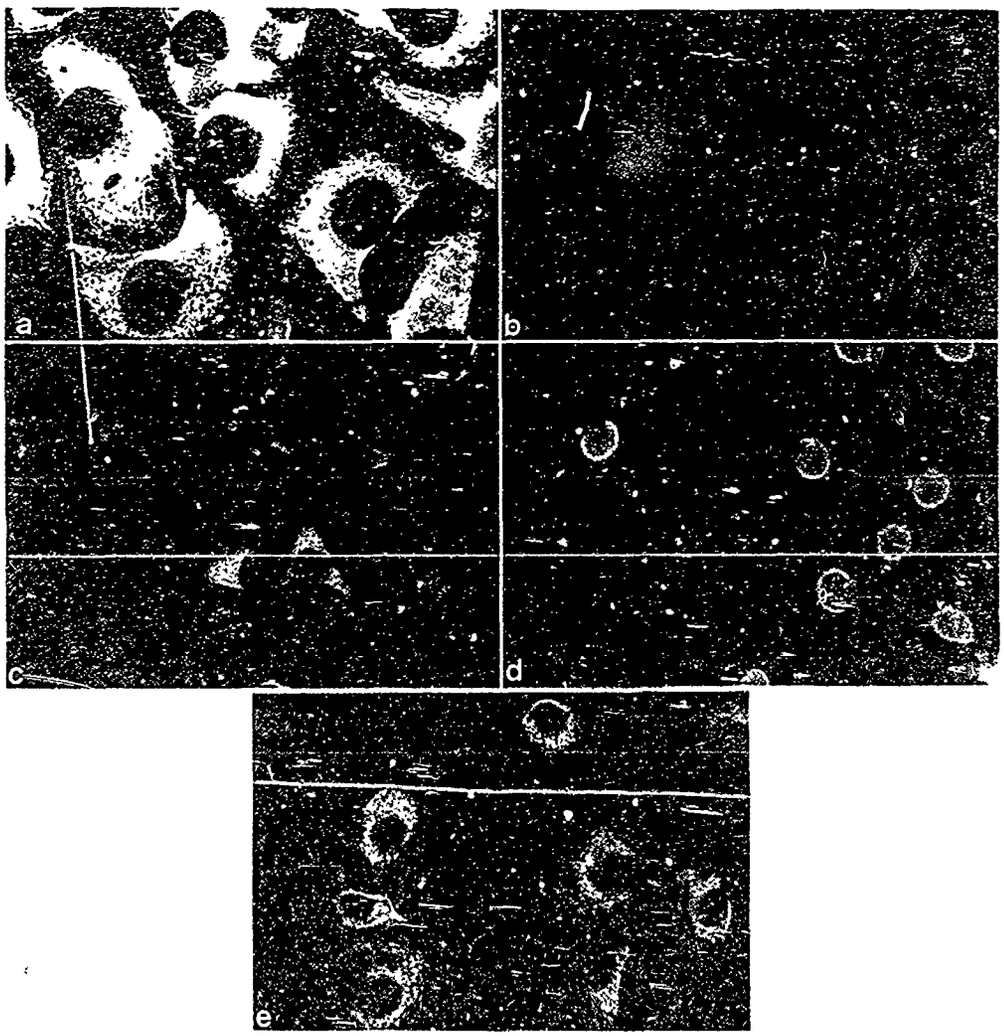

FIGURE 2. Immunofluorescent micrographs of monoclonal antibodies to *Leishmania* promastigotes × cultured bovine endothelial cells. Monoclonals: a = anti-*L. b. panamensis* flagellum, b = anti-*L. b. braziliensis* flagellum, c = anti-*L. m. amazonensis* kinetoplast, d = anti-*L. m. amazonensis* nucleus; e = anti-*L. b. panamensis* cytoplasmic granules. ×500.

conjugate was removed by another cycle of washes in PBS-Tween 20 and 100 μl of the substrate, p-nitrophenyl-phosphate in diethanolamine buffer, pH 9.8, was added. Hydrolysis was stopped after 15 min at room temperature by the addition of 50 μl 3 M NaOH and the resultant color change was quantitated at 405 nm in the Titertek Multiscan Spectrophotometer (Flow Laboratories, Alexandria, Virginia). The absorbance of wells containing the isolate against which the monoclonal antibody was produced (homologous reaction) was interpreted as 100% reactivity. Absorbance values for the remaining isolates (heterologous reactions) were then expressed as a percentage of the homologous reaction. Reactivity of <25% was interpreted as a demonstrable qualitative difference in the antigenic make-up between two isolates. Reactivity within the 25%–75% range was considered representative of a measurable quantitative difference of antigen expression. Each percentage represents a mean of at least three determinations performed with different lots of antigen on different days.

TABLE 3

Reactivity of New World Leishmania *with monoclonal antibodies specific for surface antigens*

Isolates	Monoclonal antibodies** 84C 4F4 (FS)	83T 9D3 (FS)	83U 7D5 (FS)	83J 8G10 (SG)	83T 3E9 (SG)	84C 8C7 (SG)	84G 8B10 (SS)	85C 5B2 (SS)	83U 5F3 (SS)
L. mexicana amazonensis									
WR-303	100.00*	100.00	100.00	31.44	88.06	100.00*	29.28	100.00*	100.00
WR-111	82.35	93.61	60.50	17.50	78.18	68.18	20.71	78.52	100.00
L. m. mexicana									
WR-222	98.03	100.00*	100.00*	46.25	100.00*	89.20	11.42	93.61	100.00*
L. m. aristidesi									
GML-3	60.78	66.66	62.19	0.00	54.32	46.59	15.71	49.07	26.66
L. braziliensis peruviana									
WR-140	92.15	100.00	88.41	25.00	96.29	96.02	20.00	58.28	57.94
L. b. panamensis									
WR-470	41.17	47.44	39.63	82.50	48.55	31.81	26.42	64.11	27.47
GML-1	13.72	0.00	3.76	82.50	4.11	2.84	100.00*	37.42	12.19
L. b. guyanensis									
WR-390	81.69	86.87	77.74	18.75	77.77	63.63	10.71	61.04	53.33
L. b. braziliensis									
WR-508	5.88	6.02	8.20	87.50	4.52	6.25	13.57	39.63	8.20
WR-359	100.00	76.24	72.86	73.25	59.67	86.73	35.71	64.73	83.07
GML-18	4.57	1.77	0.00	100.00*	0.00	3.97	15.00	15.33	12.30
L. donovani chagasi									
WR-484	100.00	100.00	79.26	7.50	81.48	96.82	2.85	67.48	75.89
Endotrypanum schaudinni									
GML-465	1.37	0.00	0.00	18.75	3.29	1.70	3.57	37.49	6.66
Trypanosoma cruzi									
Tulahuen	14.37	3.54	3.04	27.50	3.70	2.27	2.14	18.71	3.58

* = homologous reaction.

** = Fusions 83T, 84G and 84J were performed with splenocytes from mice immunized with saline sonicates of promastigotes, fusion 83U was against purified pellicular membranes, fusion 84C was against insect forms. (FS) = flagellum + surface as shown in Figure 1g, (SG) = surface granules as shown in Figure 1h; (SS) smooth surface as shown in Figure 1f.

fortunately, this antibody also reacted at a level of 60%–70% with *L. b. guyanensis* (WR-390), *L. d. chagasi* (WR-484) and the WR-359 isolate, presumably *L. b. braziliensis*. Monoclonal 83L-9D6, to a cytoplasmic antigen of *L. b. panamensis* (WR-470) was also *Leishmania*-specific but it did not react with three of the other *L. braziliensis* isolates (WR-508, GML-1 and GML-18). In contrast, 84G-9E2, generated against *L. b. panamensis*, was positive with all isolates.

Irrespective of immunization method, only three readily recognizable IFA patterns were produced by monoclonal antibodies to surface antigens of living and/or formalin fixed promastigotes. The reactive epitope appeared in a uniform linear distribution (Fig. 1f) which frequently included the flagellum (Fig. 1g), or it was visualized as irregularly spaced granules of varying sizes (Fig. 1h). With rare exception, however, all of these monoclonal antibodies were nonreactive with *T. cruzi* and *E. schaudinni* (Table 3). The *L. d. chagasi* isolate (WR-484) was reactive (>65%) with all monoclonals to surface antigens of the *L. mexicana* complex but consistently negative, (<10%), with those to *L. braziliensis* species (e.g., 83J-8G10). The ELISA results for the seven *L. braziliensis* isolates were the most difficult to decipher. *L. b. peruviana* (WR-140), *L. b. guyanensis* (WR-390) and the WR-359 strain of *L. b. braziliensis* all reacted at >5% with monoclonals to *L. mexicana* surface antigens. Conversely, the two presumed isolates of *L. b panamensis* (WR-470 and GML-1) and two of the presumed isolates of *L. b. braziliensis* (WR-

TABLE 2

Reactivity of New World Trypanosomatids with monoclonal antibodies specific for epitopes of the flagellum (F), flagellar pocket (P), kinetoplast (K), nucleus (N) and cytoplasmic granules (C)

Isolates	Monoclonal antibodies**						
	83Z-9C7 (F)	84G-9G3 (P)	84C-1F2 (K)	84F-4G5 (N)	83L-9D6 (C)	84G-9E2 (C)	83N-2C4 (C)
L. m. amazonensis							
WR-303	84.24	93.33	100.00*	100.00*	100.00	100.00	100.00
GML-111	100.00	73.33	100.00	87.67	100.00	71.25	82.48
L. m. mexicana							
WR-222	79.93	89.52	94.65	91.63	100.00	84.21	100.00*
L. m. aristidesi							
GML-3	98.17	48.57	97.03	72.11	100.00	58.70	33.64
L. b. peruviana							
WR-140	65.00	60.00	97.03	71.53	100.00	77.73	47.00
L. b. panamensis							
WR-470	79.76	100.00	88.72	88.61	100.00*	78.94	22.21
GML-1	77.77	100.00*	64.68	91.63	10.34	100.00*	0.00
L. b. guyanensis							
WR-390	90.21	80.95	85.45	80.42	100.00	35.46	69.12
L. b. braziliensis							
WR-508	100.00*	100.00	82.19	96.08	31.03	90.28	4.14
WR-359	86.53	100.00	100.00	85.07	100.00	73.27	65.89
GML-18	100.00	59.04	50.74	58.71	0.00	51.41	0.00
L. d. chagasi							
WR-484	91.19	76.19	100.00	66.78	100.00	68.01	64.05
E. schaudinni							
GML-465	87.39	53.33	62.90	61.74	0.00	51.51	2.30
T. cruzi							
Tulahuen	90.87	60.00	62.49	88.85	10.34	54.65	5.99

* = homologous reaction

** = Fusions 83Z, 84G, 83L and 83N were performed with splenocytes from mice immunized with saline sonicates of promastigotes. Fusions 84C and 84F were performed with splenocytes from mice exposed to infectious sand flies.

RESULTS

Approximately 25% of the monoclonal antibodies generated through a single cell fusion exhibited exclusive reactivity, at the subcellular level, with either the flagellum, flagellar pocket, kinetoplast or nucleus of air dried promastigotes (Figs. 1a–1d). Areas of red fluorescence are due to the nonspecific binding of the Evans Blue counterstain. Unfortunately, these antibodies could not discriminate between genera of the Trypanosomatidae and, in fact, recognized epitopes common to cultured mammalian cells (Figs. 2a–2d). ELISA data, expressing taxonomic specificity as percent reactivities of the 14 isolates with antibodies representative of each of these four immunofluorescent patterns, are given in Table 2. The immunogens for fusions 83Z and 84G were cultured promastigotes, whereas those for fusions 84C and 84F were insect forms.

Whenever splenocytes from BALB/c mice immunized with saline sonicates of cultured promastigotes were used as one of the fusion partners, an additional 25% of the resultant monoclonal antibodies were reactive with epitopes associated with intracellular granules (Fig 1e). While some of these epitopes were common to both protozoan and vertebrate cells (Fig. 2e), others appeared specific at the species and, possibly, the subspecies level (Table 2). Monoclonal 83N-2C4, generated against a cytoplasmic antigen of a presumed isolate of *L. m. mexicana* (WR-222), was strongly reactive (>80%) with two isolates of *L. m. amazonensis* (WR-303 and GML-111) but weakly reactive (33%) with the rodent isolate of *L. m. aristidesi* (GML-3). Un-

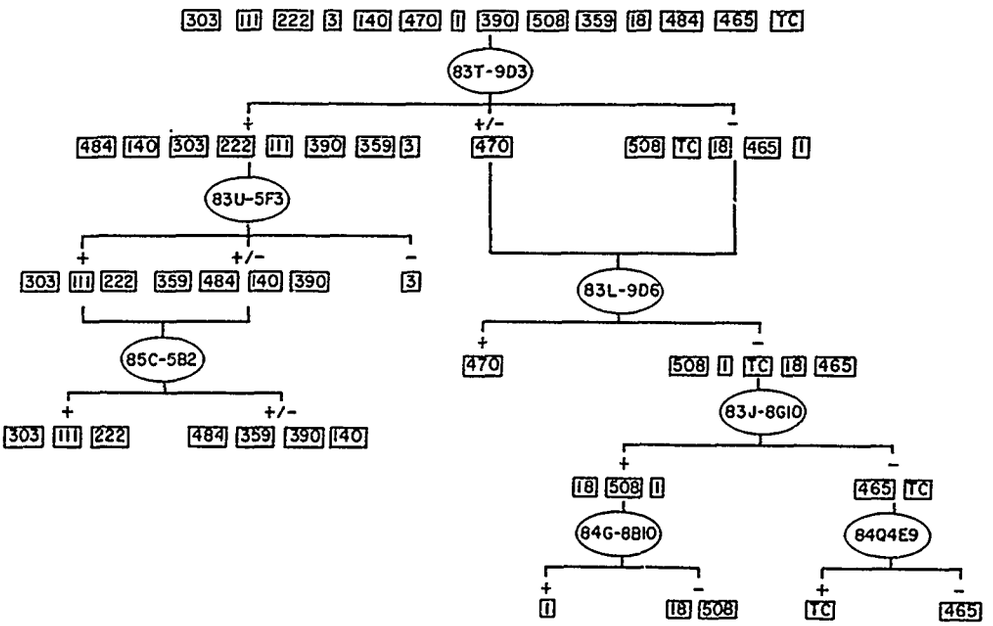

FIGURE 3. Flow sheet for separating 14 New World isolates on the basis of their reactivity in ELISA, with a selected panel of monoclonal antibodies. + = reactivity >75% of the homologous reaction, +/− = 25%–75% reactivity, − = <25% reactivity. Monoclonals: 83T-9D3 = anti-surface flagellum of *L. m. mexicana*; 83U-5F3 = anti-surface membrane of *L. m. mexicana*, 83L-9D6 = anti-cytoplasmic granules of *L. b. panamensis*; 83C-5B2 = anti-surface membrane of *L. m. amazonensis*, 83J-8G10 = anti-surface membrane of *L. b. braziliensis*; 84G-8B10 = anti-surface membrane of *L. b. panamensis*; 84Q-4E9 = anti-surface *T. cruzi*.

508 and GML-1) were either negative or weakly reactive with that same panel. On the other hand, monoclonals to surface antigens of *L. b. braziliensis* (e.g., 83J-8G10) were strongly reactive (>70%) with those four isolates but they were consistently negative with all others. Monoclonal 84G-8B10 exhibited exclusive reactivity with the isolate used for immunization (GML-1), thus suggesting recognition of an isolate-specific epitope. The *L. m. aristidesi* isolate exhibited minimal reactivity (60%–70%) with all monoclonals to surface-flagellar antigens of *L. m. amazonensis* and *L m mexicana*. Although most monoclonals included in this study were generated against surface antigens of *L. m. amazonensis* and *L m. mexicana*, we have not been able to identify epitopic differences between WR-303, WR-222 and GML-111.

Figure 3 is a flow sheet summarizing the capability of representative monoclonal antibodies to distinguish between the 14 isolates in the quantitative ELISA. Separation was based upon both qualitative (<25%) and quantitative (25%–75%) differences in antigen expression.

DISCUSSION

Presently, it is quite fashionable to identify New World *Leishmania* to the species or subspecies level on the basis of their isoenzyme profiles,[13 17 18] their capacity to hybridize with species-specific kinetoplast DNA probes[5,19] or their reactivity with species-specific monoclonal antibodies.[7–9] The sensitivity and specificity of the three procedures are probably comparable but each has, in addition to its distinct advantages, some serious limitations. Although isoenzyme electrophoresis does not require complicated preparation of species-specific reagents, at this time it is still necessary to isolate, cultivate and extract each specimen prior to the analyses. Preparation of species-specific DNA probes can be a long tedious procedure but the availability of such probes does permit the identification of as few as 1,000 organisms in a direct touch blot of the patient's lesion. The blots can be collected and air dried in the field but since the actual hybridization assay requires the incorporation of radioactive tags, their final evaluation is restrict-

at further characterization of the reactive epitopes. Sites of reactivity might be associated with any of several intracellular organelles including glycosomes, lysosomes, ribosomes and secretory granules. Nevertheless, these antibodies did exhibit a broad spectrum of specificity, ranging from cross-reactivity with mammalian cells (Fig. 2e) to capability of differentiating some isolates of *L. mexicana* at the level of subspecies (Table 2). As a rule, however, all monoclonals to leishmanial cytoplasmic granules which were negative with *Trypanosoma* and *Endotrypanum,* irrespective of species of the immunogen, were also nonreactive with WR-508, GML-1 and GML-18, all presumed members of the *L. braziliensis* complex. WR-508 and GML-18 were from cases of mucocutaneous disease.

Most monoclonal antibodies reactive with *Leishmania* surface membrane antigens, regardless of IFA patterns, were taxonomically-specific at the genus level (Table 3). Regrettably, differentiation at the species level was not so clear cut.

Although three isolates of the *L. braziliensis* complex (WR-508, GML-1 and GML-18) did not react with monoclonal antibodies to *L. mexicana* surface antigens, one isolate (WR-470) was weakly reactive and three isolates (WR-140, WR-359 and WR-390) reacted strongly. Conversely, all four of the *L. braziliensis* isolates which were reactive with the anti-*L. mexicana* antibodies were negative with monoclonals to surface epitopes of WR-508, GML-1 and GML-18. Recent analyses of isoenzyme electropherograms confirmed that all cultures used for this study were pure. Thus, if we accept reactivities with monoclonal antibodies as valid parameters for identification we must conclude that isolate WR-359 from a single cutaneous lesion, does not belong to the same subspecies as WR-508 and GML-18, both from mucocutaneous disease. Others [10] have identified WR-359 as an *L. braziliensis* spp. Its reactivity is nearly identical to that of the isolates of *L. b. peruviana* (WR-140) and *L. b. guyanensis* (WR-390) but distinctly different from that of the presumed isolates of *L. b. panamensis* (GML-1 and WR-470) and *L. b. braziliensis* (WR-508 and GML-18). Additional evidence for antigenic divergence between subspecies was provided by the two *L. b. panamensis* isolates. WR-470, strongly reactive with monoclonals produced against the GML-18 *L. b. braziliensis* isolate, was negative with the antibody to GML-1 (84G-8B10). This antibody appears to react with a surface antigen which is peculiar to a subpopulation of the GML-1 cultured promastigotes. Finally, we have not been able to differentiate the *L. d. chagasi* (WR-484) isolate from the *L. mexicana* species. We are convinced, however, that the low level reactivities of the rodent isolate of *L. m. aristidesi* (GML-3) with monoclonals to WR-303 and WR-222 reflect real quantitative differences in antigen expression.

In summation, the intracellular site of reactivity of a monoclonal antibody to New World *Leishmania,* as visualized by IFA, was used to predict the taxonomic specificity of the antibody, as measured by ELISA. Genus-specific antibodies were directed to either surface or cytoplasmic antigens but never to flagellar, kinetoplast or nuclear antigens. Although specificity at the level of species, subspecies or isolate was limited to antibodies reactive with surface antigens, both quantitative and qualitative differences in reactivities were obvious. Finally, reactivities of some monoclonals confirmed that the antigenic divergence within species and/or subspecies can be equal to or greater than that which is observed between species and subspecies. These observations make it quite apparent that universal acceptance of monoclonal antibody technology for identification of New World *Leishmania* will be dependent upon examination of many additional isolates with established panels of monoclonals. Only after that phase is completed, can one begin to think about development of simple diagnostic techniques for use in the field.

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REFERENCES

1. Peters, W., Evans, D. A., and Lanham, S. M., 1983. Importance of parasite identification in

cases of leishmaniasis. *Trans R. Soc. Trop. Med. Hyg., 77:* 540–542.
2. Shaw, J. J., and Lainson, R., 1976. Leishmaniasis in Brazil. XI. Observations on the morphology of *Leishmania* of the *braziliensis* and *mexicana*-complexes. *J. Trop. Med., 79.* 9–13.
3. Chance, M. L., Peters, W., and Schory, L., 1974. Biochemical taxonomy of *Leishmania.* I. Observations on DNA. *Ann. Trop. Med. Parasit., 68:* 307–316.
4. Decker-Jackson, J. E., Schrot, J. R., and Levin, G. V., 1977. Identification of *Leishmania* ssp. by radiorespirometry. *J. Protozool., 24.* 463–470.
5. Barker, D. C., and Butcher, J., 1983. The use of DNA probes in the identification of leishmaniasis: Discrimination between isolates of the *Leishmania mexicana* and *L. braziliensis* complexes. *Trans. R. Soc. Trop. Med. Hyg., 77:* 285–297.
6. Jackson, P. R., Wohlhieter, J. A., Jackson, J. E., Sayles, P., Diggs, C. L., and Hockmeyer, W. T., 1984. Restriction endonuclease analysis of *Leishmania* kinetoplast DNA characterizes parasites responsible for visceral and cutaneous disease. *Am. J. Trop. Med. Hyg., 33:* 808–819.
7. McMahon-Pratt, D., and David, J. R., 1981. Monoclonal antibodies that distinguish between New World species of *Leishmania. Nature, 291.* 581–583.
8. McMahon-Pratt, D., Bennett, E., and David, J. R., 1982. Monoclonal antibodies that distinguish subspecies of *Leishmania braziliensis. J. Immunol., 129:* 926–927.
9. Handman, E., and Hocking, R. E., 1982. Stage-specific, strain-specific and cross-reactive antigens of *Leishmania* species identified by monoclonal antibodies. *Infect. Immun., 37:* 28–33.
10. Beveridge, E., Caldwell, I. C., Latter, V. S., Neal, R. A., Udall, V., and Waldron, M. M., 1980. The activity against *Trypanosoma cruzi* and cutaneous leishmaniasis and toxicity of moxipraquine (349C59). *Trans. R. Soc. Trop. Med. Hyg., 74:* 43–51.
11. Miles, M. A., Lainson, R., Shaw, J. J., Povoa, M., and de Souza, A. A., 1981. *Leishmaniasis* in Brazil: XV. Biochemical distinction of *Leishmania mexicana amazonensis, L. braziliensis braziliensis,* and *L. braziliensis guyanensis*-aetiological agents of cutaneous leishmaniasis in the Amazon Basin of Brazil. *Trans. R. Soc. Trop. Med. Hyg., 75:* 524–529.
12. Miles, M. A., Povoa, M., de Souza, A. A., and Shaw, J. J., 1979. Some methods for the enzymic characterization of Latin American *Leishmania* with particular reference to *Leishmania mexicana amazonensis* and subspecies of *Leishmania hertigi. Trans. R. Soc. Trop. Med. Hyg., 64:* 243–252.
13. Kreutzer, R. D., Semko, M. E., Hendricks, L. D., and Wright, N., 1983. Identification of *Leishmania* spp. by multiple isozyme analyses. *Am. J. Trop. Med. Hyg., 32:* 703–715.
14. Dwyer, D., 1980. Isolation and partial characterization of surface membranes from *Leishmania donovani* promastigotes. *J. Protozool., 27:* 176–182.
15. Kennett, R. H., 1980. Cell fusion. Pages 345–359 *in* T. W. Jakoby and H. Pastan, eds., *Methods in Enzymology.* Academic Press, New York.
16. Oi, V. T., and Herzenberg, L. A., 1980. Immunoglobulin producing hybrid cell lines. Pages 351–372 *in* B. B. Mischell and S. M. Shiigi, eds., *Selective Methods in Cellular Immunology.* W. H. Freeman Co., San Francisco.
17. Kreutzer, R. D., and Christensen, H. A., 1980. Characterization of *Leishmania* spp. by isozyme electrophoresis. *Am. J. Trop. Med. Hyg., 29:* 199–202.
18. Evans, D. A., Lanham, S. M., Baldwin, C. I., and Peters, W., 1984. The isolation and isoenzyme characterization of *Leishmania braziliensis* subsp. from patients with cutaneous leishmaniasis acquired in Belize. *Trans. R. Soc. Trop. Med. Hyg., 78:* 35–42.
19. Wirth, D. F., and McMahon-Pratt, D., 1982. Rapid identification of *Leishmania* species by specific hybridization of kinetoplast DNA in cutaneous lesions. *Proc. Natl. Acad. Sci. USA, 79:* 6999–7003.
20. Anderson, S., David, J. R., and McMahon-Pratt, D., 1983. *In vivo* protection against *Leishmania mexicana* mediated by monoclonal antibodies. *J. Immunol., 131:* 1616–1618.
21. de Ibarra, A. A. L., Howard, J. G., and Snary, D., 1982. Monoclonal antibodies to *Leishmania tropica major*: Specificities and antigen location. *Parasitol., 85:* 523–531.
22. Piras, M. M., de Rodriguez, O. O., and Piras, R., 1981. *Trypanosoma cruzi*: Antigenic composition of axonemes and flagellar membranes of epimastigotes cultured *in vitro. Exp. Parasitol., 51:* 59–73.
23. Gallo, J. M., and Anderton, B. H., 1983. A subpopulation of trypanosome microtubules recognized by a monoclonal antibody to tubulin. *EMBO J., 2:* 479–483.
24. de Souza, W., Meza, I., Martinez-Palomo, A., Sabanero, M., Souto-Padron, T., and Meirelles, M. N. L., 1983. *Trypanosoma cruzi*: Distribution of fluorescently labeled tubulin and actin in epimastigotes. *J. Parasitol., 69:* 138–142.
25. Aarden, L. A., de Groot, E. R., and Feltkamp, T E. W., 1975. Immunology of DNA 111. *Crithidia luciliae*. A simple substrate for the detection of anti-ds DNA with the immunofluorescence technique. *Ann. N.Y. Acad. Sci. 254:* 505–515.

MEMBRANE ANTIGENS OF NEW WORLD *LEISHMANIA*

CHARACTERIZATION AND QUANTITATION OF MEMBRANE ANTIGENS OF NEW WORLD *LEISHMANIA* SPECIES USING MONOCLONAL ANTIBODIES IN WESTERN BLOT AND FLOW MICROFLUOROMETRIC ASSAYS

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ABSTRACT

Membrane-specific monoclonal antibodies generated against promastigotes of New World Leishmania species were used in Western blot, Elisa and flow microfluorometric assays to characterize their antigen specificity and to determine the external surface distribution of the reactive epitopes. Three major membrane antigens of molecular weight 72 KD, 55 KD and 42 KD, were identified as well as a dominant antigen which migrated as a broad-band on SDS-Page corresponding to a molecular weight of 10 - 15 KD. By Dot-Elisa this antigen was also found to be excreted by promastigotes into their culture medium. One minor membrane antigen of 25 KD, and a triplet component of 66, 58, and 56 KD were also identified. While assays performed on air-dried promastigotes revealed the almost ubiquitous presence of the 72 KD and 55 KD antigens, indirect immunofluorescent staining of live promastigotes followed by flow cytometric analysis revealed that these antigens had no external exposure. Antibodies binding the 55 KD component were also reactive toward purified mammalian tubulin. The remaining antigens had a variable distribution on the 8 isolates utilized and these quantitative differences could be used to distinguish isolates of the mexicana complex from those belonging to the braziliensis complex.

Strains of digenetic protozoans belonging to the genus Leishmania are responsible for a variety of localized and/or disseminated ulcerative cutaneous lesions in man. Infection is initiated when leishmania are inoculated into the skin by the bite of the phlebotomine sandfly vector, and is maintained by subsequent parasite invasion of and multiplication within cells of the reticuloendothelial system. In the Americas, strains of the L. mexicana and L. braziliensis complexes are those most often implicated in human disease. Although the severity of disease is generally determined by the adequacy of the host immune system in responding to the parasite burden, some strains may persist as chronic infections despite the presence of perceptible host humoral and cell-mediated responses (32). The differentiation of those strains which characteristically produce benign, self-limiting lesions from those capable of producing progressive disease is essential in terms of patient prognosis and choice of chemotherapy.

The surface membrane of the promastigote, which serves as a barrier between the detrimental components of the sandfly digestive tract and the extracellular milieu of the mammalian dermis, is a dynamic structure capable of high affinity interaction with its host cell, the macrophage. This membrane is also the site of multiple antigens which can elicit antibody production and alter T-cell functions in the mammalian host (2-4, 27). Qualitative and quantitative antigenic differences between membrane components of leishmania species, sub-species and isolates have been demonstrated using polyvalent antisera (10, 28) or monoclonal antibodies (1, 13, 17, 23-25). Moreover, parasite differentiation from the extracellular promastigote to the

intracellular amastigote is also accompanied by antigenic alterations (6, 14, 29). Although pathogenic mechanisms of this human leishmaniasis have yet to be clearly delineated, it would appear that the expression of different surface components and their distribution within the lipid bilayer may play a significant role in the behavior of the parasite within the insect vector and the mammalian host. Available evidence indicates that membrane antigenic proteins and glycoconjugates of Leishmania spp. function in the recognition of and adhesion to membranes of mammalian host cells (7, 11) and in the resistance of the parasite to degradation by proteolytic enzymes present in the sandfly digestive tract and in the parasitophorous vacuole (5, 15, 21).

We now report on the identification of 6 different antigenic components on the surface membrane of leishmania promastigotes using monoclonal antibodies generated against isolates of the L. mexicana and L. braziliensis complexes. Reactive antigens of isolated promastigote membranes or detergent extracts have been characterized by polyacrylamide gel electrophoresis, followed by Western blot immunoautoradiography. Most importantly, differences in the external distribution of these antigens on the surface membranes of eight New World Leishmania isolates have been quantitated by flow microfluorometry, and these differences were used to determine those antigens which are species-specific.

MATERIALS AND METHODS

Parasites. Human isolates of L. mexicana mexicana (WR 222), L. mexicana amazonensis (WR 303), L. braziliensis guyanensis (WR 390), L. braziliensis panamensis (GML 1), L. braziliensis braziliensis (WR 508, GML 18), L. species (Courtwright - WR 359) and one rodent isolate of L. mexicana amazonensis (GML 111) were obtained from the Walter Reed Army Institute of Research (WR series) and The Gorgas Memorial Laboratory (GML series) (Table I). All strains were isolated from cutaneous lesions except GML 18 and WR 508, which were isolated from advanced mucocutaneous lesions. Epimastigotes of T. cruzi (Tulahuen strain) were obtained from American Type Culture Collection (Rockville, MD). Parasites were cultivated in 650 ml screw-top tissue culture flasks at 26°C in Medium-199 with Hanks' salts, 12 mM Hepes, 20 mM L-glutamine, 20% heat inactivated fetal calf serum, 50 USP units penicillin, and 50 ug/ml streptomycin and gentamycin sulfate.

Immunization of Balb/c Mice. Female Balb/c mice, 6-8 weeks of age, were inoculated by subcutaneous injection with 15 ug of whole promastigote suspension prepared by repeated freezing (-70°C) and thawing (+40°C) and sonic disruption (6 pulses at 50 watts for 3 seconds each at 2°C) . Isolates utilized in the sensitization of mice were WR 222 (fusions 83H and 83T), and GML 1 (fusion 84G). Fusion 83U was performed using splenocytes from a mouse immunized with isolated membranes of WR 222 promastigotes which were purified on discontinuous sucrose gradients as detailed by Dwyer (8). For this fusion, injections consisted of 100 ug of purified membrane protein. Mice received injections twice weekly for 3 weeks and were rested the week prior to

splenectomy. An exception to this schedule was the 84C fusion. That mouse was sensitized with live irradiated promastigotes isolated from the digestive tracts of sandflies infected with WR 303. The mouse received a total of 3 injections of approximately 10^5 promastigotes and was rested for 1 month prior to the last injection. All mice were given a final inoculation 3 days prior to sacrifice.

Cell Fusion and Selection of Hybrid Clones. Fusion of mouse splenocytes with syngeneic plasmacytoma cells (P3X63-Ag8) was performed according to the modifications by Kennett (18) and Oi and Herzenberg (26) of methods originally described by Kohler and Milstein (19). Briefly, 10^8 spleen cells were mixed with 10^7 myeloma cells, pelleted by centrifugation, and fused at room temperature by resuspending the pellet in 0.2 ml of 30% (v/v) polyethylene glycol-1000 in Dulbecco's Modified Eagle Medium without serum. After 8 minutes the cells were washed, resuspended in 30 ml. Dulbecco's supplemented with hypoxanthine, thymidine and 20% newborn calf serum, and distributed into 96-well tissue culture plates (50 ul/well). Hybrid cells were selected by the addition of 8 mM aminopterin the following day, and were screened for antibody production 10-14 days later by indirect immunofluorescent (IFA) or enzyme-linked immunosorbent (Elisa) assays of cell culture supernatants. Antibody producing hybrids were cloned in semi-solid agarose in 48-well tissue culture plates. Cloning was initially performed using irradiated Balb/3T3-A2 cells as feeder layers but were not used in later cloning procedures as the hybrids grew equally well in their absence. For long term storage, 10^7 cells were frozen in a mixture of 0.5 ml Dulbecco's medium and 0.5 ml cryoprotective medium (M.A. Bioproducts, Walkersville, MD) and kept in liquid nitrogen.

Production of Ascites Fluid. Balb/c mice were primed with 0.5 ml Pristane (2, 6, 10, 14-tetramethylpentadecane) on day 10 and day 3 prior to receiving an injection of 10^4 antibody-producing hybridoma cells. Ascites fluid was recovered from the peritoneal cavities of Balb/c mice 2 to 3 weeks later. From 6 to 10 ml of ascites fluid per mouse could routinely be collected.

Antibody Isotyping. The isotype of each monoclonal antibody was determined by a sandwich Elisa using rabbit anti-mouse subclass specific immunoglobulins and a peroxidase-coupled goat anti-rabbit serum (Mouse Immunoglobulin Subtype Identification Kit, Boehringer-Mannheim, Indianapolis, IN).

Assays of Culture Supernatants for Monoclonal Antibody. For enzyme-linked immunosorbent assays, 2 x 10^4 intact stationary-phase promastigotes were extensively washed in phosphate-buffered saline, pH 7.2 (PBS) and pipetted into the wells of flat-bottomed polystyrene 96-well microtiter plates which were pretreated (2 hrs/room temperature) with 50 ul/well of a 0.001% solution of poly-L-lysine in PBS. Plates were centrifuged at 2000g for 5 min in microplate carriers, and excess PBS was removed by inversion. The plates were allowed to air-dry overnight. Wells were washed 2 times with PBS/0.05% Tween-20 and uncoated sites were blocked by the addition of 100 ul/well of 1% normal goat serum. One hundred ul/well of culture supernatant was added and incubated for 2 hrs at room temperature or overnight at 4°C. Plates were washed 3 tires with PBS-Tween 20 and 100 ul/well of a 1/500 dilution in PBS of alkaline phosphatase-conjugated goat anti-mouse immunoglobulin serum was added. Bound enzyme was detected by the addition of 100 ul/well of substrate, p-nitrophenyl phosphate, 1 mg/ml

in diethanolamine buffer, pH 9.8. After incubating for 15 min at room temperature, the reaction product was quantitated in a Titertek Multiskan spectrophotometer by reading absorbance at 405 nm. The non-specific myeloma (P3) immunoglobulin served as the negative control. Absorbance readings of greater than 0.1 were considered positive.

Indirect immunofluorescence was performed on 8-well microscope slides which were previously coated with PBS-washed promastigotes (500/well), air-dried, and stored at -70°C until use. The slides were incubated with 25 ul of culture media/well for 30 min at room temperature. The conjugate was an affinity purified goat anti-mouse immunoglobulin serum labelled with fluorescein isothiocyanate and diluted to 1/50 with 0.25% Evans Blue in PBS. Reactivity was graded subjectively on a scale of 1-4+.

Flow Cytometric Detection of Membrane Antigen. External orientation of membrane antigens and species-specificity of antibodies were determined by suspension staining of 10^6 live promastigotes at 4°C followed by flow cytometric analysis. Parasites were incubated in 1 ml of hybridoma culture medium in microfuge tubes with periodic agitation, and further incubated with a 1/100 dilution of FITC-conjugated goat-antimouse Ig in PBS containing 1% normal goat serum. Cells were washed 3 times in PBS between incubation steps by centrifugation at 10,000g in a Beckman microfuge. Parasites were fixed in 0.5% phosphate-buffered paraformaldehyde, pH 7.6, washed again, and resuspended in PBS to a final volume of 0.5 ml. These cells could be stored in the dark at 4°C for up to 2 weeks with negligible loss of reactivity. Simultaneous measurement of forward angle light scatter and fluorescence intensity

were made on a Beckton-Dickinson FACS IV, with an excitation wavelength of 488 nm. Green fluorescence of FITC was measured at 530 nm. Relative fluorescent intensity was calculated by multiplying the total number of cells/channel by the channel number (T2) and dividing by the number of cells analysed (T1). Experiments were done in triplicate with 10,000 cells/sample analyzed.

Detergent Extraction. 10^8 promastigotes were extracted twice in 1% Triton X-100 (v/v) buffered with 38 mM Tris, 0.1 M glycine and 2 mM phenylmethyl sulfonyl fluoride (PMSF) as described by Gardiner and Dwyer (9).

SDS-Page. Vertical slab gel electrophoresis was performed using the SDS-discontinuous buffer system of Laemmli (20) on a 12.5% resolving gel. Samples consisted of 100 ug of purified promastigote membrane or detergent extract, which were prepared under reducing (with 2-mercaptoethanol) and non-reducing (without 2-mercaptoethanol) conditions. Protein concentrations were determined by the method of Lowry, et al.(22) The molecular weight standards were phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400).

Western Blot Determination of Antibody Specificity. Proteins resolved on polyacrylamide gels were electrophoretically transferred using the technique of Towbin et al. (30) to 0.45 um nitrocellulose paper for 2 hrs. at 1 amp in a Hoefer model TE-50 Transphor Cell. Nitrocellulose strips were blocked for 30 min in 5% bovine serum albumin in PBS. Strips were then placed directly in a 1/2 dilution of hybridoma culture medium or a 1/100 dilution of ascites fluid in PBS-2%

BSA and incubated for 2 hrs at room temperature with gentle rocking. Strips were washed and placed in a 1/1000 to 1/1500 dilution (20,000 cpm/ml) of ^{125}I-labelled goat anti-mouse immunoglobulin serum in PBS-2% BSA. Large volumes of PBS-0.05% Tween 20 were used to wash between incubation steps. The strips were air-dried and exposed to X-ray film (Kodak X-OMat AR film) at -70°C for 3 to 7 days utilizing a calcium tungstate intensifying screen.

Identification of Excreted Antigen by Dot Blot Analysis. One ul of supernatant media from 4-day old promastigote cultures was dotted onto a strip of nitrocellulose paper. The paper was immersed in a 1/5 dilution of hybridoma culture medium for 1 hour, washed, and reacted with a 1/2500 dilution of peroxidase-labelled goat anti-mouse immunoglobulin serum. Color was developed by the addition of a 0.15% (w/v) solution of 4-chloro-1 naphthol with 0.015% (v/v) H_2O_2. The buffer used for antibody dilutions and washes was 20 mM Tris-500 mM saline, pH 7.5. Fresh culture media was run in parallel as a negative control.

RESULTS

Approximately 50% of the monoclonal antibodies produced against sonicated promastigotes are directed toward intracellular components. The remaining 50% react with surface antigens and can be categorized on the basis of patterns of membrane fluorescence by IFA. The panel of antibodies used in this study, and the immunofluorecent pattern which each demonstrates, are described in Table II.

83H-2D6 and 84C-5B2, generated against WR 222 and WR 303 respectively, produce the smooth, linear pattern of fluorescence, exclusive of the flagellum (Fig. 1A). The antigen recognized by these antibodies was identified in Western blots of purified promastigotes membranes as a 72,000 MW component (Fig 1B). This antigen could not be demonstrated in blots of Triton-extracted antigens. By Elisa, the presence of this antigen was demonstrated on all leishmania species isolated from cutaneous lesions, but was not present on the membrane of the mucocutaneous isolate, GML 18, or on epimastigotes of T. cruzi (Table III).

Antibodies 83T-9D3 and 83U-7D5 also demonstrate a smooth pattern of fluorescence of uniform distribution along the membrane, including the flagellum (Fig. 2A). Western blots of both Triton extracts and purified membranes verified that the reactive antigen was a diffuse, rapidly-migrating membrane component with a molecular weight ranging from 10-15,000 (Fig. 2B). Since these antibodies were strongly reactive when as little as 5 ug of crude Triton extract was loaded onto the gel, we concluded that they were directed against a major membrane

antigen. Results of the dot-Elisa using media from 4-day old promastigote cultures, revealed that this antigen is also present in spent leishmania culture media (Fig 2C). Fresh Medium-199 was non-reactive.

83U-5F2 and 83U-9B3 also display a smooth pattern of distribution by indirect immunofluorescence microscopy. The membrane component recognized by 83U-5F2 has a molecular weight of 25,000 (Fig. 3). Since it was weakly demonstrable under all electrophoretic and blotting conditions, we presume that this antigen represents a minor membrane component. Alternatively, this minimal reactivity could be due to low affinity binding of this antibody, or the result of some antigenic destruction under the electrophoretic conditions employed. Two major bands of approximate molecular weight 66,and 58,000, and one minor band of MW 56,000 were recognized by antibody 83U-9B3 (Fig 3). These bands could represent breakdown products of a single large molecule, or 3 separate molecules containing the same reactive determinant.

83T-6F11 and 84G-6B6, stain the entire surface of the parasite, but in addition display a diffuse background of extracellular fluorescence around the periphery of each organism (Fig 4A). These antibodies identified a 55,000 MW component present on intact purified membranes, but the antigen was not demonstrable in the Triton extracts. The molecular weight of this component corresponds to that of the predominant band on the Coomassie blue stained gel of resolved purified membranes (Fig 4B). The possibility that this antigen may represent the tubulin portion of the submembrane cytoskeletal network was examined by comparing its SDS-Page and Western blot profiles with that of purified mammalian (rat brain) tubulin. Fig. 4B confirms that 83T-6F11 and 84G-6B6 also bind to rat brain tubulin, although the mammalian tubulin

has a slightly higher electrophoretic mobility. Reactivity by Elisa establishes that this antigen is common to both Leishmania and Trypanosomes (Table III).

The remainder of the antibodies (84C-8C7, 83U-6F4, 83U-2F11 and 83T-10E4) show the discrete granular pattern of fluorescence, of uniform distribution along the membrane and flagellum (Fig. 5A). An antigenic component of approximately 42,000 MW was identified by these antibodies in immunoblots of both the Triton extract and purified membranes (Fig 5B). However, the antigen could only be detected when the electrophoretic separation was performed under non-reducing conditions.

Results of indirect flow microfluorometric assays designed to detect and quantitate externally oriented surface membrane antigens on 8 strains of New World Leishmania are presented in Table IV. Fluorescence intensities are reported as T2/T1 ratios, where T2 is the total number of cells/channel multiplied by the channel number, and T1 is the total number of cells analyzed. It can be seen that the antigens recognized by 4 antibodies (83H-2D6, 84C-5B2, 83T-6F11, 84G-6B6) could not be demonstrated on live organisms stained in suspension. Since these antigens were readily detected on air-dried organisms by Elisa and IFA, they must be embedded in the membrane such that their reactive epitopes are not exposed in the intact organism. The remaining antibodies display high levels of binding to live parasites of the homologous strain, thus indicating that the antigens which they recognize are located on the external side of the bilayer. Representative fluorescent histograms are presented in Fig. 6. The variability of expression of any one membrane antigen permits quantitative differentiation of isolates of the braziliensis from those

of the mexicana complex. Externally-oriented antigens recognized by 83T-9D3, 83U-7D5, 83U-2F11, 83U-6F4, 83T-10E4 and 84C-8C7 are most heavily concentrated on the surface of L. mexicana strains (WR 222, WR 303, GML 111), are present in moderate amounts on the L. b. guyanensis (WR 390) membrane and are minor membrane constitutents of L. species (WR 359). Antigens recognized by 83U-5F2 and 83U-9B3 were undetectable on this isolate. Using this assay, all antibodies were negative on both isolates of L. b. braziliensis (GML 18 and WR 508) and on the single L. b. panamensis (GML 1) isolate tested.

DISCUSSION

Species-specific monoclonal antibodies can be used to differentiate New World Leishmania isolates belonging to the mexicana and braziliensis complexes (1, 23-25). The value of these antibodies as reagents for diagnostic assays and epidemiologic surveys cannot be disputed. The fusion of immune splenocytes from mice inoculated with whole parasite homogenates results in the production of monoclonal antibodies which encompass a wide range of specificities for membrane, flagellar and intracellular antigens (1). Although some monoclonal antibodies to cytoplasmic constituents can differentiate isolates at the sub-species level, most which are directed against intracellular or flagellar epitopes lack specificity at the genus level (1). Antigens associated with the surface membrane show a higher degree of species heterogeneity, but whether these differences parallel differences in pathogenicity and tissue tropism is yet to be determined.

Biologic membranes are asymmetric in terms of lipid, protein and carbohydrate composition. Surface membrane macromolecules with an external orientation are in contact with extracellular substances as well as other cell membranes. Such components of the leishmania membrane play a dominant role in the recognition of its host cell, the macrophage (7, 11). We have now identified and quantitated some of these surface molecules by measuring their reactivity with selected monoclonal antibodies in flow microfluorometric assays using eight isolates of New World Leishmania. Since we had difficulties in the identification of some antigens using enzymatically-mediated membrane iodination and immunoprecipitation techniques, we concluded that the

relevant antigens were either associated with the cytoplasmic surface of the membrane, or, if exposed, lacked the tyrosine or histidine residues required for iodination. In contrast, all antigens were identified using an immunoblot procedure. Interestingly, relatively few groups of antigens were identified when categorized by apparent molecular weight.

Antibodies 83U-7D5 and 83T-9D3 bind strongly to externally-disposed antigens, which migrated by SDS-Page as a broad-band component ranging in apparent molecular weight from 10-15,000. This antigen is present in large quantity along the membrane as it could be detected by Western Blot when only 5 ug of sample protein was loaded onto the gel. Dot blots of media supporting L. mexicana promastigotes confirmed that this antigen is an excreted factor, and periodic-acid schiff staining of antigen purified by immunoaffinity chromatography confirmed the presence of a carbohydrate moiety (not shown). Such diffusely migrating surface components have been identified in other leishmania species, and seem to represent a class of strongly immunogenic macromolecules present in large quantity along the promastigote membrane. For example, a carbohydrate-containing, acid-labile glycoconjugate extracted from L. donovani promastigotes migrated as a polydisperse band by SDS-Page (31). An antigenic component of L. m. pifanoi membranes visualized by SDS-Page/Western Blot analysis using specific monoclonal antibodies (23) had a similar diffuse appearance. Most recently, Handman et al. (12), using a 2-site immunoradiometric Western Blot and a specific monoclonal antibody, identified a major broad band glycoconjugate on the membrane of L. major which was also detected in culture supernatants. Biosynthetic labelling confirmed that this

antigen incorporated both radioactive carbohydrate and lipid precursors. We have not yet performed lipid analysis of the antigen recognized by 83U-7D5 and 83T-9D3. Although this antigen is not stained by Coomassie blue, the presence of minor protein constituents cannot be ruled out.

A major externally oriented antigen of an approximate molecular weight of 42 KD was identified by 4 of 12 antibodies. The reactive epitope of this antigen is obviously sensitive to thiol reagents, as it was undetectable when the SDS-Page sample buffer contained 2-mercaptoethanol. By radioiodination of live leishmania promastigotes of L. m. amazonensis followed by immunoprecipitation of surface antigens, Chang identified a major membrane glycoprotein with a molecular weight of 43 KD (5). While we cannot assume that this antigen is identical to that recognized by our 4 antibodies, we can conclude that the 42 KD membrane component identified in the present study is a dominant antigen of the mexicana isolates utilized.

83H-2D6 and 84C-5B2 recognize a 72 KD component which is a dominant component of most strains of leishmania tested. This antigen could not be identified by Western Blot analyses of Triton extracts, but it was quite distinct using intact membranes. It is possible that antigenicity was destroyed during extraction, but Triton-X and other polyethoxy-type nonionic detergents are nondenaturing and are therefore preferred where retention of biological activity is critical (16). Furthermore, denaturation of the intact membrane preparation by boiling in SDS did not destroy its reactive epitope. Triton-X is not efficient at breaking protein-protein interactions, so this antigen simply may not have been extracted. Flow cytometry displays little external

exposure of the antigen, whereas strong antibody binding was seen when promastigotes were air-dried to allow antibody penetration through the membrane. This antigen could be deeply embedded within the bilayer or it may be associated with the cytoplasmic surface of the membrane.

Similarly, antigens recognized by 83T-6F11 and 84G-6B6 were not present on the external side of the membrane and were not demonstrated using Triton-extracted promastigotes by Western Blot immunostaining. However, both recognized a 55,000 MW component of isolated intact promastigote membranes. Dwyer (8) identified the dominant 55 KD component of L. donovani pellicular membranes as tubulin, and demonstrated the subpellicular mitrotubule network remains attached during the isolation procedure. These antibodies were shown to cross-react with purified rat brain tubulin by immunoblot staining, and by Elisa are strongly positive on all strains tested, including T. cruzi.

In summary, we have used 12 monoclonal antibodies to identify asymetrically disposed proteins and glycoconjugates of the leishmania membrane. The surface expression of these antigens on promastigote membranes was quantitated in flow microfluorocytometric assays. On the basis of the number of antibodies which recognize components of the same molecular weight, it would seem that the L. mexicana membrane consists of at least 4 dominant and 2 minor antigens. The 72 KD membrane antigen detected by 83H-2D6 and 84C-5B2 and the 55 KD component recognized by 83T-6F11 and 84G-6B6 probably serve as structural molecules, such as tubulin, and show little species diversity. The remaining antigens, which have an external distribution, show species heterogeneity and probably serve as molecules of recognition. Studies designed to confirm this suspicion are in progress.

LITERATURE CITED

1. Anthony, R.L., Williams, K.M., Sacci, J.B. & Rubin, D.C. 1985. Subcellular and taxonomic specificity of monoclonal antibodies to New World Leishmania. Am. J. Trop. Med. Hyg., (in press).

2. Arredondo, B. & Perez, H. 1979. Alterations of the Immune Response Associated with Chronic Experimental Leishmaniasis. Infec. Immun., 25: 16-22.

3. Castes, M., Agnelli, A. & Rondon, A.J. 1984. Mechanisms associated with immunoregulation in human American cutaneous leishmaniasis. Clin. Exp. Immunol., 57: 279-286.

4. Castes, M., Agnelli, A., Verde, O., & Rondon, A.J. 1983. Characterization of the cellular immune response in American cutaneous leishmaniasis. Clin. Immunol. & Path., 27: 176-186.

5. Chang, K.P. 1983. Cellular and Molecular Mechanisms of Intracellular Symbiosis in Leishmaniasis. Int. Rev. Cytol., Suppl. 14: 267-305.

6. Chang, K.P. & Fong, D. 1982. Antigenic changes during intracellular differentiation of Leishmania mexicana in cultured macrophages. Infect. Immun., 36: 430-431.

7. Chang, K.P. & Fong, D. 1983. Cell biology of host-parasite membrane interactions in leishmaniasis. in Cytopathology of parasitic disease (Ciba Foundation symposium 99), Pitman Books, London: 113-137.

8. Dwyer, D.M. 1980. Isolation and partial characterization of surface membranes from Leishmania donovani promastigotes. J. Protozool., 27: 176-182.

9. Gardiner, P.R. & Dwyer, D.M. 1983. Radioiodination and identification of externally disposed membrane components of Leishmania tropica. Mol. Biochem. Parisitol., 8: 283-295.

10. Gardiner, P.R., Jaffe, C.L. & Dwyer, D.M. 1984. Identification of cross-reactive promastigote cell surface antigens of some Leishmania stocks by ^{125}I-labelling and immunoprecipitation. Infect. Immun., 43: 637-643.

11. Handman, E. & Goding, J.W. 1985. The Leishmania receptor for macrophages is a lipid-containing glycoconjugate. EMBO J., 4: 329-336.

12. Handman, E., Greenblatt, C.L. & Goding, J.W. 1984. An amphipathic sulphated glycoconjugate of Leishmania: characterization with monoclonal antibodies. EMBO J., 3: 2301-2306.

13. Handman, E. & Hocking, R.E. 1982. Stage-specific, strain-specific, and cross-reactive antigens of Leishmania species identified by monoclonal antibodies. Infect. Immun., 37: 28-33.

14. Handman, E., Jarvis, H.M. & Mitchell, G.F. 1984. Leishmania major: identification of stage-specific antigens and antigens shared by promastigotes and amastigotes. Parasit. Immunol., 6: 223-233.

15. Handman, E., Mitchell, G.F., & Goding, J.W. 1981. Identification and characterization of protein antigens of Leishmania tropica isolates. J. Immunol., 126:508-512.

16. Helenius, A. & Simons, K. 1975. Solubilization of membranes by detergents. Biochim. et Biophys. Acta, 415: 29-79.

17. Jaffe, C.L. & McMahon-Pratt, D. 1983. Monoclonal antibodies specific for Leishmania tropica. I. Characterization of antigens associated with stage- and species-specific determinants. J. Immunol., 131: 1987-1993.

18. Kennett, R.H. 1979. Cell fusion. in Jakoby, W.B. & Pastan, H., eds., Methods in Enzymology, Vol 68, Academic Press, New York, 68: 345-359.

19. Kohler, G. & Milstein, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature, 256: 495-497.

20. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680.

21. Lewis, D.H. & Peters, W. 1977. The resistance of intracellular Leishmania parasites to digestion by lysosomal enzymes. Ann. Trop. Med. Parasitol., 71: 295-312.

22. Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.

23. McMahon-Pratt, D., Bennett, E., Grimaldi, G. & Jaffe, C.L. 1985. Subspecies- and species-specific antigens of Leishmania mexicana characterized by monoclonal antibodies. J. Immunol., 134: 1935-1940.

24. McMahon-Pratt, D., Bennett, E. & David, J..R. 1982. Monoclonal antibodies that distinguish subspecies of Leishmania braziliensis. J. Immunol., 129: 926-927.

25. McMahon-Pratt, D. and David, J.R. 1981. Monoclonal antibodies that distinguish between New World species of Leishmania, Nature, 291: 581-583.

26. Oi, V.T. & Herzenberg, L.A., 1980, Immunoglobulin-producing hybrid cell lines. in Mishell, B.B. & Shiigi, S.M., eds., Selected Methods in Cellular Immunology, W.H. Freeman, Co., San Francisco: 351-372.

27. Perez, H. 1983. Factors influencing the host response to Leishmania mexicana. in Cytopathology of parasitic disease (Ciba Foundation symposium 99), Pitman Books, London: 157-173.

28. Ramasamy, R., Kar, S.K. & Jamnadas, H. 1983. Cross reacting surface antigens on Leishmania promastigotes. Internat. J. Parasitol., 13: 337-341.

29. Sadick, M.D. & Raff, H.V. 1985. Differences in expression and exposure of promastigote and amastigote membrane molecules in Leishmania tropica. Infect. Immun., 47: 395-400.

30. Towbin, H., Staehelin, T. & Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA, 76: 4350-4354.

31. Turco, S. J., Wilkerson, M.A. & Clawson, D.R. 1984. Expression of an unusual acidic glycoconjugate in Leishmania donovani, J. Biol. Chem., 259: 3883-3889.

32. Walton, B.C., Brooks, W.H. & Arjora, I. 1972. Serodiagnosis of American leishmaniasis by the indirect fluorescent antibody test. Am. J. Trop. Med. Hyg., 21: 296-299.

Fig. 1: Immunofluorescent pattern (A) and Western blot characterization (B) of 72 KD membrane component. Monoclonal antibodies specific for these determinants were 83H-2D6 (Lane A) and 84C-5B2 (Lane B). Micrograph magnification = 600x.

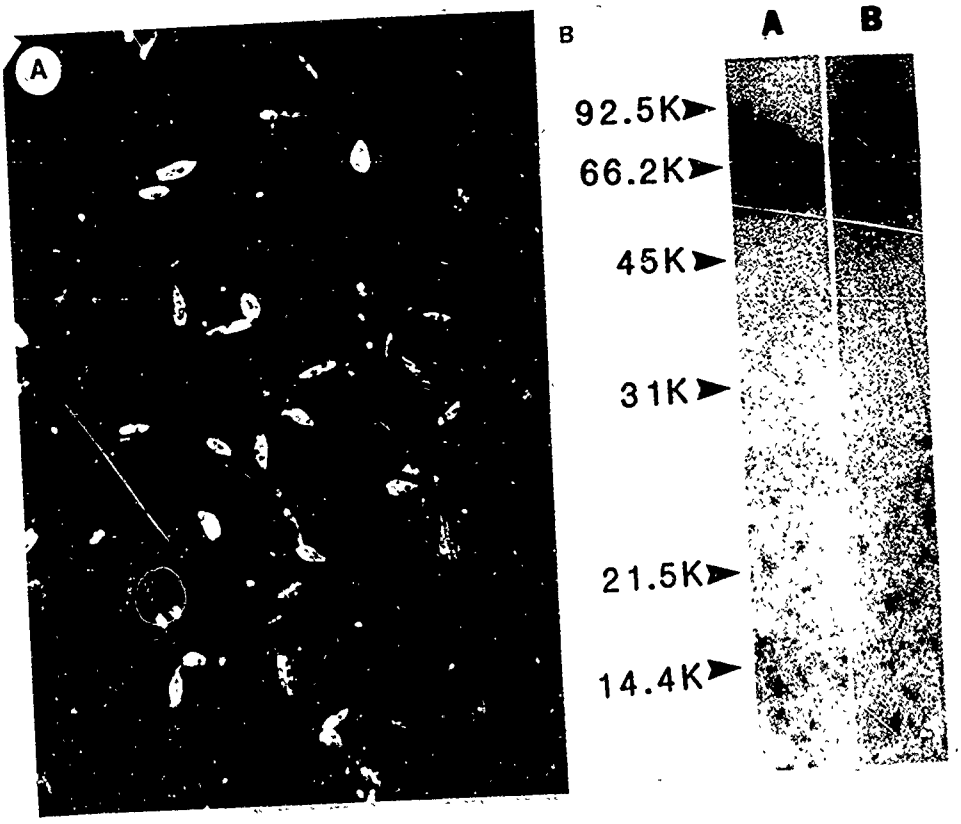
A
B
A
B
92.5K
66.2K
45K
31K
21.5K
14.4K

Fig. 2: Immunofluorescent pattern (A) and Western blot characterization (B) of polydisperse membrane component with molecular weight of 10-15 KD. Monoclonal antibodies specific for these determinants were 83T-9D3 (Lanes A and B) and 83U-7D5 (Lanes C and D). Dominant antigen was identified when as little as 5ug of protein was loaded onto gel (Lanes B and D). Results of the Dot-Elisa (C) demonstrate that these antigens are also excreted by promastigotes into their culture media. Micrograph magnification = 600x.

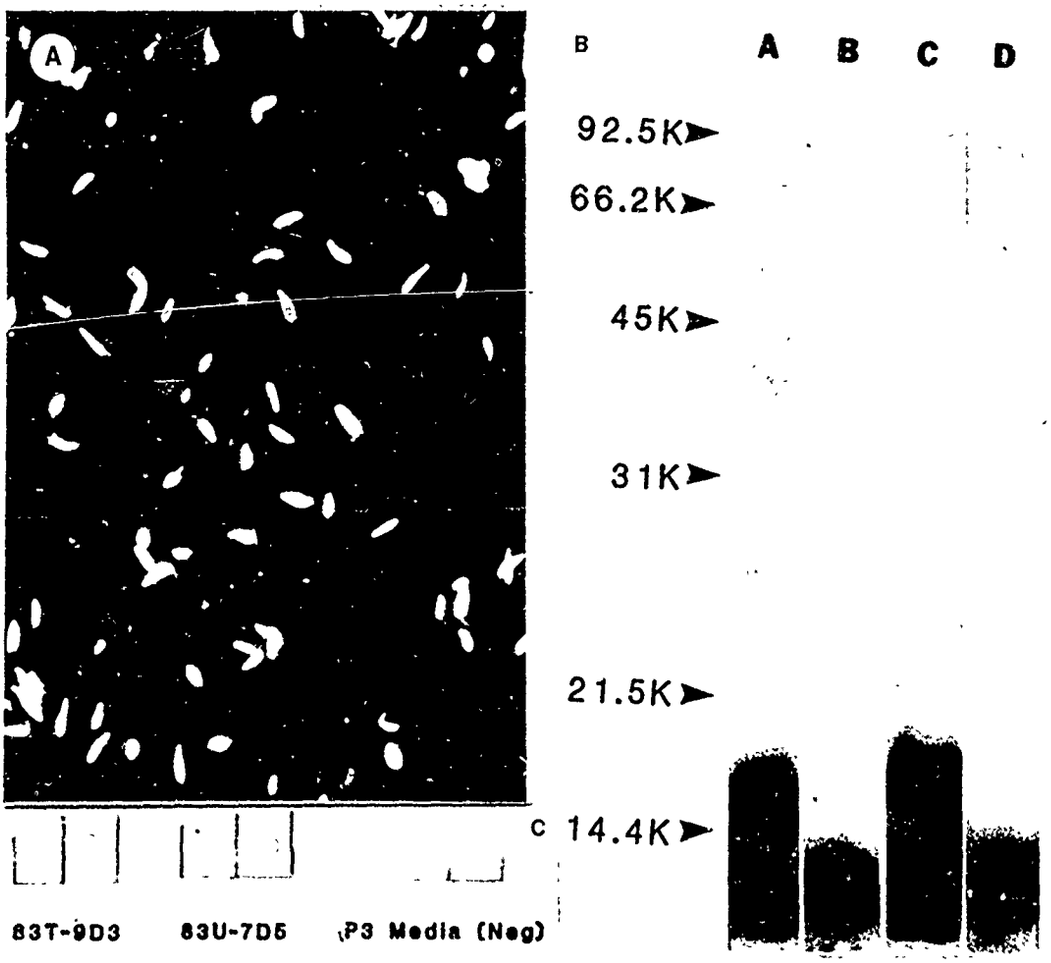
A
B
A B C D
92.5K
66.2K
45K
31K
21.5K
14.4K
C
83T-9D3
83U-7D5
P3 Media (Neg)

Fig. 3: Western blot characterization of membrane antigens recognized by 83U-9B3 (Lane A) and 83U-5F2 (Lane B).

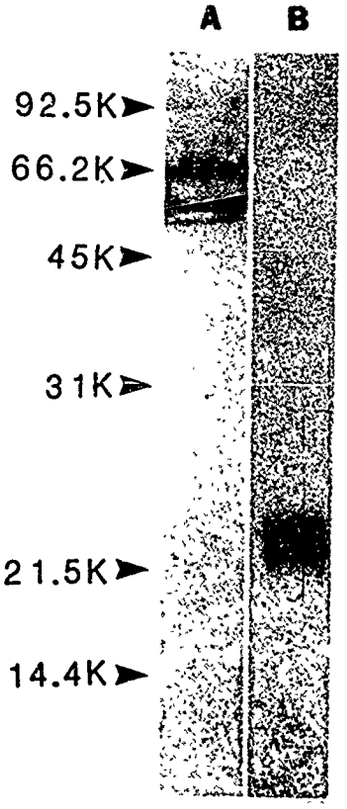
A
B
92.5K
66.2K
45K
31K
21.5K
14.4K

Fig. 4: Immunofluorescent pattern (A) and Western blot characterization (B) of 55 KD membrane antigen. Monoclonal antibodies specific for this determinant were 84G-6B6 (Lane B) and 83T-6F11 (Lane D). This membrane component appears as the dominant band on a Coomassie blue stained gel of resolved membrane proteins (Lane A), and was presumptively identified as tubulin on the basis of the reactivity of 84G-6B6 and 83T-6F11 with purified mammalian tubulin (Lanes C and E, respectively). Micrograph magnification = 900x.

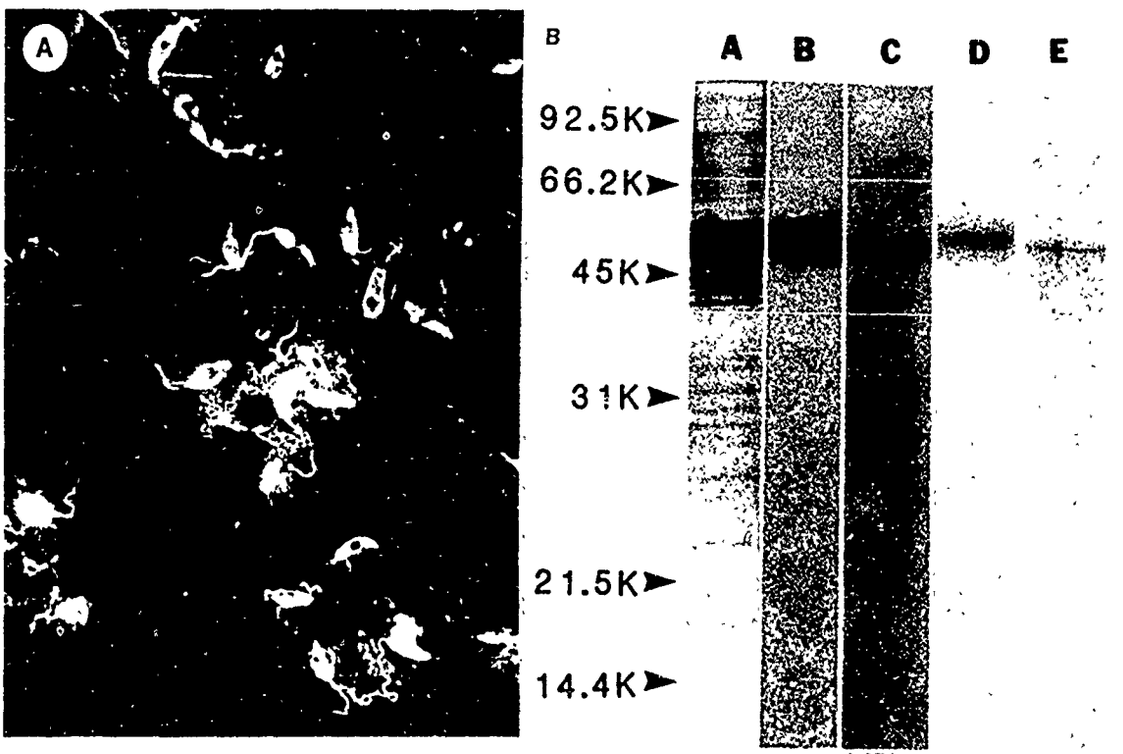
A
B
A
B
C
D
E
92.5K
66.2K
45K
31K
21.5K
14.4K

Fig. 5: Granular pattern of immunofluorescence (A) demonstrated by monoclonal antibodies specific for 42 KD membrane component (B). Antibodies recognizing this component were 84C-8C7 (Lane A), 83U-6F4 (Lane B), 83U-2F11 (Lane C) and 83T-10E4 (Lane D). Micrograph magnification = 600x.

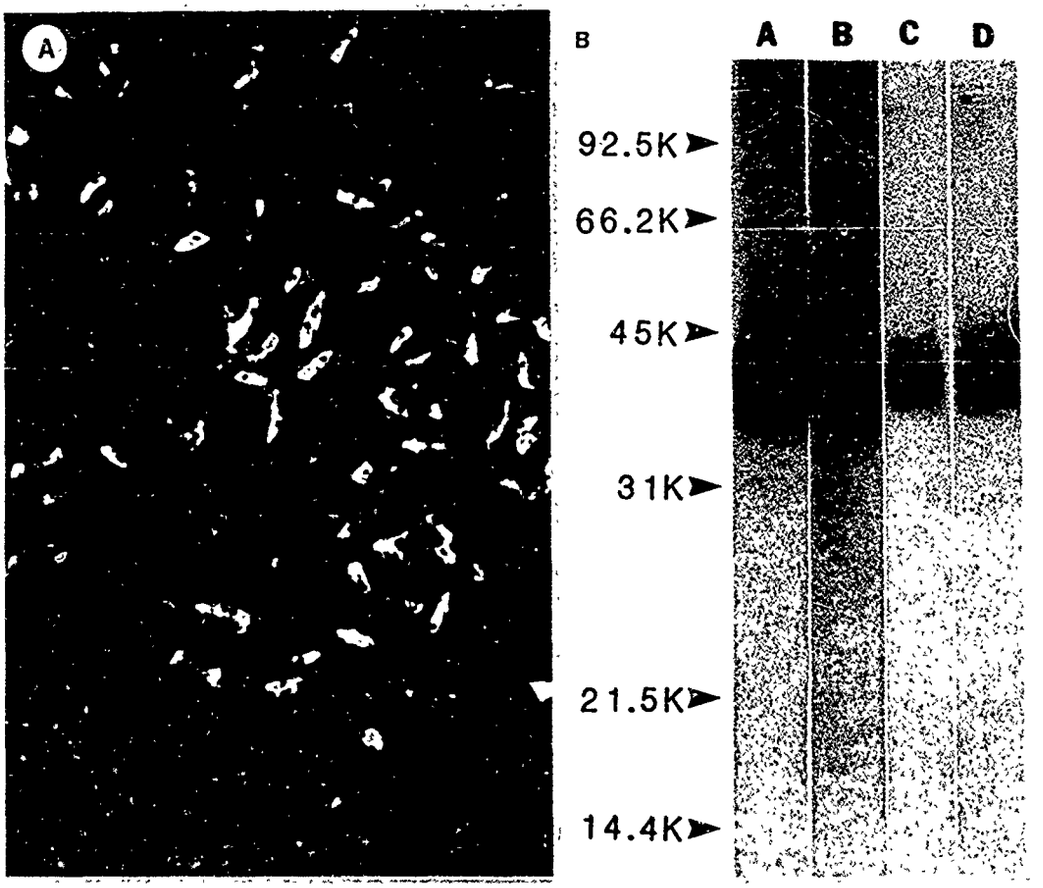
A
B
A
B
C
D
92.5K
66.2K
45K
31K
21.5K
14.4K

Fig. 6: Fluorescent histograms demonstrating strong binding of monoclonal antibody 83T-9D3 with L. mexicana mexicana (A) and L. mexicana amazonensis (B) promastigotes. Negative control photographed simultaneously (upper histogram) for comparison.

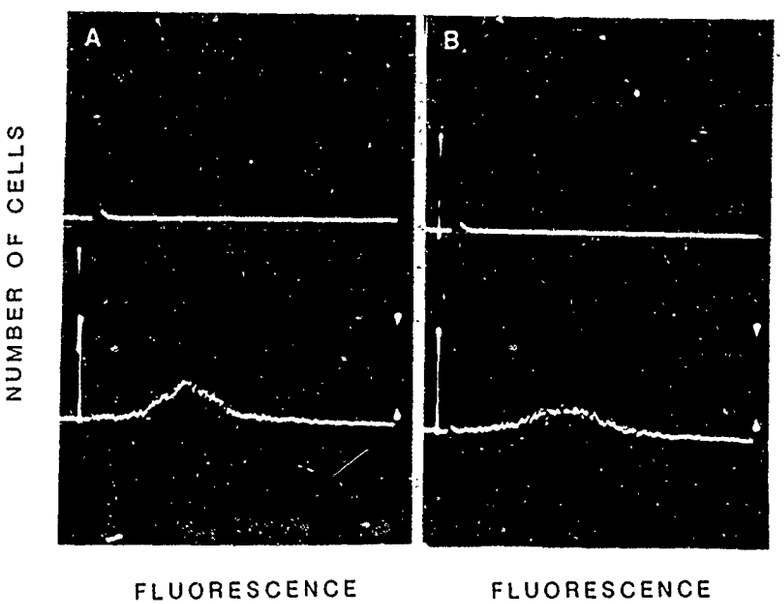
A
B
NUMBER OF CELLS
FLUORESCENCE
FLUORESCENCE

TABLE I.

NEW WORLD LEISHMANIA ISOLATES USED IN THIS STUDY

Isolate Identification	Numerical Designation	Country of Origin	Host	Type of Lesion
L. mexicana mexicana	WR 222	Panama	Human	Cutaneous
L. mexicana amazonensis	WR 303 (LV 72)	Brazil	Human	Cutaneous
L. mexicana amazonensis	GML 111	Brazil	Rice Rat	Cutaneous
L. braziliensis guyanensis	WR 390*	Brazil	Human	Cutaneous
L. species (Courtwright)	WR 359 (LV 185)	Panama	Human	Cutaneous
L. braziliensis panamensis	GML 1	Panama	Human	Cutaneous
L. braziliensis braziliensis	WR 508	Brazil	Human	Mucocutaneous
L. braziliensis braziliensis	GML 18	Peru	Human	Mucocutaneous
T. cruzi (Talahuen strain)	No. 30266, ATCC**	Chile	Human	Cardiac

* Designated WHO International Reference Strain MHOM/BR/75/M4147

** American Type Culture Collection, Rockville, Md.

TABLE II.

CHARACTERISTICS OF MONOCLONAL ANTIBODIES PRODUCED AGAINST NEW WORLD LEISHMANIA SPECIES

Antibody	Isotype	Elisa Titre[a]	IFA. Titre[b]	Pattern of Fluorescence[c]	Immunogen
P3 (Neg)	IgG1	0	0	Negative	N/A
83H-2D6	IgG1	256	128	SS	WR 222 Sonicate
84C-5B2	IgG1	128	128	SS	WR 303 Insect Isolate
84C-8C7	IgG1	128	64	SG	WR 303 Insect Isolate
84G-6B6	IgM	2048	512	SE	GML 1 Sonicate
83T-6F11	IgM	512	256	SE	WR 222 Sonicate
83T-9D3	IgG2b	2048	1024	SF	WR 222 Sonicate
83T-10E4	IgA	256	128	SG	WR 222 Sonicate
83U-2F11	IgG1	128	64	SG	WR 222 Purified Membrane
83U-5F2	IgG1	8	16	SF	WR 222 Purified Membrane
83U-6F4	IgG2a	128	64	SG	WR 222 Purified Membrane
83U-7D5	IgG3	2048	1024	SF	WR 222 Purified Membrane
83U-9B3	IgG1	512	128	SF	WR 222 Purified Membrane

[a] Highest dilution giving an absorbance reading of >0.1 at 405 nm with homologous antigen.
[b] Highest dilution showing at least 1+ fluorescence with homologous antigen.
[c] SS = smooth surface exclusive of flagellum (Fig. 1A).
SF = smooth surface including flagellum (Fig. 2A).
SE = smooth surface plus diffuse extracellular (Fig 4A).
SG = surface granules including flagellum (Fig. 5A).

*TABLE III.

REACTIVITY OF MONOCLONAL ANTIBODIES TO
NEW WORLD LEISHMANIA PROMASTIGOTES AND T. CRUZI EPIMASTIGOTES BY
ENZYME-LINKED IMMUNOSORBENT ASSAYS[a]

Antibody	*L.m.mexicana* WR 222	*L.m.amazonensis* WR 303	*L.m.amazonensis* GML 111	*L.b.guyanensis* WR 390	*L.species* WR 359	*L.b.panamensis* GML 1	*L.b.braziliensis* GML 18	*L.b.braziliensis* WR 508	*T.cruzi*
P3 (Neg)	.001	.002	.001	.002	.001	.001	.002	.001	.002
83H-2D6	.261	.336	.258	.363	.229	.183	.081	.221	.038
83C-5B2	.314	.326	.246	.299	.211	.122	.050	.227	.061
83T-6F11	.319	.337	.354	.288	.397	.218	.171	.277	.278
84G-6B6	.465	.500	.453	.534	.414	.426	.370	.569	.374

[a] Mean optical density at 405 nm of a minimum of 3 assays.

TABLE IV.

QUANTITATION OF LEISHMANIA SURFACE MEMBRANE ANTIGENS
ON THE BASIS OF THEIR REACTIVITY WITH MONOCLONAL ANTIBODIES
IN FLOW CYTOMETRIC ANALYSES[a]

Antibody	*L.m.mexicana* WR 222	*L.m.amazonensis* WR 303	*L.m.amazonensis* GML 111	*L.b.guyanensis* WR 390	*L.species* WR 359	*L.b.panamensis* GML 1	*L.b.braziliensis* WR 508	*L.b.braziliensis* GML 18
P3 (Neg)	10.4	10.9	10.9	10.6	9.7	10.2	10.2	9.7
83H-2D6	13.3	13.7	14.7	13.4	12.8	12.9	13.8	12.0
84C-5B2	13.3	14.1	15.5	13.9	12.9	13.0	14.1	11.3
83T-6F11	10.1	10.4	10.6	9.7	9.8	10.0	10.1	10.4
84G-6B6	10.2	10.6	10.5	10.7	9.8	10.1	9.9	10.1
83T-9D3	95.6	106.4	19.1	54.2	21.8	10.1	10.9	9.7
83U-7D5	74.4	75.4	87.3	51.6	19.9	10.0	10.4	9.7
83U-2F11	66.0	85.5	84.8	46.7	14.9	10.1	9.9	9.7
83U-6F4	82.8	67.6	91.4	45.5	18.9	10.2	10.2	10.3
84C-8C7	74.4	96.4	82.5	53.3	18.4	10.3	10.9	9.5
83T-10E4	66.9	85.5	84.8	46.7	15.9	10.0	9.9	9.7
83U-5F2	36.8	38.3	32.4	20.3	10.8	10.0	9.8	9.7
83U-9B3	78.2	76.4	62.6	24.8	10.1	9.8	9.8	9.7

[a] Mean T2/T1 of 3 samples with 10,000 cells/sample analyzed.

Values > 16.0 considered significantly different from negative control by paired t-test.

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12/23/85

FLOW CYTOMETRIC ANALYSIS OF THE EFFECTS EXERTED BY MONOCLONAL ANTIBODIES ON BINDING AND UPTAKE OF LEISHMANIA MEXICANA MEXICANA PROMASTIGOTES BY MURINE PERITONEAL MACROPHAGES.

BINDING AND UPTAKE OF LEISHMANIA PROMASTIGOTES

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Abstract:

A flow cytometric assay was developed to quantitate the binding kinetics of Leishmania mexicana mexicana promastigotes to murine peritoneal macrophages, and to determine if selected membrane-specific monoclonal antibodies would exert an effect on the binding process. Three monoclonal antibodies, all reactive with a similar 42 kd surface membrane component by Western Blot, enhanced parasite-macrophage binding at levels greater than 45%. Three additional monoclonal antibodies which identified low molecular weight antigens of the promastigote (15-20 kd), had no effect on the binding process. One of these antibodies, however, did appear to inhibit internalization of the parasites subsequent to attachment to the macrophage membrane.

Introduction:

Pathogenic species of dimorphic protozoans belonging to the genus Leishmania are responsible for a spectrum of disfiguring cutaneous lesions which may spread locally or metastasize to the viscera or mucocutaneous junctions of the oropharyngeal cavity. Infection is initiated by direct inoculation of the flagellated promastigote into the host skin by the phlebotomine sandfly vector. Promastigotes are then phagocytized by dermal macrophages, where they differentiate into the intracellular, or amastigote form. Once infection is established, parasites are restricted to cells of the reticuloendothelial system. Amastigote replication continues to the point of cell rupture, at which time the released amastigotes are phagocytized by proximal macrophages which have been recruited to the inflammatory focus.

Since promastigotes are susceptible to lysis by components of the extracellular fluid (25,29), prompt attachment to and uptake by the macrophage is required for successful maintenance of infection. Leishmania-macrophage membrane interactions have been examined extensively using phagocytes from humans or rodents in combination with amatigotes or promastigotes of various Leishmania species (5,7,10,11,24,30,33,34). While these studies have established that optimum conditions for in vitro demonstration of leishmania-macrophage binding may vary depending on the assay system and the particular species of parasite and host involved, several characteristics are common to all models. These characteristics include specificity, saturability, and the involvement of glycosylated antigenic components of the parasite surface membrane (8,35). Although binding readily occurs in the absence of

opsonins or cytophilic antibody, humoral factors are known to influence this interaction and the subsequent survival of leishmania within the parasitophorous vacuole (5,17,18).

We now report on the use of a rapid and sensitive flow cytometric assay to monitor the binding kinetics of L. m. mexicana promastigotes to murine peritoneal macrophages, and to determine if this process is affected by the presence of murine monoclonal antibodies. Results confirmed that three monoclonal antibodies, reactive with a 42 kd moiety on the parasite surface membrane, greatly enhanced promastigote binding to macrophages. Another monoclonal antibody, which identified a low molecular weight component of the parasite membrane, had no effect on parasite-macrophage binding but inhibited subsequent phagocytosis of the promastigote.

Materials and Methods:

Parasites. A human isolate of L. m. mexicana (WR 222) was obtained from the cryobank at The Walter Reed Army Institute of Research. Promastigotes were maintained in 650 ml screw top tissue culture flasks at 26C in liquid Medium 199 containing 12 mM Hepes buffer, 20% heat-inactivated fetal calf serum and antibiotics.

Peritoneal exudate cells. Cells, isolated by lavage from the peritoneal cavities of Balb/c mice which had been primed 7 days previously with 2 ml of sterile 4% thioglycollate broth, were washed twice by centrifugation for 5 min at 500g, ard resuspended in Eagle's Minimum Essential Medium with Hanks' salts base containing 10 % heat-inactivated fetal calf serum (hereafter called cell media). Macrophages were enumerated by total and differential cell counting and the cell suspension was standardized to contain 1.25×10^6 macrophages/ml.

Monoclonal antibodies. Balb/c mice, 6-8 week of age, were immunized by subcutaneous inoculation of purified promastigote membranes (Fusion U), sonicated whole promastigotes (Fusions L and T), or irradiated promastigotes isolated from the digestive tract of infected phlebotomine sandflies (Fusion C). Protocols for inoculum preparation and immunization schedules were outlined previously (1). Immune splenocytes were recovered and fused with 10^7 syngeneic P3X63-Ag8 plasmacytoma cells for 8 min at room temperature in the presence of 30% polyethylene glycol 1000. Methods for the performance of fusions and selection of aminopterin-resistant hybridomas have been described by Kohler and Milstein (20) and modified by Oi and Herzenberg (27). Anti-

body-secreting hydridomas were identified by indirect immunofluorescent (IFA) or enzyme-linked immunosorbent assays (ELISA) using stationary phase promastigotes air-dried onto 8-well microscope slides or poly-l-lysine treated 96-well polyvinyl chloride microtiter plates, respectively (19). Antibody-producing hybridomas were cloned by limiting dilution in soft agarose. Taxonomic and subcellular specificities of these antibodies have been described elsewhere (1).

Antibody purification and characterization. 10 hybridoma cells secreting selected monoclonal antibodies were injected into the peritoneal cavities of Balb/c mice which had been primed with 0.5 ml of Pristane (2,6,10,14-tetramethyl pentadecane) 10 and 3 days previously. The resultant ascites fluid was clarified by ultracentrifugation at 100,000g for 30 min at 4C, and then dialysed against 100 volumes of 0.02M Tris-HCl buffer, pH 7.2. Monoclonal antibodies were purified from ascites fluid by gradient elution (0-100 mM NaCl) through a DEAE-Affigel blue (Biorad, Richmond, CA) column (6). After the purity of the IgG fraction was confirmed by SDS-PAGE, antibodies were concentrated by ultrafiltration in an Amicon cell fitted with a PM-10 membrane. IgM monoclonal antibodies were purified by overnight dialysis against 5 mM Tris-HCl buffer, pH 7.5 (28). The resultant precipitate was washed twice in dialysis buffer by centrifugation at 10,000 g for 30 min at 4C and then redissolved in 1 mM Tris-HCl, pH 8.6. Prior to macrophage binding studies, all purified monoclonal antibodies were dialysed overnight against cell media without serum and then standardized to a final concentration of 2 mg/ml.

Isotypes of the monoclonal antibodies were determined by a sandwich ELISA using rabbit anti-mouse subclass specific immunoglobulins and a peroxidase coupled goat anti-rabbit serum (Boehringer-Mannheim, Indianopolis. IN).

Fluorescent labelling of promastigotes. Fifty milliliters of stationary phase promastigotes (approximately 10^7/ml) were washed twice by centrifugation at 5,000 g for 10 min in sterile Ca^{++}-Mg^{++} free Hanks' balanced salt solution. They were then resuspended in 20 ml of a saturated solution of fluorescein isothiocyanate isomer I (Sigma Chemcal Co., St. Louis, MO) in Hanks' balanced salt solution (HBSS), pH 7.2. After a 30 min incubation at 35C, the parasites were washed 4 times in HBSS, and resuspended in cell media. Alternatively, for incubation mixtures with monoclonal antibodies, parasites were resuspended in cell media containing the pre-determined dilution of antibody. The post-staining viability of promastigotes was verified by microscopic assessment of flagella motility.

Parasite-macrophage binding studies. Kinetics of parasite attachment to macrophages was assessed by the addition of FITC-labelled L. m. mexicana promastigotes to a suspension of unlabelled peritoneal exudate cells. The increase in fluorescence of the macrophage population, as monitored by flow cytometry, was a direct measure of the binding of promastigotes to their surface. A 0.4 ml volume of the exudate cell suspension was pipetted into polypropylene tubes (5 X 10^5 macrophages per tube). FITC-labelled promastigotes were suspended to the appropriate concentration and added in a 0.1 ml volume so that the final ratio of parasites to macrophages was either 2:1, 5:1, 10:1 or 20:1. Cell mixtures were incubated at 35C in a humidified

atmosphere containing 5 % CO. At sequential time intervals following infection, 10 ul was removed for microscopic examination by IFA and the remainder was fixed with 0.5 % cold paraformaldehyde for 10 min, washed once in PBS, and resuspended in 0.5 ml PBS for flow cytometric analysis. All experiments were performed at least twice using triplicate specimens at each time point.

Effect of monoclonal antibodies on binding kinetics. Fluoresceinated parasites were suspended in cell media containing the appropriate dilution of the purified monoclonal antibody and were added to macrophages at a 10:1 parasite to macrophage ratio. Cell mixtures were incubated for 1 hr in the continuous presence of antibody. Alternatively, the opsonizing activity of monoclonal antibodies was determined by allowing them to react with the promastigotes for 1 hr at 37C prior to addition of macrophages. Conversely, the cytophilic property of the monoclonals was assessed by their incubation with macrophages before addition of the parasites. In both cases, unbound antibody was removed by washing twice with fresh media before cell mixtures were prepared. Tubes were then incubated as before for 1 hr at 35C and analysed by flow cytometry.

Flow cytometry. Simultaneous measurement of forward angle light scatter at 488 nm and green fluorescence of FITC at 530 nm were performed on a Becton-Dickinson FACS IV Cell Sorter/Analyser equipped with a argon laser light source. The fluorescent signals of the parasite-macrophage mixture were electronically gated to include only those signals emitted by cells within the size range of macrophages, as determined by light scatter. An increase in

fluorescence in these channels was a direct measurement of the attachment of fluoresceinated promastigotes to the macrophage membrane. The negative controls were: (a) a mixture of fixed peritoneal exudate cells and fixed FITC-labelled promastigotes incubated in cell media or in the presence of an irrevalent antibody at the same dilution as that of the test samples, and (b) peritoneal exudate cells incubated with media from the last washing of the promastigote staining procedure. Those cells deflected into channels higher than the negative gated values were counted, electronically, as positive cells. The percentage positive cells in a particular sample was then calculated by dividing the positive count by the total number of cells analysed (e.g. 10,000 cells/tube). Photographs of histograms were taken directly from the monitor using Polaroid ASA 3000 film.

Parasite membrane extraction. 5×10^8 L. m. mexicana promastigotes were washed 4 times by centrifugation at 5,000 g for 10 min at 4C in 38 mM Tris, 0.1M glycine, pH 8.7. The parasites were resuspended in 1 ml of the same buffer containing 1 % Triton-X 100 and 2 mM phenyl-methylsulfonyl fluoride (PMSF), and were extracted by stirring at 4C for 1 hr. Parasites were centrifuged for 30 min at 48,000 g and the supernatant was stored in 1 ml aliquots at -20 C untli needed.

SDS-PAGE. Polyacrylamide gel electrophoresis of immunoglobulins and parasite membrane extracts was performed according to the discontinuous buffer system of Laemmli (21) utilizing a 12.5% total acrylamide concentration in the

resloving gel. Appropriate molecular weight standards were included. Resolved proteins were stained by diffusion with 0.25 % Coomassie Brilliant Blue or were electroblotted onto nitrocellulose membranes.

Western blot analysis. Resolved parasite membrane proteins were transferred from polyacrylamide gels onto nitrocellulose strips for 2 hrs at 1 amp using the buffer system of Towbin et al. (32). After electroblotting, susbsequent non-specific protein binding was blocked by placing the strips in 3 % BSA in PBS for 30 min at room temperature. After 3 washes in large volumes of PBS-0.5 % Tween 20, pH 7.6, strips were placed in either a 1:2 dilution of hybridoma culture medium or a 1:100 dilution of ascites fluid in PBS for 2 hr at room temperature. Nitrocellulose membranes were again washed and then placed in a 1:2500 dilution of I^{125} conjugated goat anti-mouse immunoglobulins (New England Nuclear). After another 2 hr incubation and wash cycle, the strips were air-dried and autoradiographed for 3-5 days at -70 C using Kodak X-Omat AR film and a calcium tungstate intensifying screen.

Results:

Fluorescent labelling of promastigote membranes. Direct labelling of promastigotes with fluorescein isothiocyanate resulted in uniform intense fluorescent staining of the surface membrane with minimal toxicity to the parasite. Since there was little loss of membrane fluorescence, as quantitated by flow cytometry, after washing the parasites 15 times, or after storing of fixed promastigotes for up to 3 weeks in the dark at 4c (data not shown), tight bonds were apparently formed between the dye and the parasite membrane proteins. Post-staining viability of the promastigotes was generally greater than 95% as judged by flagellar motility.

Parasite-macrophage binding kinetics. Flow cytometric analyses and concomitant examination by indirect immunofluorescent microscopy confirmed that promastigotes rapidly bind to the surface of macrophages in the absence of specific antibodies. When a 20:1 ratio of promastigotes to macrophages was used, 70% of the macrophages exhibited one or more parasites on their surface within one hour (Fig. 1). Our inability to increase this level of saturation by using higher parasite to macrophage ratios was attributed to the differences in susceptibility of peritoneal exudate cells which are at various stages of differentiation (14). In fact, some cells appeared to be resistant to infection (Fig. 2). Using a 10:1 parasite to macrophage ratio, maximum binding approached that of the 20:1 curve but a additional 3 hrs of incubation was required. Conversely, when lower ratios were tried, the number of cells emitting a fluorescent signal plateaued at <25 % within 30 min. We suspect that the majority of these cells were binding only one or two parasites which

were rapidly internalized and thus, the level of surface fluorescence promptly fell below the limits of detection. Unfortunately, the cytometer cannot distinguish between attached and internalized parasites. Nevertheless, the slight decrease in the fluorescence signal occurring at six hours post infection, in all likelihood, represents phagocytosis of the fluoresceinated promastigotes.

Effect of monoclonal antibodies on the binding kinetics. Three monoclonal antibodies, 84C-4F4, 84C-8C7 and 83L-5G9, all of which recognized a 42 kd molecule of the parasites surface membrane (Fig. 3), enhanced the binding of promastigotes to macrophages, with 84C-4F4 producing the maximum effect. In fact, when a 1:10 dilution of 84C-4F4 was incubated in the presence of a 1:10 mixture of parasites to macrophages, binding was enhanced immediately and actually surpassed that of the antibody-free experiments using the 20:1 ratio (Fig. 4). Since parasite binding in the tube containing 84C-4F4 was nearly double that of the corresponding 1:10, antibody- free, control tube after only one hour of incubation, those parameters were selected for all subsequent experiments. Interestingly, the binding curve of 84C-4F4 shows a gradual decrease in fluorescence as the incubation time progresses, and actually meets that of the 20:1 binding curve after 6 hrs. It seems that even though the initial increase in binding surpasses that of the 20:1 mixture, the number of promastigotes which the macrophage can accomodate is limited as the parasites are internalized.

The dose response curve demonstrating the binding enhancement produced by incubating cell mixtures in the presence of serial dilutions of 84C-4F4 is presented in Figure 5. While an antibody dilution of 1:1280 caused significant enhancement (10.8 %) of parasite binding, the maximum enhancement was achieved at an antibody dilution of 1:10. These data are observed on the fluorescence histograms as a gradual shift to the right of the macrophage population as increasing concentrations of the antibody are included in the parasite-macrophage mixture (Fig. 6).

When promastigotes were opsonized with a 1:10 dilution of 84C-4F4 for 1 hr and then washed with fresh media, their attachment to macrophages was also greatly enhanced. In fact, no difference in macrophage binding was observed between opsonized promastigotes and those incubated in the continuous presence of antibody. Macrophages preincubated with 84C-4F4 also bound a significantly greater number of parasites as compared to controls. However, maximum binding obtained was somewhat less than that acheived by promastigote opsonization. The results of these experiments, as well as those performed in the continuous presence of the other membrane-specific monoclonal antibodies, are summarized in Table 1.

Although binding was enhanced by the three monoclonals which recognized the 42 kd component of the parasite membrane, three others which exhibited exclusive reactivity with a low molecular weight (15-20 kd) polydisperse molecule (Fig. 7) had no effect. However, when these antibody-parasite-macrophage mixtures were held for an additional 24 hrs and then examined by immunofluorescent microscopy, it was discovered that one monoclonal antibody,

83L-2D3, actually prohibited phagocytosis of the promastigotes by the macrophage (Fig.8). After an additional day of incubation, these parasites still remained paralyzed at the macrophage surface.

Discussion:

Conclusions based on previous studies of the interactions of leishmania with isolated macrophages seem to depend on the pecularities of the particular parasite-macrophage pairing and the variability in the assays conditions employed. These studies have also been hampered by difficulties in distinguishing the initial process of parasite-macrophage membrane interaction from subsequent parasite phagocytosis and intracellular multiplication. Quantitation of macrophage parasitization has involved microscopic enumeration of Giemsa stained preparations (3,26) or solubilization and scintillation counting of macrophage cultures infected with radiolabelled promastigotes (8). Although valuable information has been obtained utlizing these methods, the extensive handling and manipulation of cultures which is required and the tedious and subjective nature of microscopic counting limits their applicability. The success of others (2,5),in using flow cytometric techniques to measure parameters of phagocytosis of bacteria and yeasts, prompted the current effort.

The mechanism of promastigote binding to macrophages is generally believed to occur via the attachment of leishmania surface glycoproteins or glycolipids to lectin-like antigenic components on the macrophage membrane. The process can be completely inhibited by incubating in the presence of certain saccharides (5,10,34) or can be reduced by pretreatment of parasites with various glycosidases (8,34). Although humoral factors are not a prerequisite for efficient binding to occur, Herman (18) demonstrated that sera from infected mice did enhance the binding of L. donovani to both resident

and thioglycollate induced murine macrophages. This enhancement, however, could be abrogated by pretreatment of the macrophages with trypsin. More recently, Bray (5) reported that sera from leishmania infected humans, guinea pigs and rabbits contained both cytophilic and opsonic antibodies which enhanced phagocytosis of L. mexicana mexicana promastigotes by murine, starch elicicted. macrophages. In those studies, however, pretreatment of macrophages with trypsin greatly enhanced the adherence of the promastigotes. In contrast, Chang (9) demonstrated an inhibition of parasitization of human phagocytes by L. donovani amastigotes in the presence of a polyvalent rabbit anti-amastigote serum, but he could not show a comparable effect with rabbit anti-promastigote serum.

These discrepant observations are not surprising when one attempts to reconcile data obtained using different species of leishmania and various sources of macrophages as binding partners. The surface membrane of macrophages from different mammlian species display heterogeneity with respect to their composite of Fc receptors for Ig isotypes, association constants and sensitivity to trypsin (22). Moreover, the specificities and isotypes of antibodies in polyvalent antisera vary with respect to the animals used, the route of inoculation and the schedual of bleeding. Perhaps most importantly, macrophage receptors show species variation in affinity and mechanism of binding to heterologous Fc (23). Finally, the obvious antigenic differences between the surface membranes of the two leishishmania forms make evaluations of the effects of antibodies on parasite-macrophage interactions

even more complicated. In an effort to eliminate most of these variables, we have examined the effect of antibodies on parasite-macrophage binding by using standardized amounts of monoclonals of known isotype and specificity.

Of the three monoclonal antibodies which enhanced parasite binding to macrophages, 84C-4F4 was an IgG2a whereas 84C-8C7 and 83L-5G9 were of the IgG1 isotype. Although all three of these antibodies recognized epitopes on what appeared to be the same 42 kd component of the parasite membrane, the superior capability of the 84C-4F4 monoclonal to enhance parasite-macrophage binding was obvious. We suspect that this higher level of enhancement can be attributed to the greater affinity of the mouse macrophage FcR1 receptor for IgG2a than that which occurs with the FcR2 receptor for IgG1 (12). Moreover, since 84C-4F4 clearly has opsonic reactivity, its presence in the form of an immune complex at the parasites surface will also contribute to a greater avidity for FcR2 (31).

Three additional monoclonal antibodies which recognized a polydisperse, low molecular weight component of the promastigote membrane had no measurable effect on parasite-macrophage binding. However, subsequent immunofluorescent microscopy revealed that the IgM monoclonal, 83L-2D3, inhibited the internalization of the parasites once they had attached to the macrophage surface. Since mouse macrophages do not display a receptor for IgM (28), we can not attribute the obvious adherence and ensuing paralysis to the cytophilic mechanism described by Farah (13). We suspect that the mutilvalent IgM molecule causes a blockage, either steric or antigen-specific, of the triggering sequence required for phagocytosis. Other authors (11,17) have

suggested that the reduced number of amastigotes in macrophages infected with opsonized promastigotes is a consequence of enhanced intracellular killing. Unfortunately, the assays used by these authors could not distinguish between inhibition of binding, blockage of internalization and enhancement of intracellular killing.

Finally, Handman et al (16) have reported that the Fab fragment of a monoclonal antibody generated against a glycoconjgate of L. tropica inhibits the binding of promastigotes to mouse macrophages in vitro. This glycoconjugate, which has an electrophoretic mobility and size congruent with the moiety identified by 83T-9D3, 83U-7D5 and 83L-2D3, can be purified from medium supporting stationary phase promastigotes. Moreover, this 15-20 kd molecule binds to the surface of macrophages and thus, it has been regarded as the leishmania ligand responsible for attachment (15). Although we have not used Fab fragments in our binding studies, we suspect that any potential inhibition of binding through antibody-mediated blocking of the glycoconjugate ligand may be counterbalanced by macrophage Fc receptor-mediated adherence of opsonized parasites.

In summary, we have applied the technique of fluorescent activated cell sorting to study the kinetics of leishmania binding to mouse macrophages. This technology is sensitive, detecting as few as one promastigote per macrophage, and it can count and sort large populations of cells in a short period of time. Most importantly, flow cytometry can be used to assess the effects of highly specific monoclonal antibodies on the events of adherence and

internalization and, in conjuction with electroimmunoblotting, identify those surface antigens of leishmania which contribute to the immunopathology of the disease.

Literature Cited:

1. Anthony, R.L., K.M. Williams, J.B. Sacci, and D.C. Rubin. 1985. Subcellular and taxonomic specificity of monoclonal antibodies to New World Leishmania. Am. J. Trop. Med. Hyg. 34: 1085-1094.

2. Bassoe, C.L., L.J. Glette, G. Hopen, B. Haneberg, and C.O. Solberg. 1983. Simultaneous measurement of phagocytosis and phagosomal pH by flow cytometry: Role of polymorphonuclear neutrophilic leukocyte granules in phagosome acidification. Cytometry 4: 254-262.

3. Berman, J.D., D.M. Dwyer, and D.J. Wyler. 1979. Multiplication of Leishmania in human macrophages in vitro. Infect. Immun. 26: 375-379.

4. Bjerknes, R. 1984. Flow cytometric assay for combined measurement of phagocytosis and intracellular killing of Candida albicans. J. Immunol. Methods 72: 229-241.

5. Bray, R.S. 1983. Leishmania mexicana mexicana: Attachment and uptake of promastigotes to and by macrophages in vitro. J. Protozool. 30: 314-322.

6. Bruck, C., D. Portetelle, C. Glineur, and A. Bollen. 1982. One-step purification of mouse monoclonal antibodies from ascitic fluid by DEAE Affigel Blue chromatography. J. Immunol. Meth. 53: 313-319.

7. Chang, K.P. 1983. Cellular and molecular mechanisms of intracellular symbiosis in leishmaniasis. Int. Rev. Cytol. (Suppl. 14): 267-305.

8. Chang, K.P. 1981. Leishmania donovani-macrophage binding mediated by surface glycoprotein/antigens: Characterization in vitro by a radioisotopic assay. Mol. Biochem. Parasitol. 4:67-76.

9. Chang, K.P. 1981. Antibody-mediated inhibition of phagocytosis in Leishmania donovani-human phagocyte interactions in vitro. Am. J.Trop. Med. Hyg. 30: 334-339.

10. Chang, K.P. 1979. Leishmania donovani: Promastigote-macrophage surface interactions in vitro. Exper. Parasitol. 48: 175-189.

11. Chang, K.P., and D. Fong. 1983. Cell biology of host-parasite membrane interactions in leishmaniasis, p. 113-137 in Cytopathology of Parasitic Diseases (Ciba Foundation Symposium 99), Pitman Books, London.

12. Ezekowitz, R.A.B., M. bampton, and S. Gordon. 1983. Macrophage activation selectively enhances expression of Fc receptors for IgG2a. J. Exp. Med. 157:807-812.

13. Farah, F.S., S.A. Samra, and N. Nuwayri-salti. 1975. The role of the macrophage in cutaneous leishmaniasis. Immunol. 29: 755-764.

14. Fortier, A.H., D.L. Hoover, and C.A. Nacy. 1982. Intracellular replication of Leishmania tropica in mouse peritoneal macrophages: amastigote infection of resident cells and inflammatory exudate macrophages. Infect. Immun. 38: 1304-1308.

15. Handman, E., and J.W. Goding. 1985. The Leishmania receptor for macrophages is a lipid-containing glycoconjugate. EMBO J. 4:329-336.

16. Handman, E., C.L. Greenblat, and J.W. Goding. 1984. An amphipathic sulphated glycoconjugate of Leishmania: characterization with monoclonal antibodies. EMBO J. 3: 2301-2306.

17. Handman, E., and R.E. Hocking. 1982. Stage-specific, strain-specific and cross-reactive antigens of Leishmania species identified by monoclonal antibodies. Infect. Immun. 37:28-33.

18. Herman, R. 1980. Cytophilic and opsonic antibodies in visceral leishmaniasis in mice. Infect. Immun. 28:585-593.

19. Kennett, R.H. 1980. Enzyme-linked antibody assay with cells attached to polyvinyl chloride plates, p. 367-377. in R.H. Kennett, R.J. McKearn, and K.B. Bechtol (ed.), Monoclonal Antibodies. Hybridomas: A new dimension in biological analyses. Plenum Press, New York.

20. Kohler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256: 495-497.

21. Laemmli, U.K. 1979. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227-680-685.

22. Leslie, R.G.Q. 1984. Immunoglobulin and soluble immune complexes binding to phagocyte Fc receptors. Biochem. Soc. Trans. 12: 743-745.

23. Leslie, T.G.Q., and A.H. Niemetz. 1979. Species-specificity in the binding of IgG to macrophages. Immunol. 37:835-840.

24. Merino, F., E. Ajjam. A. Hernandez, K. Dawidowicz, and E.J. Merino. 1977. In vitro infection of murine macrophages by Leishmania braziliensis. Mechanisms of penetration. Int. Arch. Aller. Appl. Immunol. 55:487-495.

25. Mosser, D.M., and P. J. Edelson. 1984. Activation of the alternate complement pathway by Leishmania promastigotes: Parasite lysis and attachment to macrophages. J. Immunol. 132:1501-1505.

26. Nacy, C.L., and C.L. Diggs. 1981. Intracellular replication of Leishmania tropica in mouse peritoneal macrophages: comparison of amastigote replication in adherent and nonadherent macrophages. Infect. Immun. 34: 310-313.

27. Oi, V.T. and L.A Herzenberg. 1980. Immunoglobulin-producing hybrid cell lines. p. 351-372 in B.B. Mishell and S.M. Shiigi (ed.), Selective methods in cellular immunology, W.H. Freeman Co., San Francisco.

28. Parham, P. 1983. Monoclonal antibodies against HLA products and their use in immunoaffinity purification, p. 124. In J.J. Langone and H. Van Vunakin (ed.), Methods in enzymology, Vol. 92, Academic Press, New York.

29. Pearson, R.D. and R.T. Steigbigel. 1980. Mechanism of lethal effect of human serum upon Leishmania donovani. J. Immunol. 125:2195-2201.

30. Pearson, R.D., J.A. Sullivan, D. Roberts, R. Roseann, and G.L. Mandell. 1983. Interaction of Leishmania donovani promastigotes with human phagocytes. Infect. Immun. 40:411-416.

31. Segal, D.M., and E. Hurwitz. 1977. Binding affinity of cross-linked oligomers of IgG to cells bearing Fc receptors. J. Immunol. 118:1338-1347.

32. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc. Natl. Acad. Sci. USA. 76:4350-4354.

33. Wyler, D.J. 1982. In vitro parasite-monocyte interactions in human leishmaniasis. J. Clin. Invest. 70:82-88.

34. Zehavi, U., J. El-On, E. Pearlman, J.C. Abrahams, and C.L. Greenblatt. 1983. Binding of Leishmania promastigotes to macrophages. Z. Parasitenkd. 69:405-414.

35. Zenian, A. 1981. Leishmania tropica: biochemical aspects of promastigotes attachment to macrophages in vitro. Exper. Parasitol. 51:175-187.

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TABLE I

EFFECT OF MONOCLONAL ANTIBODIES ON PARASITE-MACROPHAGE BINDING

Antibody Treatment	% Positive Macrophages ± SEM	% Change	Ig Isotype
No antibody	46.2 ± 2.30	--	--
84C-4F4 (CP)[a]	78.9 ± 4.28[d]	↑70.7%	IgG2a
84C-4F4 (Opson)[b]	77.5 ± 4.31[d]	↑68.2%	IgG2a
84C-4F4 (Cytophil)[c]	69.1 ± 5.29[d]	↑49.6%	IgG2a
84C-8C7 (CP)	67.5 ± 6.97[d]	↑46.1%	IgG1
83L-5G9 (CP)	70.1 ± 4.34[d]	↑51.8%	IgG1
83L-2D3 (CP)	48.0 ± 3.76	↑3.8%	IgM
83T-9D3 (CP)	49.3 ± 4.97	↑6.7%	IgG2b
83U-7D5 (CP)	43.9 ± 3.26	↓4.9%	IgG3

[a] Cell mixtures incubated in the continuous presence of antibody.

[b] Promastigotes opsonized with antibody prior to incubation with macrophages.

[c] Macrophages incubated with antibody prior to addition of promastigotes.

[d] Considered significant change from control, $p < 0.01$.

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FIG. 1. Flow cytometric analyses of promastigote-macrophage binding kinetics. Percent positive macrophages = number of cells emitting a fluorescent signal/total number of cells passing through that particular channel.

KINETICS OF PARASITE BINDING TO MACROPHAGES

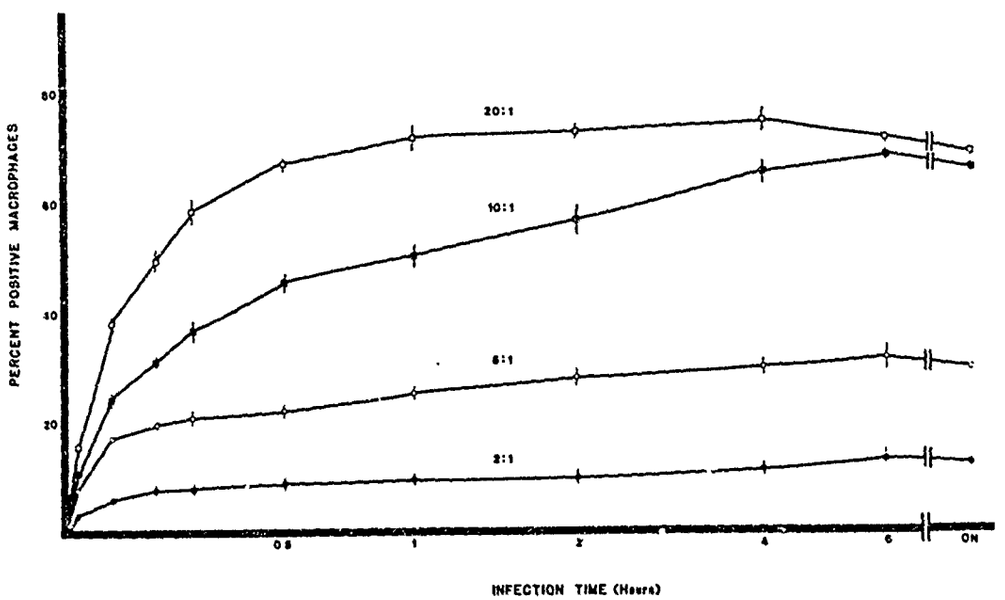

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FIG. 2. Immunofluorescent micrograph confirming differential susceptibility of murine peritoneal macrophages to infection with promastigotes of Leishmania mexicana mexicana. Arrows indicate uninfected cells.

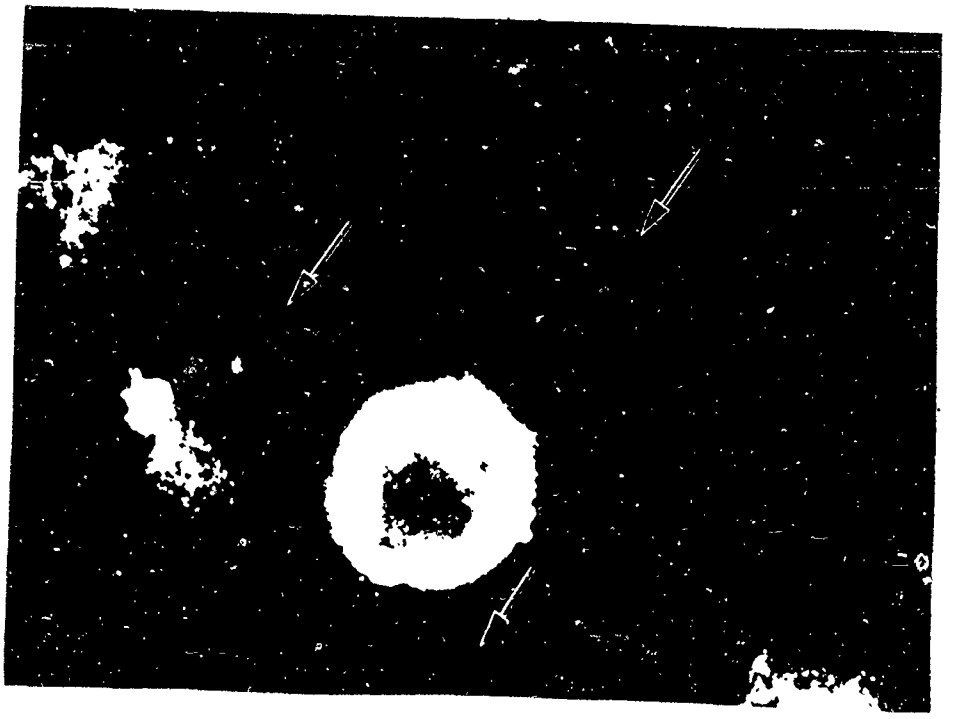

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FIG. 3. Western blot identification of the 42 kd surface membrane antigen of Leishmania mexicana mexicana promastigotes recognized by monoclonal antibodies which enhance parasite-macrophage binding. Lane A = 84C-4F4; Lane B = 84C-8C7; Lane C = 83L-5G9.

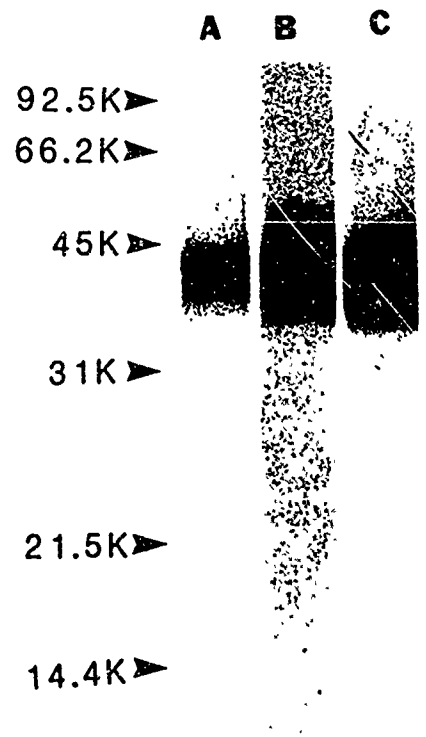
A
B
C
92.5K
66.2K
45K
31K
21.5K
14.4K

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FIG. 4. Effect of monoclonal antibody, 84C-4F4, on promastigote-macrophage binding kinetics.

EFFECT OF ANTIBODY ON BINDING KINETICS

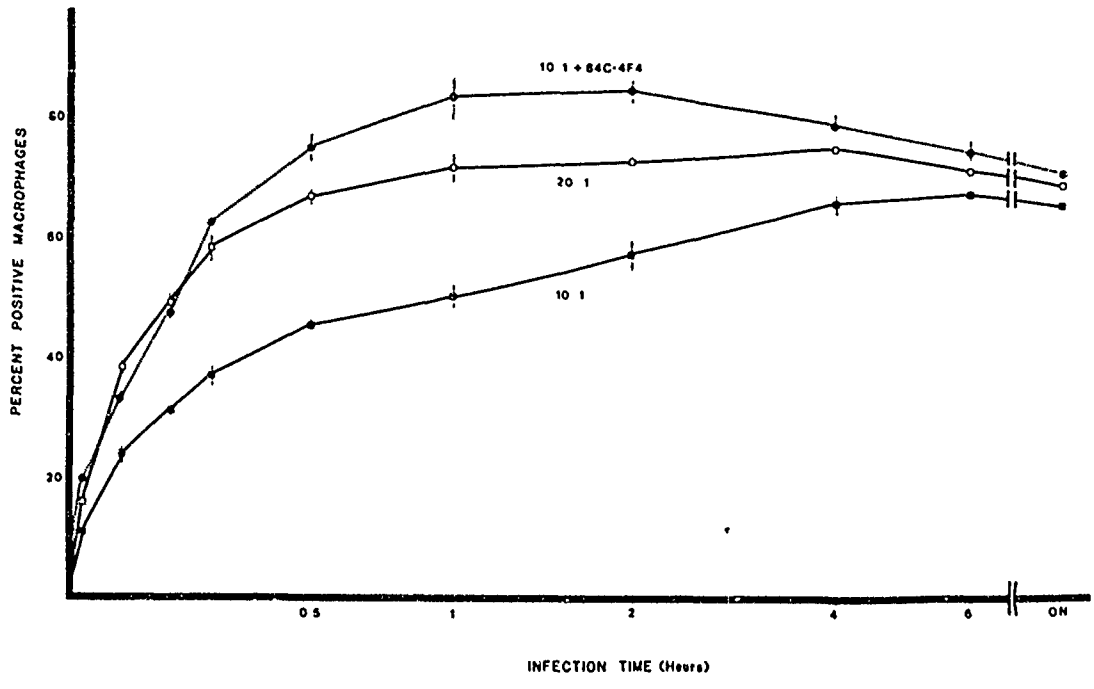

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FIG. 5. Dose response curve of the effect of monoclonal antibody 84C-4F4 on the parasite-macrophage binding kinetics.

DOSE RESPONSE OF BINDING ENHANCEMENT

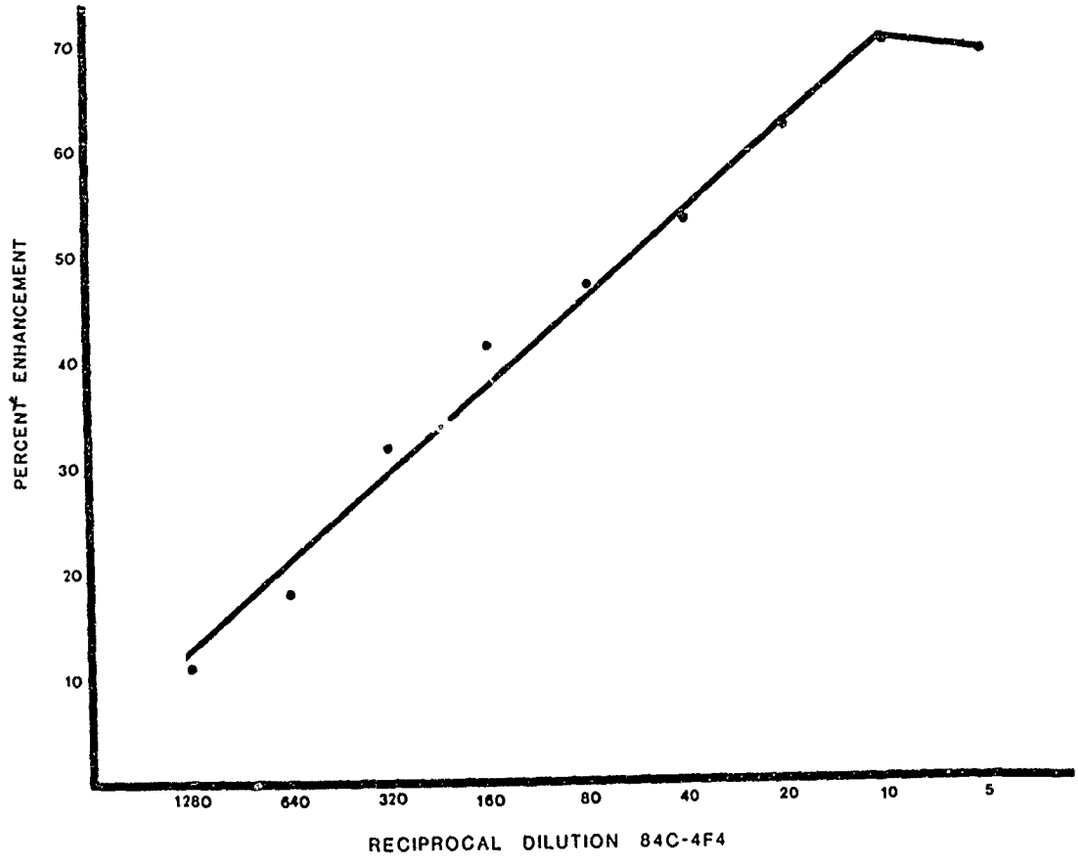

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FIG 6. Histograms comparing the surface fluorescence of macrophages which have bound the fluoresceinated promastigotes in the absence of antibody (upper scans on panels A to F) to surface fluorescence of macrophages which have bound fluoresceinated promastigotes in the presence of serial dilutions of 84C-4F4 (lower scans on panels A to F). Antibody dilutions: A=1:1280; B=1:320; C=1:80; D=1:40; E=1:20; F=1:10. The gradual shift to the right of the peak in the lower scans, with higher concentration of antibody, indicates increased fluorescence at the macrophage surface as a consequence of enhanced binding of the fluoresceinated parasites.

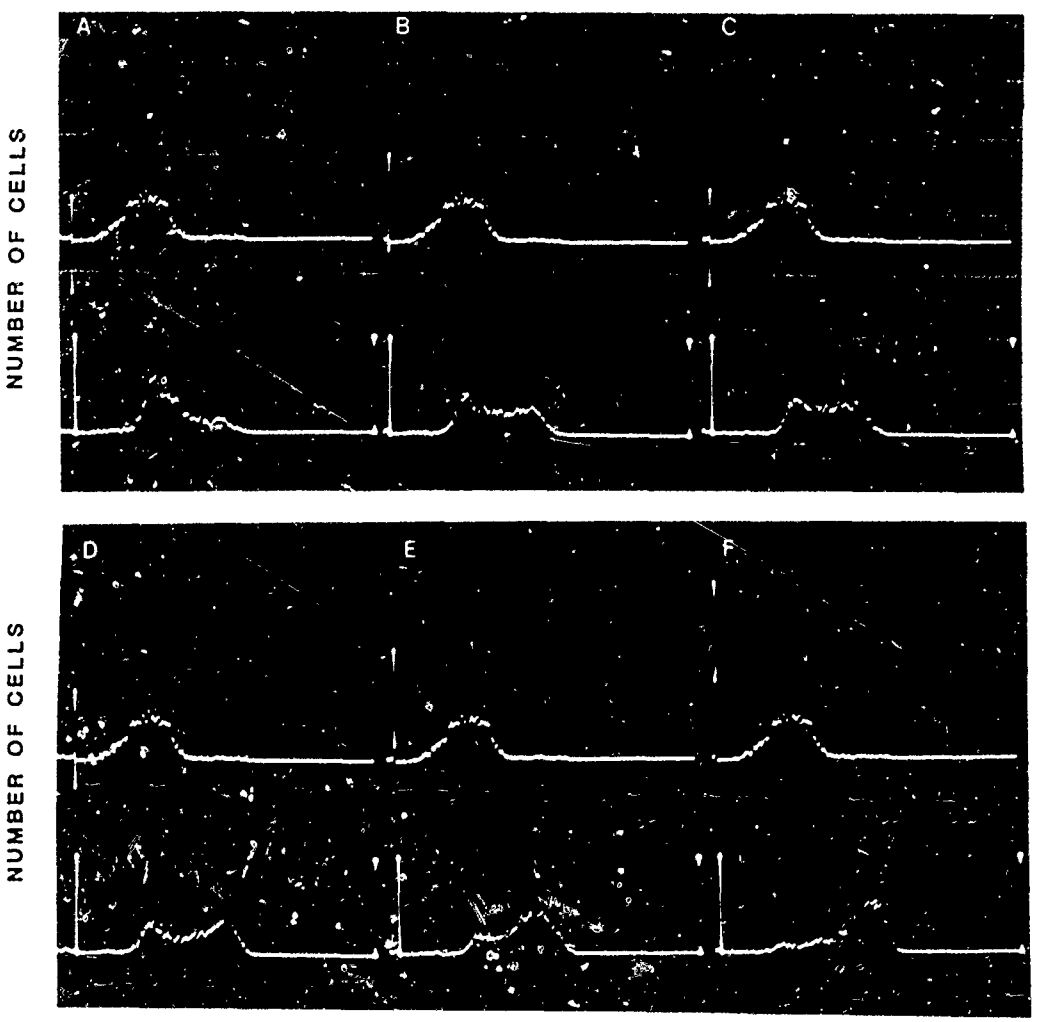
A
B
C
NUMBER OF CELLS
D
E
F
NUMBER OF CELLS
FLUORESCENCE
FLUORESCENCE
FLUORESCENCE

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FIG. 7. Western blot identification of the low molecular weight, surface membrane antigen of Leishmania mexicana mexicana promastigotes recognized by monoclonal antibodies 83T-9D3 (Lane A), 83U-7D5 (Lane B) and 83L-2D3 (Lane C).

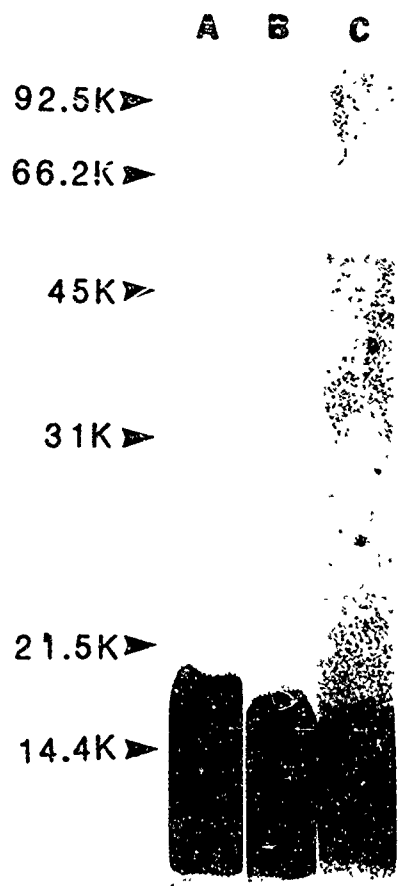
A
B
C
92.5K
66.2K
45K
31K
21.5K
14.4K

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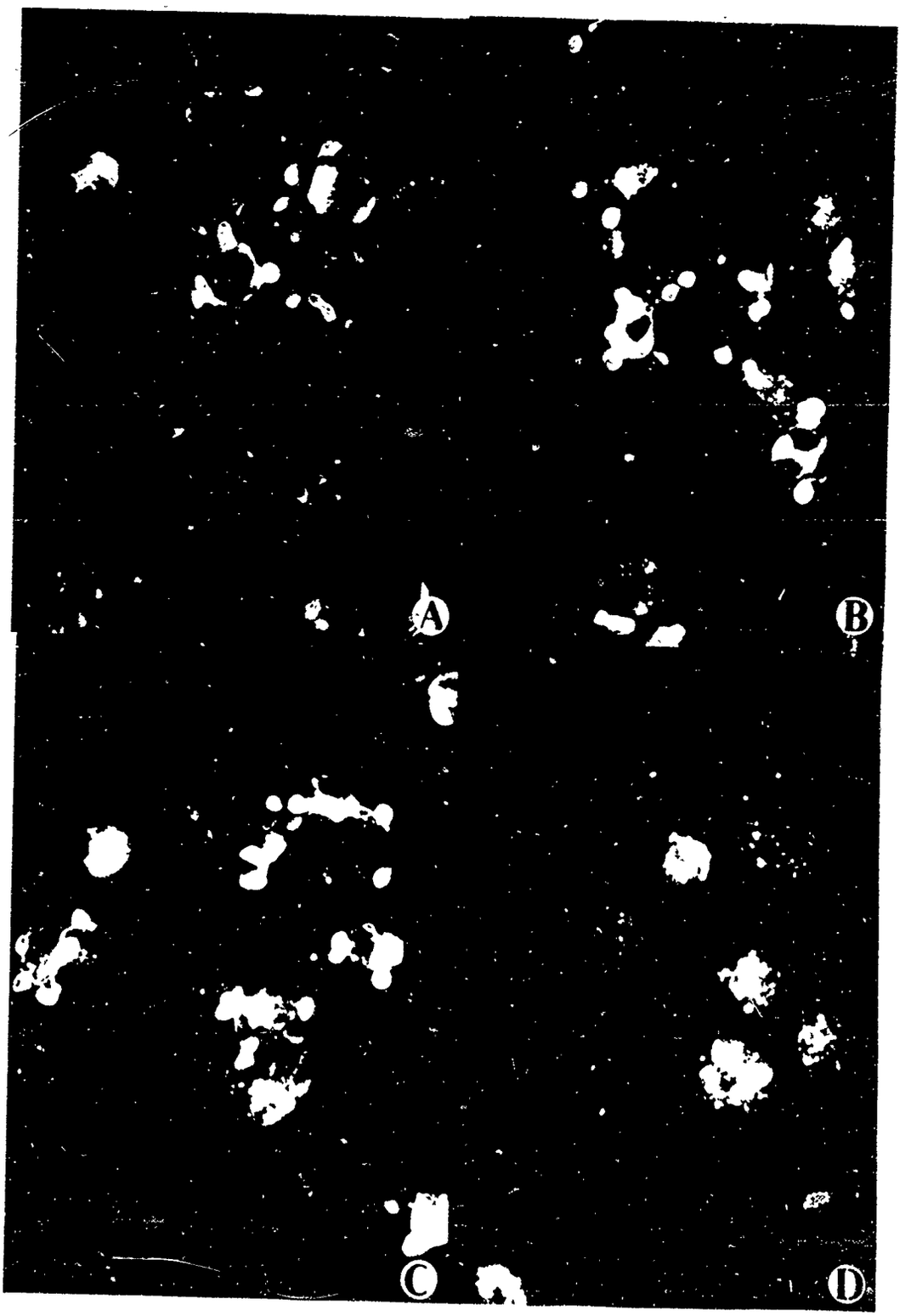
FIG. 8. Immunofluorescent micrograph demonstrating the capability of monoclonal antibody 83L-2D3 to immobilize parasites at the macrophage surface after incubation at 6 hours (Panel A), 24 hours (Panel B) and 48 hours (Panel C). Panel D represents the control where promastigotes were incubated and internalized in the presence of 84C-4F4.


A
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Leishmania Antigen on Surface of Mouse Macrophages

Identification and Recovery of Leishmania Antigen Displayed on the Surface Membrane of Mouse Peritoneal Macrophages Infected *in vitro*.[1]

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Footnotes:

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Abstract:

A murine monoclonal antibody (83L-2D3/a) to a dominant surface antigen of Leishmania braziliensis panamensis (WRAIR-470) recognized a determinant expressed on the surface membrane of mouse peritoneal macrophages infected in vitro. This determinant, also demonstrable on the surface membrane of intracellular amastigotes, was not displayed by the macrophage until at least 6 hours post infection. This delay in expression and the obvious negativity of the occasional uninfected macrophage inherent to infected cultures implied that the leishmania determinant had an intracellular origin. Furthermore, expression was dependent upon maintenance of macrophage function. When the parasite burden became overwhelming additional antigen processing ceased and that which had accumulated was either shed into the medium or internalized. Immunochemical analyses revealed that the 83L-2D3/a reactive epitope was part of a 15 kd molecule which, in all likelihood, represents a breakdown product of a major surface glycoconjugate which had been degraded in the phagolysosome.

Introduction:

Protozoan parasites of the genus Leishmania survive and replicate within the phagosome-lysosome vacuolar system of macrophages (1). Although precise mechanisms by which these intracellular parasites, amastigotes, escape the arsenal of microbicidal factors of the phagolysosome have not been delineated, it appears that leishmanial cell surface antigens (2) and excretory factors (3) play a significant role. In fact, blockage of these cell surface determinants with specific monoclonal antibodies to promastigotes resulted in inhibition of intracellular differentiation (4) and in partial abrogation of the amastigote's resistance to enzymatic degradation (5). Furthermore, it is well established that the surface membrane of resistant amastigotes is comprised of several stage specific antigens which must be synthesized during residency within the phagolysosome (6). At least one of these amastigote-specific antigens seems to be in close association with the inner lining of the parasitophorous vacuole and it is subsequently expressed at the surface of infected macrophages (7). We now report on the identification and characterization of a dominant promastigote surface antigen of Leishmania braziliensis panamensis which is conserved throughout intracellular differentiation into amastigotes, is evidently resistant to lysosomal degradation and, within six hours post infection of in vitro cultures, is displayed on the surface membrane of mouse peritoneal macrophages. This antigen, recognized by monoclonal antibody 83L-2D3/a, is a glycoconjugate which is common to at least three species and eight sub-species on New World Leishmania but which is not present on the surface membrane of Endotrypana and Trypanosoma.

Materials and Methods:

Murine peritoneal exudate cells.

Two milliliters of sterile 4% thioglycollate broth were injected into the peritoneal cavity of female BALB/c mice. Five days after injection, the mice were sacrificed by cervical dislocation and 5 ml sterile Hanks' balanced salt solution containing penicillin (100 units/ml), streptomycin (50 ug/ml) and heparin (50 units/ml) were introduced into the peritoneal cavity. After gentle massage, the peritoneal exudate was aspirated into a sterile syringe fitted with an 18 gauge needle and the cells were pelleted by centrifugation at 200g for 5 min. The cells were washed and resuspended at a concentration of 10^6 cells/ml in Eagles' MEM with Hanks' salt base, sodium bicarbonate, 2mM glutamine, 1 ug/ml insulin, 10 mM Hepes, 10% fetal calf serum and antibotics. Macrophages were dropped onto glass slides for immunofluorescent microscopy, plated into permanox tissue culture dishes for immunoelectronmicroscopy, or suspended in polypropylene tubes according to methods outlined by Nacy (8).

Parasites.

Promastigotes of Leishmania braziliensis panamensis (WRAIR isolate 470) were maintained at 26C in Medium 199 with Hanks' salts, containing 12mM Hepes, 20mM L-glutamine, 20% heat inactivated new born calf serum, 50 units penicillin/ml, 50 ug streptomycin/ml and 50ug gentamycin sulfate/ml. Organisms, harvested in the stationary growth phase, were used to infect cultures of adherent macrophages, on glass microscope slides or tissue culture dishes, at a ratio of 10 parasites/macrophage. Infection was permitted to proceed for 2 hr at 35C and then all extracellular organisms which failed to parasitize the macrophage were eliminated by washing. Replication of the

intracellular amastigotes was allowed to continue for 4 days at 35C. Every six hours post infection, selected slides were washed in media, rinsed in cold acetone and set aside for use as substrates in indirect immunofluorescent antibody assays (IFA).

Monoclonal antibodies.

Eight-week old female BALB/c mice were inoculated, subcutaneously, into the axillary area with 0.1 ml (150 µg/ml) of a suspension of stationary phase promastigotes of isolate WRAIR 470 which had been rendered non-infectious by repeated freezing (-70C) and thawing (+40C) and by sonic disruption at 6 pulses of 50 watts for 30 sec at 2C. The inoculation was repeated on every fifth day until a serum sample, taken via the tail vein, produced a 4+ immunofluorescence by IFA (titer > 1:800) and an absorbance value of > 0.500 at 405 nm by enzyme linked immunosorbent assays (ELISA). One final inoculation was given 3 days prior to splenectomy. The splenocytes were then recovered and fused with P3X63-Ag8 plasmacytoma cells in the presence of 30% polyethylene glycol for 8 min at room temperature. Precise protocols for performing cell fusions and for selection of hybridomas in HAT medium have been provided by Kennett (9). Hybridomas synthesizing antibodies reactive with L. b. panamensis promastigotes were selected by IFA and ELISA and subsequently cloned in a semi-solid agarose medium. All monoclonal antibodies were isotyped by immunoprecipitation with goat antisera specific for each class and subclass of mouse immunoglobulin (Litton Bionetics, Charleston, SC).

Indirect Immunofluorescent Antibody Assays (IFA).

Promastigotes, washed in phosphate buffered saline (PBS pH 7.2) and standardized to a concentration of 1 X 10^4 organisms/ml, were air-dried, in 25 µl volumes, on eight-well microscope slides. Each well was then covered with 25 µl of medium from the 83L-2D3/a hybridoma for 30 min at room temperature. The unreactive protein was removed by three washes in phosphate buffered saline (PBS, pH 7.2) and 25 µl goat anti-mouse immunoglobulin serum labeled with fluorescein isothiocyanate and diluted 1:50 in 0.25% Evans Blue was added. Incubation was continued for an additional 30 min at room temperature. Unreactive conjugate was then removed by another cycle of washes in PBS and a coverslip was mounted with buffered glycerol. Sites of reactivity were visualized using a 50X water immersion lens mounted on a Leitz Ortholux immunofluorescent microscope. Medium from unfused P3X63-Ag8 cells served as the negative control.

Enzyme linked immunosorbent assay (ELISA).

The 96 flat bottom wells of polyvinyl microtitration plates were coated with 25 µg poly-l-lysine for 30 min at room temperature. The wells were then emptied and 10^4 promastigotes, in 100 ul PBS, were placed into each well. After centrifugation at 2500 rpm for 5 min at room temperature, the plates were inverted to remove the liquid phase and permitted to air-dry overnight. One hundred microliters of the 83L-2D3/a monoclonal antibody was put into each well. Medium from unfused P3X63-Ag8 plasmacytoma cells served as the negative control. After 2 hr at room temperature, the unreactive protein was removed by three washes in PBS containing 0.025% Tween 20 and then 100 ul of goat anti-mouse immunoglobuln serum labeled with alkaline phosphatase and diluted

1:500 in PBS-Tween 20 was added. Incubation was continued for 2 hr at room temperature. Unreactive conjugate was eliminated by a second cycle of washes in PBS-Tween 20 and 100 ul of the substrate, p-nitrophenyl phosphate in diethanolamine buffer, pH 9.8, was added. Hydrolysis was stopped after 15 min at room temperature by the introduction of 3M NaOH and the resultant color change, indicative of bound 83L-2D3/a monoclonal antibody, was measured spectrophotometrically at 405 nm. Absorbance values of <0.100 were interpreted as negative reactions.

Ascitic fluid.

BALB/c mice were primed with 0.5 ml Pristane, intraperitoneally, on days -10 and -3. On day 0, the mice received an intraperitoneal injection of 10^4 cells from the 83L-2D3/a hybridoma clone. After 10 days, ascitic fluid was collected and clarified by centrifugation at 100,000g for 30 min at 4C. Since the monoclonal 83L-2D3/a was an IgM antibody, it could be recovered from the ascitic fluid by dialysis against 1000ml 5mM Tris-HCl buffer, pH 7.5 (10). The resultant precipitate, regarded as purified IgM, was washed 2 times in cold dialysis buffer, redissolved in 1mM Tris-HCl (pH 8.6), dialysed against PBS and lyophilized. Protein content was determined by the Bio-Rad assay.

Immunochemical characterization of the 83L-2D3/a reactive antigen.

The reactivity of monoclonal antibody 83L-2D3/a with unfractionated and purified antigens of the WR-470 promastigote was assessed by the Western Blot. Antigens (100 µg), separated by vertical electrophoresis in a sodium dodecyl sulfate-polyacrylamide gel gradient, were electrophoretically transferred onto a nitrocellulose sheet. Subsequent to blocking with 3% bovine serum albumin for 30 min at room temperature, the sheet was covered with a 1:50 dilution of

83L-2D3/a monoclonal antibody in 1% normal goat serum (NGS), for twelve hours at 4C. Unreactive protein was eliminated by thorough washing in PBS containing 0.025% Tween 20. Sites of reactivity were developed by the addition of 1:2500 dilution of goat anti-mouse immunoglobulin serum labeled with I^{125} and exposure, for 3 days at -70C, to Kodak X-Omat-AR film on a calcium tungstate intensifying screen.

Immunoelectronmicroscopy.

5 X 10^7 stationary phase promastigotes, or 10^6 adherent macrophages containing amastigotes, were washed in PBS and fixed for 15 min at 4C in 20 ml 4% formaldehyde/1% gluteraldehyde in Millonig phosphate buffer. The parasites and/or infected cells were then examined for their reactivity with the 83L-2D3/a monoclonal antibody by applying a modification of the unlabeled peroxidase-antiperoxidase (PAP) technique described by Sternberger (11). After a 30 min incubation with 10% NGS, the fixed cells were submerged in a 1:1000 dilution of the 83L-2D3/a IgM recovered from ascitic fluid. Incubation proceeded for 2 hr at room temperature and then overnight at 4C. Unreactive protein was removed by thorough washing in PBS and a 1:50 dilution of goat anti-mouse serum in 1% normal goat serum was added. After an additional 30 min at room temperature, unreactive goat serum was eliminated by another cycle of washes in PBS and a 1:100 dilution of mouse anti-peroxidase serum + peroxidase (PAP) in 1 % NGS was added. At the completion of 30 min at room temperature, unbound PAP was removed by another wash, and areas of peroxidase activity were developed by a 10 minute exposure to 0.05% diaminobenzidine tetrahydrochloride in Tris-HCl buffer, pH 7.4, containing 0.01% hydrogen peroxide. The parasites and/or infected cells were given a final wash in Tris-HCl, post-fixed with 2%

osmium tetroxide, dehydrated, and embedded in Epon 812 for thin sectioning. Negative controls included both uninfected macrophages incubated with monoclonal 83L-2D3/a and infected macrophages incubated with a monoclonal antibody to a surface antigen of Trypanosoma cruzi.

Kinetics of antigen expression on the macrophage membrane as quantitated by relative fluorescence in flow cytometric analyses.

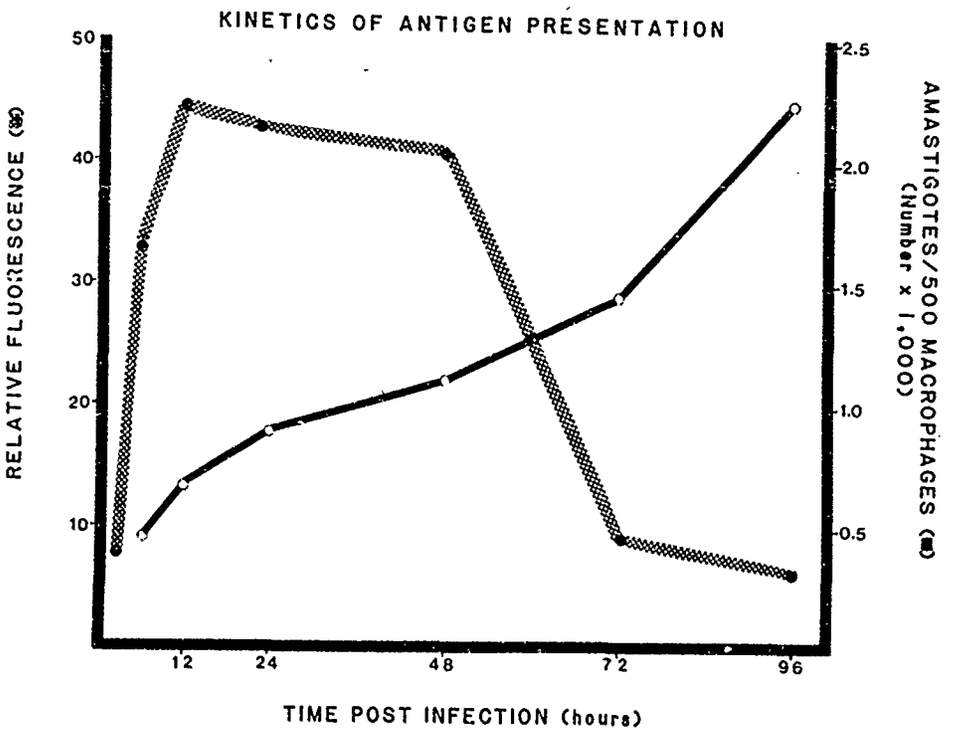
Triplicate suspension cultures of macrophages were infected for 2hr at 34C with WR-470 promastigotes at a ratio of 10 parasites/macrophage. Unattached parasites were then removed by slow speed centrifugation at 50g. The pellet of infected cells was resuspended in 0.5 ml culture medium (described above) and incubated for an additional 94 hrs at 34C. At 6 hr intervals, however, 100 ul aliquots, from triplicate cultures, were put aside for light microscopic enumeration (Geimsa stain) of intracellular amastigotes. The remaining 400 ul was incubated with 10% NGS for 30 min, washed thrice in cell culture medium, and resuspended in a 1:150 dilution of the 83L-2D3/a purified IgM in 1% NGS. After contiuned incubation at room temperature for 2 hr, the unreactive protein was removed by a cycle of washes in PBS and 100ul of goat anti-mouse immunoglobulin serum, labeled with fluorescein isothiocyanate and diluted 1:50 in 1% NGS, was added. The unreactive conjugate was eliminated by a final wash in PBS and each macrophage was then analysed for its light scatter and fluorescence signals in a Becton-Dickinson FACS IV flow cytometer. The cytometer had been gated so that the only cells assayed by fluorescence were those within the size range of macrophages, as determined by light scatter.

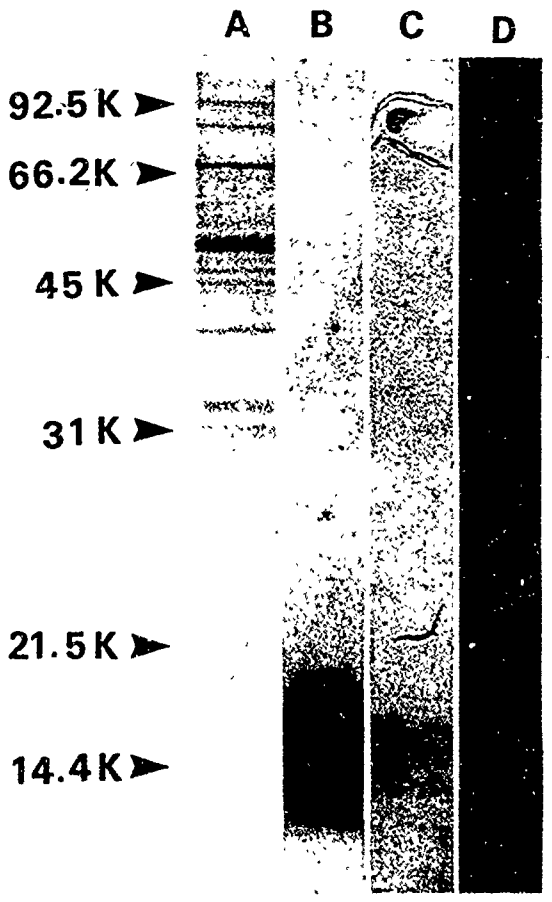
Relative fluorescence, calculated by the cytometer, was defined as the sum of the total number of cells per channel multiplied by the channel number (T-2) divided by the total number of cells analysed (T-1).

Recovery and characterization of the 83L-2D3/a reactive antigen.

10^8 stationary phase promastigotes of isolate WR-470 were pelleted by centrifugation at 5000 g and extracted in 10mM CHAPS (Sigma Chemical Co., St. Louis, MO) containing 0.05M glycine, 2mM phenyl methyl sufonyl fluoride and 5 mM EDTA (pH 7.2). The extraction was performed for 2 hr at 4C with continuous rocking. After insoluble material was removed by centrifugation at 100,000g for 30 min at 4C, the resultant supernatant was regarded as crude antigen extract.

Protocols for coupling purified monoclonal antibodies, reconstituted in 0.1M NaHCO (pH 7.5), to Affi-Gel 10 (Bio-Rad, Rockville Center, NY) have been outlined elsewhere (12). After coupling for 4 hr at 4C, remaining active esters were blocked by the addition of 100 ul of 0.1M glycine ethyl ester and the gel was transferred to a 0.7 x 15 cm column. The gel was washed with 0.05M glycine, pH 7.2, until the transmittance, at 280 nm, of the effluent reached 100%. Washing was continued with 0.05M glycine-HCl buffer, pH 2.5, and then repeated with 0.05M glycine, pH 7.2. The solubilized crude antigen (500 µg/ml) was applied to the column in a volume equal to the columns void volume (2-3 ml). The outlet was closed and the antigen was permitted to react with the immunoabsorbent, bound 83L-2D3/a monoclonal IgM, for 2 hr at 4C. The outlet was then opened and the unreactive protein was eliminated by elution with 10mM CHAPS in 0.05M glycine buffer, pH 7.2. Washing continued until a 100% transmittance was acheived. The bound antigen was eluted with 0.05M


KINETICS OF ANTIGEN PRESENTATION
RELATIVE FLUORESCENCE (%)
AMASTIGOTES/500 MACROPHAGES (■)
(Number x 1,000)
50
40
30
20
10
2.5
2.0
1.5
1.0
0.5
12
24
48
72
96
TIME POST INFECTION (hours)


A
B
C
D
92.5 K
66.2K
45 K
31 K
21.5 K
14.4K

References.

1. Alexander, J., and K. Vickerman. 1975. Fusion of host cell secondary lysosomes with the parasitophorous vacuoles of Leishmania mexicana infected macrophages. J. Protozool. 22: 502.

2. Chang, K.P., 1983. Cellular and molecular mechanisms of intracellular symbiosis in leishmaniasis. Int. Rev. Cytol. (Suppl. 14): 267.

3. El-On, J., D.J. Bradley, and J.C. Freeman. 1980. Leishmania donovani: action of excreted factor on hydrolytic enzyme activity of macrophages from mice with genetically different resistance to infection. Exp. Parasitol. 49: 167.

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

5. Chang, K.P., and D. Fong. 1983. Cell biology of host-parasite membrane interactions in leishmaniasis. in Cytopathology of Parasitic Disease, Ciba Foundation Symposium 99. Pitman Press, Bath, Great Britain. pp. 113.

6. Chang, K.P., and D. Fong. 1982. Antigenic changes during intracellular differentiation of Leishmania mexicana in cultured macrophages. Infect. Immun. 36: 430.

7. Berman, J.D., and D.M. Dwyer. 1981. Expression of Leishmania antigen on the surface membrane of infected human macrophages in vitro. Clin. Exp. Immunol. 44: 342.

8. Nacy, C.A., and C.L. Diggs. 1981. Intracellular replication of Leishmania tropica in mouse peritoneal macrophages: comparison of amastigote replication in adherent and nonadherent macrophages. Infect. Immun.34:310.

9. Kennett. R.H. 1979. Cell Fusion. in Methods in Enzymology, Vol. 68, T.W. Jakoby and H. Pastan eds. Academic Press, New York. p. 345.

10. Parham, P. 1983. Monoclonal antibodies against HLA products and their use in immunoaffinity purification. in Methods in Enzymology Vol. 92, J.J. Langone and H. Van Vunakin eds. Academic Press, New York, p. 124.

11. Sterneberger, L.A. 1979. Immunocytochemistry (2nd. ed). John Wiley and Sons Inc. New York, New York. p 130-145.

12. Caldwell, H.D., and C.C. Kuo. 1977. Purification of a Chlamydia trachomatis specific antigen by immunoabsorption with monospecific antibody. J.Immunol. 118: 437.

13. Handman, E., R. Ceredig, and G.F. Mitchell. 1979. Murine cutaneous leishmaniasis: Disease patterns in intact and nude mice of various genotypes and examination of some differences between normal and infected macrophages. Aust. J. Exp. Biol. Med. 57: 9.

14. Anthony, R.L., K.M. Williams, J.B. Sacci, and D.C. Rubin. 1985. Subcellular and taxonomic specificity of monoclonal antibodies to New World Leishmania. Am J. Trop. Med. Hyg.(in press).

15. Silverstein, S. 1982. Membrane receptors and the regulation of the mononuclear phagocyte effector function. Adv. Exp. Med. Biol. 155: 21-31.

16. Dzandu, J.K., M.E. Deh, D.L. Barratt, and G.E. Wise. 1982. Detection of erythrocyte membrane proteins, sialoglycoproteins and lipids in the same polyacrylamide gel using a double-staining technique. Proc. Nat. Acad. Sci. 81: 1733.

17. Turco, S. J., M.A. Wilkerson, and D. R. Clawson. 1984. Expression of an unusal acidic glycoprotein in Leishmania donovani. J. Biol. Chem. 259:3383.

18. Benchimol, M. and W. de Souza. 1981. Leishmania mexicana amazonensis: attachment to the membrane of the phagocytic vacuole of macrophages in vivo. Z. Parasiten kd. 66:25.

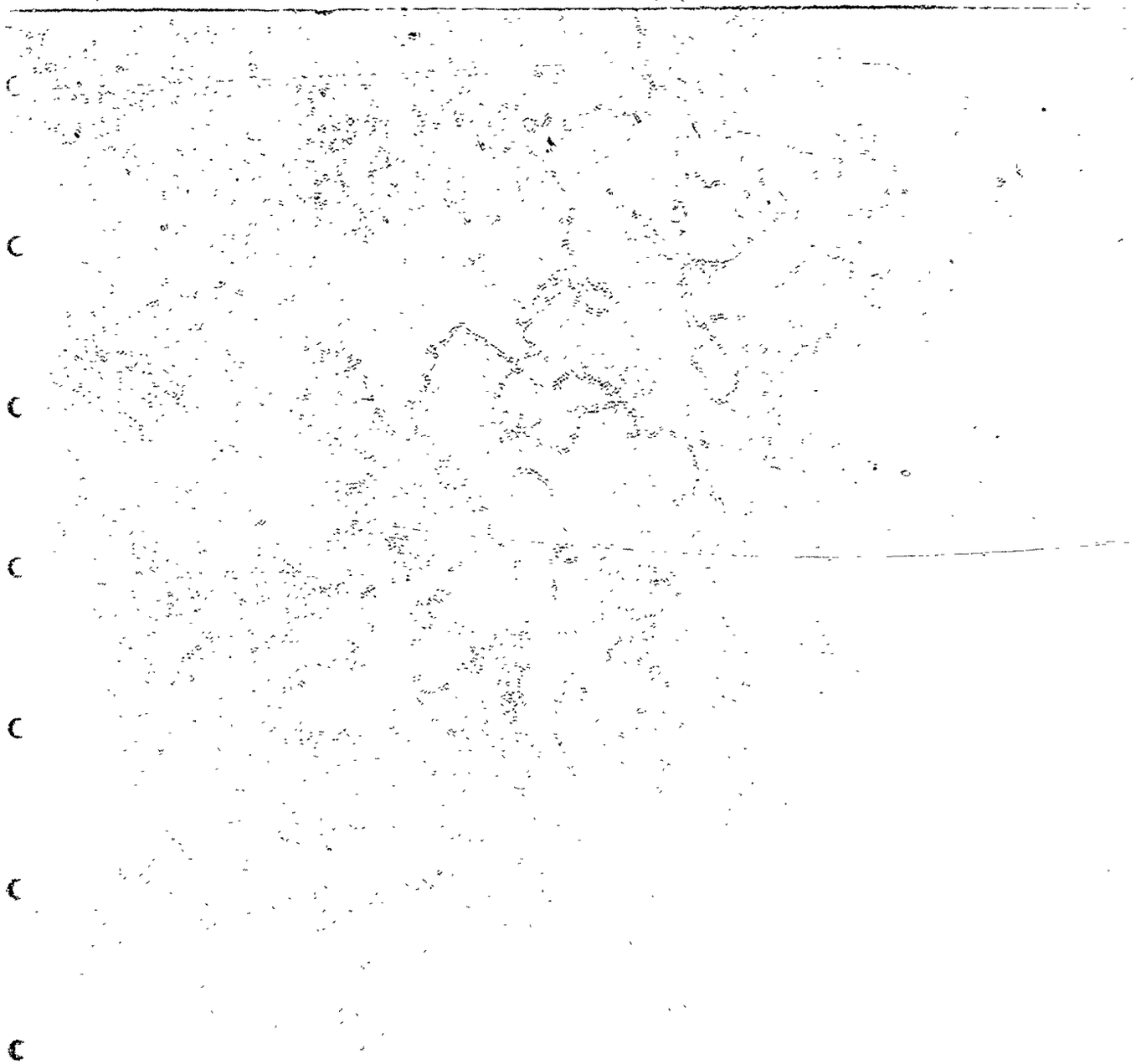
19. Dwyer, D.M. 1978. Leishmania- host cell membrane cycling in vitro. J. Protozool. 25: 28A.

20. Dwyer, D.M. 1979. Membrane interactions between Leishmania and host cells. in Microbiology -1979, D. Schlessinger, ed., Am. Soc. Microbiol., Washington, D.C. p.130.

21. Nacy, C.A., M.S. Meltzer, L.J. Leonard, and D.J. Wyler. 1981. Intracellular replication and lymphokine-induced destruction of Leishmania tropica in C3H/HeN mouse macrophages. J. Immunol. 127: 2381.

22. Nacy, C.A., A.H. Forteir, M.G. Pappas, and R.R. Henry. 1983. Susceptibility of inbred mice to infection with Leishmania tropica: correlation of susceptibility with in vitro macrophage defects for intracellular destruction of Leishmania tropica amastigotes. Cell Immunol. 123:2544.

23. Oster, C.N., and C.A.Nacy. 1984. Macrophage activation to kill Leishmania tropica: kinetics of macrophage response to lymphokines that induce antimicrobial activities against amastigotes. J. Immunol. 132:1494.



Antigen common to T. cruziand vertebrate cell matrix

Identification, Recovery and Immunogenecity of an Antigen Common toTrypanosoma cruziand the Vertebrate Cell Matrix.

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Abstract: A murine monoclonal antibody, 84B-1E3, recognizes a dominant epitope on the flagellum and surface membrane of both cultured epimastigotes and infectious, metacyclic trypomastigotes of Trypanosoma cruzi. Competitive binding assays (ELISA), whereby Chagasic sera were examined for their capacity to inhibit the binding of 84B-1E3 to epimastigotes, established that this epitope elicits a humoral antibody response in naturally occurring human infections. Immunochemical characterizations (Western Blots) of a flagella-membrane enriched fraction revealed that the parasite moiety expressing the immunogenic determinant has a molecular size and electrophoretic mobility congruent with mammalian brain tubulin. Additional analyses confirmed that this determinant has been highly conserved throughout phylogeny and, in fact, is displayed on a large array of different polypeptides which comprise the vertebrate cell matrix. These findings suggest that this conserved epitope, exposed on the host cell cytoskeleton as a consequence of parasitization, contributes to the complex antigenic stimulus which induces the sustained autoantibody response prevalent in chronic Chagas' disease.

The advent of hybridoma technology has provided investigators with an arsenal of monoclonal antibodies which can be used for the antigenic dissection of the parasitic Protozoa. In addition to having the capacity to discriminate between the antigen profiles of closely related taxons (4,5,9,27,28), these antibodies can identify unique surface membrane epitopes for each developmental stage of the organism (6,18,21). Most importantly, selected monoclonals have been used for the passive immunization of rodents against malaria (25,40), leishmaniasis (1) and toxoplasmosis (20) and they have served as ligands, in immunoaffinity chromatography, for the recovery of the protective immunogen (35).

Our prior examinations of the taxonomic and subcellular specificity of monoclonals to the parasitic trypanosomatids (5) confirmed that the surface and flagellar membranes of these protozoans also display several epitopes which have been highly conserved throughout phylogeny. Since these cross-reactive antigenic determinants are common features on polypeptides of the host cell matrix (17), neurones (36,39), and muscle cells (34), we suspected that their presence on infectious forms of the parasites might result in an antibody response which is deleterious to the host. We now report on the identification and immunogenecity of a dominant epitope of infectious metacyclic trypomastigotes of Trypanosoma cruzi recovered from the hind-guts of wild-caught Rhodnius pallescens. This epitope is common to a variety of polypeptides which comprise the vertebrate cytoskeleton and it elicits an antibody response in naturally occurring human infections. We contend that exposure to this determinant, initially as a trypanosomal antigen

and then as an antigen of host cells exposed as a consequence of parasitization, contributes to the sustained autoimmune pathology characterisitc of chronic disease.

Materials and Methods.

Human sera. Sera were obtained from 48 Bolivians with acute Chagas' disease, which had been confirmed by xenodiagnosis, antibody titers (3), and clinical evaluations. Sera from 48 healthy Baltimorians, without any history of exposure to parasitic trypanosomatids, served as negative controls.

Parasites. Stationary phase epimastigotes of the Tulahuen strain of Trypanosoma cruzi were maintained, at 26C, in Medium 199 containing 10 mM Hepes, 20% newborn calf serum and antibiotics. Metacyclic trypomastigotes were recovered from the hind-gut contents of Rhodnius pallescens collected at various sites throughout the Republic of Panama.

Monoclonal antibodies. Balb/c mice, 8 week-old females, were inoculated bi-weekly for 3 weeks with epimastigotes which had been washed in phosphate buffered saline (PBS, pH 7.2),rendered non-infectious by repeated freezing (-70C) and thawing (+4C), dispersed by ultrasonication at 6 pulses of 50 watts for 30 sec at 2C, and standardized to contain 250 ug protein/ml. Inoculations of 0.1 ml were administered, subcutaneously, into the axilla. Three days after the final inoculation, splenocytes were recovered and fused with P3X63-Ag8 plasmacytoma cells. Detailed methods for cell-fusion in the presence of 30% polyethylene glycol and the subsequent selection of hybridomas by exposure to 8 um aminopterin have been described elsewhere (22). At 15 days post-fusion, medium supporting healthy hybridomas was screened for antibody to epimastigotes by IFA. Positive hybridomas were cloned in a semi-solid agarose medium over feeder layers of Balb/3T3-A2 cells (9).

Indirect immunofluorescent microscopy (IFA). Stationary phase epimastigotes (10,000/ml) were washed thrice in PBS and then air dried, in 25 ul volumes, on 4-well microscope slides. Alternatively, the hind-gut content of a wild-caught vector, confirmed for the presence of trypanosomes by light microscopy, was sub-divided and air-dried on the 4 wells. Each well was flooded with 25 ul of the monoclonal antibody . After 30 min at room temperature and 3 washes in PBS, 25 ul goat anti-mouse immunoglobulin serum, labeled with fluorescein isothiocyanate and diluted 1:100 in 0.5% Evans Blue, was added. Incubation was continued for an additional 30 min at room temperature, unbound conjugate was eliminated by a second cycle of washes in PBS and a cover slip was added. Reactivity was visualized with a 50X water immersion objective mounted on a Leitz Ortholux immunofluorescent microscope. Medium from unfused plasmacytoma cells served as the negative control.

Recovery and identification of the reactive antigen. A flagella-rich fraction was recovered from a suspension of stationary phase epimastigotes by applying methods outlined by Pereira et.al. (31). Briefly, epimastigotes were de-flagellated in 1% Lubrol PX (Sigma Chemical Co., St. Louis, Missouri). After recovery of the flagellum-rich fraction by centrifugation on a sucrose density gradient, it was washed in carbonate-bicarbonate buffer, pH 9.5, and stored at -20C.

The polypeptide of the flagella-rich fraction recognized by the monoclonal antibody was identified by a Western Blot. One hundred micrograms of the fraction, separated by vertical electrophoresis in a sodium dodecyl sulfate polyacrylamide gel gradient (SDS-PAGE), were electrophoretically transferred onto a sheet of nitrocellulose. Subsequent to blocking with 3%

bovine serum albumin (BSA) for 30 min at room temperature, the sheet was immersed in a 1:50 dilution of the monoclonal antibody in 1% BSA for 12 hr at 4 C. Unreactive protein was eliminated by thorough washing in PBS containing 0.025% Tween 20 (PBS-T 20). Sites of reactivity were developed by the addition of a 1:2500 dilution of goat anti-mouse immunoglobulin serum labeled with I^{125}. After extensive washing, the sheet was exposed, for 3 days at -70°C, to Kodak X -Omat-AR film on a calcium tungstate intensifying screen.

Enzyme linked immunosorbent assays (ELISA). Antigens for the ELISA, (e.g. the flagella enriched fraction and the electrophoretically pure proteins derived from the vertebrate cytoskeleton) were coated onto the flat surface of 96 wells of polyvinyl microtitration plates. Each well received 10 ug protein diluted in carbonate-bicarbonate buffer, pH 9.8. After an overnight incubation at 4C, unbound protein was eliminated by three washes in PBS-T 20 and 100 ul of the murine monoclonal antibody, or a 1:10 dilution of human serum, was added. Incubation was continued for 2 hr at room temperature. Unbound components were then removed by another cycle of washes and 100 ul of goat anti-mouse immunoglobulin serum labeled with alkaline phosphatase and diluted 1:500 in PBS-T 20 was added. At the completion of an additional 2 hr incubation at room temperature, unreactive conjugate was removed by a final series of washes and 100 ul of para-nitrophenyl-phosphate in diethanolamine buffer (5 mg/ml) was added. Hydrolysis of the substrate was stopped after 15 min at room temperature and the resultant color change was quantitated spectrophotometrically at 405 nm.

Competitive assay. Wells of the polyvinyl titration plates were coated with 25 ug poly-l-lysine (Sigma Chemical Co., St. Louis, MO) for 30 min at room temperature. The wells were then dried by inverting, and 10,000 epimastigotes, in 100 ul PBS, were added to each. The plates were centrifuged at 2500 rpm for 5 min at room temperature and allowed to air dry. Each well then received 100 ul of a 1:10 dilution of the human serum. After an overnight incubation at 4 C, unbound serum was removed by 3 washes in PBS-T 20 and 100 ul of the monoclonal antibody was added. The ELISA was then completed as described above. A decrease in the absorbance value in a well which received human serum, as compared to a unblocked control well, was then interpreted as inhibition of reactivity and expressed as a percentage. Significance of the differences of the results for the Bolivian versus the Baltimore sera was verified by the F-test.

Results.

Indirect immunofluorescent antibody assays revealed that monoclonal antibody 84B-1E3, generated against stationary phase epimastigotes of T. cruzi, recognizes a dominant determinant on the flagellum and surface membrane (Fig. 1a). Subsequent demonstration of similar reactivity on infectious, metacyclic trypomastigotes recovered from the hind-guts of wild-caught reduviids (Fig. 1b) verified that the epitope had been conserved throughout parasite differentiation and, in all liklehood, is expressed as a major surface antigen in naturally occurring human infections.

The flagellar component reactive with 84B-1E3 was recovered in a Lubrol extract of stationary phase epimastigotes (Fig.2). Sequential analyses of that extract by SDS-PAGE, Western Blot and immunoautoradiography revealed that the reactive epitope was part of a 52-54 kd polypeptide (Fig. 3, lane A). Quite unexpected however, was the finding that 84B-1E3 reacted with an even greater intensity with a 54 kd tubulin recovered from rat brain (Fig.3, lane B). Because the parasite moiety and the mammalian molecule were indistinguishable with respect to size and electrophoretic mobility, we assumed that the monoclonal antibody had been elicited in response to trypanosomal tubulin. IFA, where cultured human breast cells were used as the antigenic substrate (Fig. 4) appeared to verify this assumption. However, additional attempts to confirm monoclonal antibody specificity, by ELISA, revealed that the reactive epitope was not only common to microtubules, but also to actin filaments, myosin, trypomyosin, filamin and the actin anchorage proteins, vinculin and alpha actinin (Fig. 5).

The remaining question was whether this conserved epitope, as displayed on the infectious form of the parasite, elicits an antibody response in naturally occurring human disease. Although it could be clearly demonstrated that sera from patients with Chagas' disease contain a surfeit of antibodies reactive with antigens of the flagella-enriched fraction (Fig.6), we could not be certain that these antibodies were recognizing the determinant identified by 84B-1E3. As a matter of fact, these human antibodies could have been produced in response to proteins of the host's cells damaged by parasitization. However, results of the competitive ELISA resolved that uncertainty. Since 23 of the 48 Bolivian sera inhibited the binding of the monoclonal antibody to cultured epimastigotes at a level of greater than 50% (Fig. 7), we feel confident in stating that the 84B-1E3 reactive epitope does act as an immunogen in human infections and it probably accounts for some of the anti-cytoskeletal protein antibodies seen in the acute phases of the disease. Antibody activity in the Baltimore controls, in both the direct and inhibition assays, can be attributed to the low levels of naturally occuring anti-cytoskeletal antibodies in healthy sera (15).

Discussion.

The etiologic agent of Chagas' disease is the flagellated protozoan, Trypanosoma cruzi. Infectious parasites, metacyclic forms, are transmitted to man through the feces of insect vectors belonging to the Family Reduviidae. Infection, through an open wound or across mucus membranes, leads to an acute phase of disease wherein an inflammatory reaction to the trypanosome results in cell injury. While cardiac fibers are a frequent target, parasites will infect skeletal and smooth muscles, macrophages, nerve cells, fibroblasts, and epithelial cells. When the parasite load decreases, the inflammatory response subsides and may even disappear completely (2). However, the disease may progress to a chronic heart condition with death occurring as a result of severe arrhythmias followed by cardiac failure and embolism. Gastrointestinal involvement with the development of mega-syndromes is common in some areas of Brazil and Chile (7).

Although precise mechanisms of the pathogenesis remain poorly understood, there is an increasing accumulation of evidence which implies that damage to myocardial fibers during the acute phase of American trypanosomiasis is the consequence of an autoimmune response. Investigators have suggested that autoantibodies reacting with the endocardium, vascular structures and interstitium of striated muscles (EVI antibodies) are directed against host antigens which are exposed following cell injury by the parasite (11). However, the appearance of these autoantibodies in acute phase serum and the observation that they can be absorbed with cultured forms (epimastigotes) of T. cruzi (37) implies that they are produced in response to parasite antigens.

glycine-HCl buffer, pH 2.5, and the acid eluate was immediately neutralized to pH 7.2 with 0.1M Tris. Purity and molecular weight of the antigen was verified by SDS-PAGE. Reactivity with the monoclonal antibody was confirmed by the Western Blot and presence of a carbohydrate moiety was established by staining with the Periodic Acid-Schiff Reagent.

Results.

Monoclonal antibody 83L-2D3/a, isotyped as IgM, was strongly reactive, by ELISA, with air-dried, stationary phase promastigotes of 3 species and 8 subspecies of New World Leishmania. Absorbance values (Table 1) were all >1.000 with the exception of the rodent isolate of L. m. aristidesi. Isolates of Endotrypana and Trypanosoma were non-reactive.

Reactivity of monoclonal antibody 83L-2D3/a with the surface membrane and flagellum of stationary phase promastigotes of the WRAIR-470 isolate is illustrated in Figures 1 and 2. Figure 3 confirms that this dominant surface epitope is conserved throughout intracellular differentiation of the promastigote and is subsequently displayed on the amastigote membrane (arrows). Although a variety of such epitopes have been visualized using different monoclonal antibodies, the additional stippled reactivity on the surface membrane of the parasitized macrophage (Figure 4) was limited to 83L-2D3/a. Unfortunately, the intracellular amastigotes and the macrophage membrane were usually at two different planes of focus, thus making it difficult to clearly visualize both sites of reactivity on a single cell simultaneously. Nevertheless, immunoelectronmicrographs of macrophages containing amastigotes, Figures 5a and 5b, provided convincing evidence that the antigen recognized by 83L-2D3/a was indeed expressed at the surface of these infected cells.

The kinetics of leishmanial antigen expression on the surface membrane of mouse peritoneal macrophages infected in vitro, as determined by flow cytometric analyses, is presented in Figure 6. The antigen reactive with monoclonal antibody 83L-2D3/a could be demonstrated at the cell surface within

6 hours after infection. It continued to accumulate for an additional 42 hours, at which time the intracelluar parasite load reached 1.2 X 10^3/500 macrophages. As the parasite burden continued to increase, the macrophage evidently lost its capability to process additional antigen and the membrane reactivity diminished rapidly. By 72 hours post-infection all antigen had either been shed into the medium or internalized as a normal consequence of membrane turnover. Medium from these 72 hr cultures did not bind to the surface membrane of normal macrophages.

Immunochemical characterization of the 83L-2D3/a reactive antigen is presented in Figure 7. To date, one other monoclonal antibody, generated against the WR 222B isolate of L. mexicana mexicana, recognizes this epitope. It should be noted that the 15 kd component identified by the silver stain (panel b) was not discernible, in the crude extract, with the less sensitive Coomassie Blue stain (panel a). Proof of recognition of the 15 kd antigen by the monoclonal antibody is supplied by the immunoautoradiograph of the Western Blot (panel c). The presence of a carbohydrate moiety is confirmed in panel d.

Discussion:

Handman et.al (13) reported that a polyvalent mouse antiserum to promastigotes of L. tropica recognized a leishmania antigen on the plasma membrane of infected mouse macrophages in vitro. Although the origin of that antigen could not be established, its appearance on the surface of the occasional parasite-free macrophages inherent to infected cultures suggested that it was an excretory product derived from extracellular promastigotes. Unfortunately, reactivity of this anti-promastigote serum with the intracellular amastigotes was not investigated. More recently, Berman and Dwyer (7), used a rabbit polyclonal antiserum specific for L. donovani amastigotes to demonstrate leishmania antigens of the plasma membrane of infected human macrophages in vitro. Since this antiserum also bound to the surface of uninfected macrophages cultured in medium recovered from infected cells, the reactive epitope was allegedly associated with an amastigote moiety which, after being processed in the phagolysosome, had been released and readsorbed.

It is necessary to note, nevertheless, that when extracts of whole promastigotes are used as immunogens the resultant polyclonal antisera will contain a surfeit of anti-flagellar antibodies which will cross-react with cytoskeletal and membranous proteins of uninfected mammalian cells (14). The reactive epitopes, evidently conserved throughout evolution, are associated with a large array of proteins including tubulin, actin, alpha actinin, tropomyosin, myosin, vinculin and filamin (unpublished observations). We suspect that at least part of the surface reactivity observed between uninfected cells and polyvalent antisera can be attributed to the presence of such antibodies.

We now report on the identification and recovery of yet another antigen which is common to promastigotes and amastigotes of New World Leishmania and which is expressed on the surface membrane of mouse peritoneal macrophages infected in vitro. In contrast to the earlier studies, however, this antigen was discovered by using a purified murine monoclonal antibody which had been generated to a dominant antigen on the surface membrane of L. braziliensis panamensis promastigotes. Since this monoclonal antibody, 83L-2D3/a, was an IgM, we promptly excluded the possibility that its reactivity with the infected mouse macrophage membrane was due to adsorption via Fc receptors (15). Moreover, unlike the polyclonal antisera, this monoclonal antibody did not react with the surface membrane of parasite-negative macrophages co-cultured with the infected cells.

Although the 83L-2D3/a reactive antigen had a dense distribution on the surface of both promastigotes and amastigotes (Figs.1,2 and 3), the epitope expressed on the macrophage (Figs.4 and 5) seemingly had an intracellular origin. If it had been left behind by the promastigote during attachment and penetration, we would have observed increased immunofluorescence within the first few hours post infection. That was not the case (Fig.6). In fact, the antigen did not appear on the macrophage membrane until several hours after parasitization was complete. This delay supports the belief that the amastigote antigen had to be degraded in the phagolysosome prior to deposition at the macrophage surface. Furthermore, the amount of antigen expressed appeared to be dependent upon the number of amastigotes present. As long as the parasite burden was low ,<1000 amastigotes/500 macrophages, the macrophage continued to process additional antigen and maximum levels of expression, as

measured by relative surface immunofluorescence, persisted. However, as the amastigote load continued to increase, the macrophage quickly lost its capacity to process additional antigen and surface reactivity declined rapidly. We suspect that the antigen which had accumulated on the membrane was lost as a result of extracelllar shedding and/or internalization.

The 15 kd glycoconjugate recognized by monoclonal antibody 83L-2D3/a (Fig.7, panel c) is most likely a breakdown product which happens to contain the reactive epitope of a much larger surface membrane antigen of promastigotes. Even though protease inhibitors were included in the extraction buffers, degradation during the harsh solubilization routines prerequisite to SDS-PAGE analyses could not be discounted. Alternatively, the 15 kd subunit visualized with the silver stain (Fig. 7, panel b) could represent a much larger carbohydrate molecule (Fig.7, panel d) which , because of its predominant negative charge, has an extreme anodic migration. Reactivity of such glycoconjugates with silver stains has been described by others (16).

At this time we can only speculate on the sequence of events which leads to expression of the 83L-2D3/a reactive epitope on the surface of the infected macrophage. The native surface glycoconjugate (Fig.1), conserved throughout differentiation into the amastigote (Fig.3), would be exposed to the proteolytic enzymes and acid environment of the phagolysosome. The carbohydrate moiety would not be broken down by the acid hydrolyases but it could be degraded into low molecular products upon exposure to acid pH. Such acid labile glycoconjugates have been recovered and characterized from extracts of L. donovani (17). The carbohydrate moiety, possibly a polysaccharide, which contains the reactive epitope could be incorporated into the phagolysosome

membrane (2,18). Subsequent fusion of that membrane with the macrophage surface membrane (19,20) would result in extracellular display of the epitope (Figs.4 and 5).

Although the mechanisms of macrophage activation for intracellular killing of leishmania have been reasonably defined at the cellular level (21-23), the possible contribution of parasite antigens in that process has, for the most part, been ignored. One would expect, nevertheless, that parasite antigens displayed at the surface of the macrophage would participate in the T-lymphocyte sensitization required for macrophage activation. Now that such antigens can be identified and recovered with the aid of monoclonal antibodies, an assesment of their role in regulation of the immune response in leishmaniases must be given top priority.

TABLE 1

Taxonomic Specificity of Monoclonal Antibody 83L-2D3/a as Assessed by Enzyme Linked Immunosorbent Assays.

Isolate	Identification	Absorbance (405 nm)
WR-470*	L.b.panamensis	1.041
WR-359	L.b.braziliensis	1.452
WR-140	L.b.peruviana	1.120
WR-390**	L.b.guyanensis	1.108
WR-222	L.m.mexicana	1.436
WR-303	L.m.amazonensis	1.580
GML-3***	L.m.aristidesi	0.753
WR-484	L.d.chagasi	1.387
GML-465****	E.schaudinni	0.048
Tulahuen	T.cruzi	0.046

* = homologous reaction

** = WHO reference strain

*** = rodent isolate

**** = sloth isolate

Legends for figures.

Figure 1. Immunofluorescent micrograph depicting the distribution of the 83L-2D3/a reactive epitope at the surface of stationary phase promastigotes of Leishmania braziliensis panamensis. Magnification = 1600x.

Figure 2. Immunoelectron micrograph confirming the presence of the 83L-2D3/a reactive epitope on the surface membrane of L. b. panamensis. Magnification =15,900x.

Figure 3. Immunofluorescent micrograph confirming the presence of the 83L-2D3/a reactive epitope of the surfce of intracellular amastigotes. Magnification = 1600x

Figure 4. Immunofluorescent micrograph illustrating the reactivty of monoclonal antibody 83L-2D3/a with the surface membrane of mouse peritoneal macrophages infected with L. b. panamensis in vitro. Magnification =1600x.

Figure 5(a). Immunoelectron micrograph (cross-sections) confirming replication of WR-470 in mouse periotneal macrophages.

Figure 5(b). Presence of the 83L-2D3/a reactive antigen on the surface membrane of mouse peritoneal macrophages infected with L. b. panamensis (WR-470) in vitro, longitudinal section. Magnification= 5800x

Figure 6. Kinetics of expression of the 83L-2D3/a reactive antigen on the surface membrane of mouse peritoneal macrophages infected with L. b. panamensis in vitro.

Figure 7. SDS-PAGE analyses of the 83L-2D3/a reactive antigen. Panel a = Coomassie Blue Stain of the crude antigen extract of L. b panamensis stationary phase promastigotes. Panel b = silver stain of the L. b. panamensis antigen recovered from the 83L-2D3/a affinity column. Panel c = immunoautoradiograph confirming the reactivity of the 83L-2D3/a monoclonal antibody with a 15 kd component. Panel d = Periodic acid-Shiff reaction of the affinity purified antigen confirming the presence of a carbohydrate moiety.

FIGURE 1

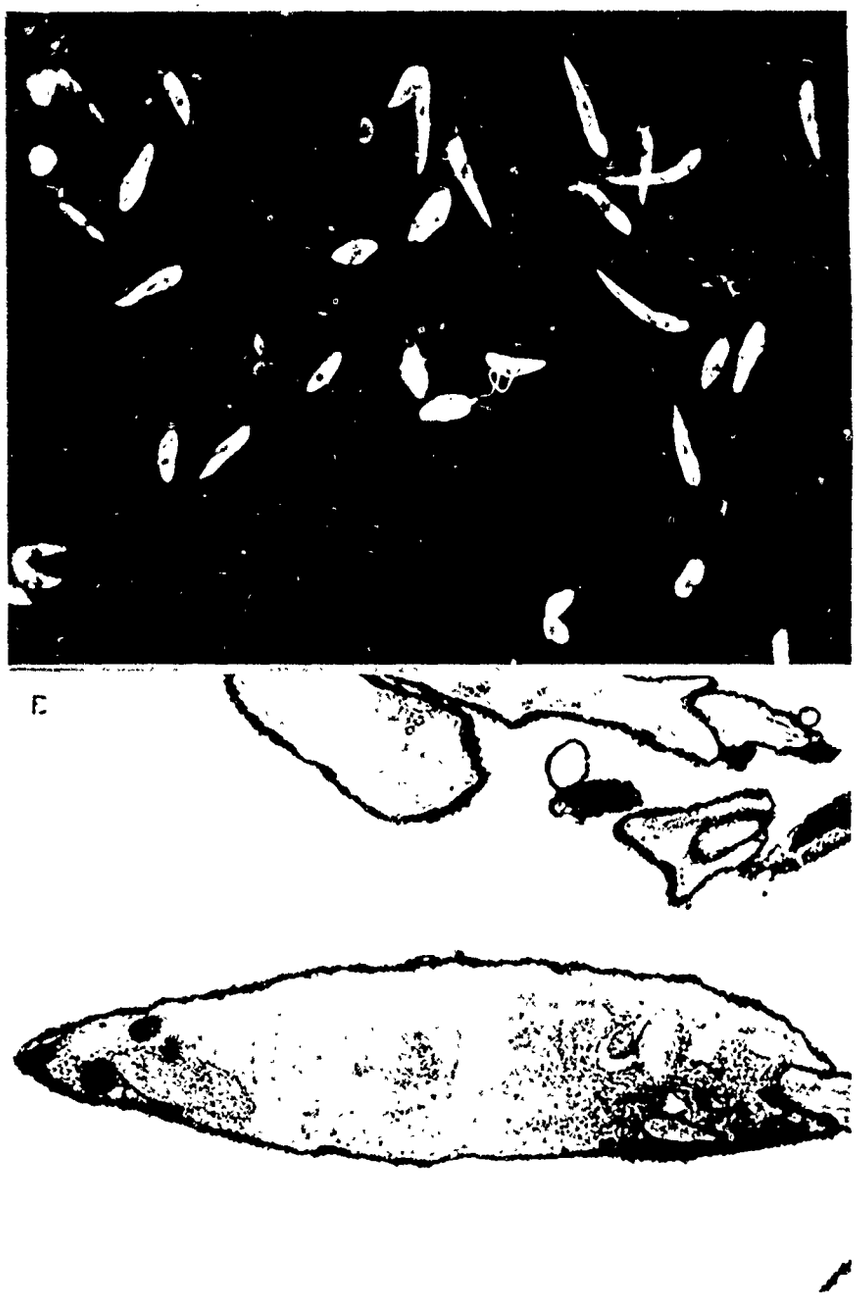

FIGURE 2

FIGURE 3

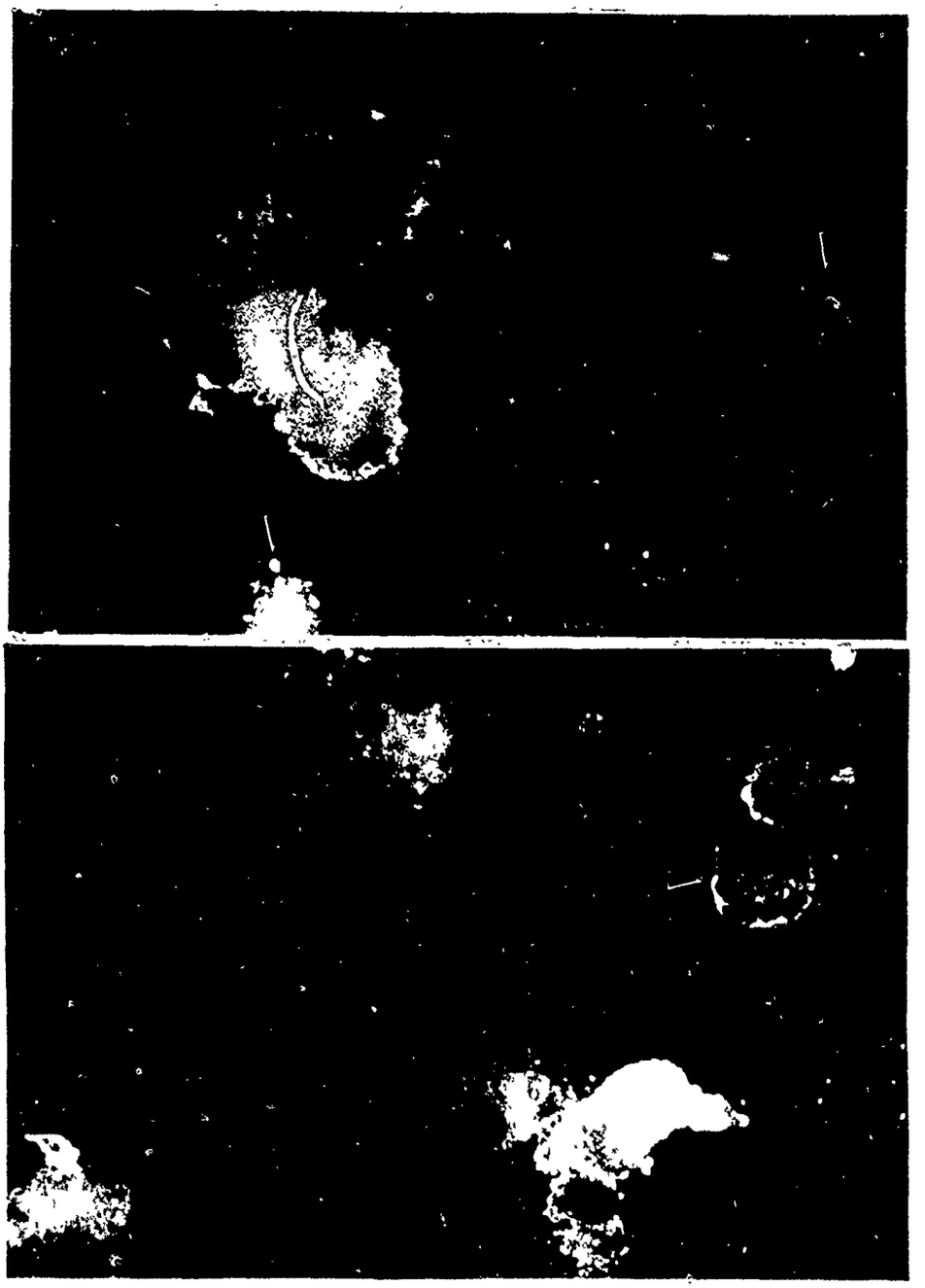

FIGURE 4

Some researchers have demonstrated that these antigens are parasite specific proteins absorbed onto the surface of infected cells (16) while others contend that autoantibodies are synthesized in response to T. cruzi antigens which display epitopes identical to those of the target tissues (12).

In contrast to classical autoimmune diseases (10), the autoimmune response in Chagas' disease occurs, concomitantly, with an increase, rather than a decrease, in suppressor T- lymphocytes (13). In other words, the exaggerated state of immunosuppression which is so characteristic of American trypanosomiasis does not abrogate the production of acute phase autoantibodies. Although definitive evidence is not in the realm of this paper, we suspect that the increase in T-suppressor cells might actually be a compensatory effort on the part of the host to regulate the production of antibodies to parasite antigens which express deteminants common to those of the host's cells.

We now present evidence that one such antigen, common to both cultured epimastigotes and infectious trypomastigotes (Figs. 1a and 1b), is a 54 kd polypeptide (Fig. 3) with an epitope which has been highly conserved throughout evolution and which appears, subsequently, as part of the vertebrate cytoskeleton (Fig. 4). This sort of phylogenetic conservation of antigenic determinants, especially among the proteins of the animal cell matrix, is not unusual (38). Furthermore, the many different filaments and microtubules which make-up the vertebrate cytoskeleton also share the same epitopes (32). Thus, the reactivity of the monoclonal antibody, 85B-1E3, with both the T. cruzi flagellum and a variety of structural proteins of vertebrate cells (Fig. 5) was not surprising. In fact, we suspect that the trypanosomal

antigens which are expressed in mammalian neurones (19,36,39) and skeletal and cardiac muscle (34) display the same epitope as that which is identified by 85B-1E3.

Perhaps more important is the observation that sera from acute cases of Chagas' disease contain antibodies reactive with the parasite-host cell epitope identified by 85B-1E3 (Fig. 7). Although others may contend that these anti-cell matrix antibodies represent a response to host cell proteins exposed as a result of cell injury or are the consequence of polyclonal B-cell activation, we contend that the antigenic challenge provided by the shared epitope on the metacyclic trypomastigote (Fig. 1b) can no longer be refuted.

If we accept the possibility that antibodies elicited in response to conserved epitopes contribute to the autoimmune pathology of Chagas' disease, then we must address the fact that similar, shared epitopes occur between host and nearly all cellular infectious agents (14,23,29) including the African trypanosomes (8,24,26,30,33). However, the diseases produced by most of these organisms do not include an autoimmune component equivalent to that which occurs following infection with T. cruzi. Although definitive evidence is not provided in this communication, we suspect that the autoimmune pathogensis of Chagas' disease is related to the tissue tropism of the parasite and, in particular, to the density and distribution of shared epitopes within the target cells of those tissues. Moreover, it appears reasonable to assume that the host's primary response to the parasite epitope would lead to acute phase antibodies while the sustanied anamnestic response to the cross-reactive host cell antigens, exposed as a consequence of parasitization, would result in the autoimmune pathology characteristic of

chronic disease. This assumption is supported by the many reports which confirm that autoantibodies produced as a consequence of polyclonal B-cell activation do not cross-react with parasite antigens (24,26,30,33) and they do not cause any direct pathologic changes (8).

In summation, Chagas' disease is the most common infectious disease in the Western Hemisphere and it is the leading cause of myocarditis in the world. Consequently, this infection, targeted by the World Health Organization's Special Program of Tropical Diseases, has a profound influence upon the socio-economic status of most Latin American countries. Although current efforts directed towards improvements in vector control, chemotherapy and diagnosis must receive continued support, further elucidation of the pathogenic progression of the disease must be ranked as a top priority. We contend that studies on the immunogenic behavior of specific parasite antigens, such as that which is identified by 85B-1E3, will eventually solve this complex conumdrum.

Acknowledgments:

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Literature cited:

1. Anderson, S., J. R. David, and D. Mc Mahon-Pratt. 1983. In vitro protection against Leishmania mexicana mediated by monoclonal antibodies. J. Immunol. 85:523-531.

2. Angrade, Z. A. 1983. Mechanisms of myocardial damage in Trypanosoma cruzi infection. p. 214-223. In Cytopathology of Parasitic Diseases, Ciba Foundation Symposium 99. Pitman Press, Bath, Great Britain.

3. Anthony, R. L., C. M. Johnson, and O. E. Sousa. 1979. Use of micro-ELISA for quantitating antibody to Trypanosoma cruzi and Trypanosoma rangeli. Am. J. Trop. Med. Hyg. 28:969-973.

4. Anthony, R. L., T. S. Cody, and N. T. Constantine. 1981. Antigenic differentiation of Trypanosoma cruzi and Trypanosoma rangeli by means of monoclonal antibodies. Am. J. Trop. Med. Hyg. 30:1192-1197.

5. Anthony, R. L., K. M. Williams, J. B. Sacci, and D. C. Rubin. 1985. Subcellular and taxonomic specificity of monoclonal antibodies to New World Leishmania. Am. J. Trop. Med. Hyg. 34:1085-1094.

6. Arajo, F. G., S. D. Sharma, V. Tsai, P. Cox, and J. S. Remington. 1982. Monoclonal antibodies to stages of Trypanosoma cruzi: Characterization and use for antigen detection. Infect. Immun. 37:344-349.

7. Atias, A. 1964. Enfermedad de Chagas digestiva en Chile. Bol. Chil. Parasit. 24: 70.

8. Boreham, P. F. L., and C. A. Facer. 1974. Autoimmunity in Trypanosome infections. II. Anti-fibrin/fibrinogen (anti-F) autoantibody in Trypanosoma (Trypanozoon) brucei infections in the rabbit. Internat. J. Parasitol. 4:601-607.

9. Constantine, N. T., and R. L. Anthony. 1983. Antigenic differentiation of the Kinetoplastids Leishmania braziliensis and Trypanosoma cruzi by means of monoclonal antibodies. J. Protozool. 30:346-350.

10. Coovadia, H. M., I. R. MacKay, and A. J. D'Apice. 1981. Suppressor cells assayed by three different methods in patients with chronic hepatitis and systemic lupus erythematosis. Clin. Immunol. Immunopath. 18:268-275.

11. Cossio, P. M., R. P. Laguens, C. Diez, A. Szarfman, A. Segal, and R. M. Arana. 1979. Chagasic cardiopathy. Antibodies reacting with plasma membrane and striated muscle of endothelial cells. Circulation. 50: 1252-1259.

12. Cossio, P. M., C. Diez, A. Szarfman, E. Kreutzer, B. Candidolo, and R. M. Arana. 1979. Chagasic cardiopathy. Demonstration of a serum gammaglobulin factor which reacts with endocardium and vascular structures. Circulation. 49:13-21.

13. Cunningham, D. S., M. Grogl, and R. E. Kuhn. 1980. Suppression of antibody responses in humans infected with Trypanosoma cruzi. Infect. Immun. 30:496-499.

14. Dales, S., R. Fujinami, and M. Oldstone. 1983. Infection with vaccinia virus favors the selection of hybridomas synthesizing autoantibodies against intermediate filaments, one of them cross reacting with the virus hemagglutinin. J. Immunol. 131:1546-1553.

15. Dighiero, G., B. Guilbert, and S. Avrameas. 1982. Naturally occurring antibodies against nine common antigens in humans sera. II. High incidence of monoclonal Ig exhibiting antibody activity against actin and tubulin and sharing antibody specificities with natural antibodies. J. Immunol. 128:2788-2792.

16. Dos Santos, R. R., and L. Hudson. 1980. Trypanosoma cruzi: Immunologic consequences of parasite modification of host cells. Clin. Exp. Immunol. 40:36-41.

17. Gallo, J., and B. H. Anderson. 1983. A subpopulation of trypanosome microtubules recognized by a monoclonal antibody to tubulin. EMBO 2:479-483.

18. Handman, E., and R. E. Hocking. 1982. Stage-specific, strain-specific and cross-reactive antigens of Leishmania species identified by monoclonal antibodies. Infect. Immun. 37:28-33.

19. Hudson, L. 1983. Immunopathogenesis of experimental Chagas' disease in mice; damage to autonomic nervous system. p 234-251. In Cytopathology of Parasitic Diseases, Ciba Foundation Symposium 99. Pitman Press, Bath, Great Britain.

20. Johnson, A. M., P. J. Mc Donald, and S. H. Neoh. 1983. Monoclonal antibodies to Toxoplasma cell membrane surface antigens protect mice from toxoplasmosis. J. Protozool. 30:351-356.

21. Kasper, L. H., M. S. Bradley, and E. R. Pfefferkorn. 1981. Identification of stage specific sporozoite antigens of Toxoplasma gondii by monoclonal antibodies. J. Immunol. 132:443-449.

22. Kennett, R. H. 1980. Cell Fusion. p.345-349. In T.W. Jakoby and H. Pastan (eds.), Methods in Enzymology. Academic Press, New York.

23. Linder, E., P. Kurki, and L. Anderson. 1979. Autoantibody to intermediate filaments in infectious mononucleosis. Clin. Immunol. Immunopath. 14:411-417.

24. Mac Kenzie, A. R., and P. F. L. Boreham. 1974. Autoimmunity in Trypanosome infections. I Tissue autoantibodies in Trypanosoma (Trypanozoon) brucei infections of the rabbit. Immunology 26:1225-1238.

25. Majarian, W. R., T. M. Daly, W. P. Weidanz, and C. A. Long. 1984. Passive immunization against murine malaria with an IgG3 monoclonal antibody. J. Immunol. 132:3131-3137.

26. Mansfield, J. M., and J. P. Kreier. 1971. Autoimmunity in experimental Trypanosoma congolense infections of rabbits. Infect. Immun. 5:648-656.

27. Mc Mahon-Pratt, D., and J. R. David. 1981. Monoclonal antibodies that distingusih between New World species of Leishmania. Nature 291:581-583.

28. Mc Mahon-Pratt, D. Bennett, and J. R. David. 1982. Monoclonal antibodies that distinguish subspecies of Leishmania braziliensis. J. Immunol. 129: 926-927.

29. Mortazavi-Milani, S. M., S. S. Badakers, and E. J. Holborow. 1984. Antibody to intermediate filaments of the cytoskeleton in sera of patients with acute malaria. Clin. Exp. Immunol. 55:177-182.

30. Mortazavi-Milani, S. M., C. A.Facer, and E. J. Holborow. 1984. Induction of anti-intermediate filament antibody in rabbits experimentally infected with Trypanosoma brucei brucei. Immunology 52:423-426.

31. Pereira, N. M., W. De Souza, R. D. Machado, and F. T. De Castro. 1977. Isolation and properties of flagella of trypanosomatids. Protozool. 24:511-514.

32. Pruss, R. M., R. Mirsky, and M. C. Raff. 1981. All classes of intermediate filaments share a common antigenic determinant defined by a monoclonal antibody. Cell 27:419-428.

33. Seed, J. R., and A. A. Gam. 1967. The presence of antibody to a normal rabbit liver antigen in rabbits infected with Trypanosoma gambiense. J. Parasit. 53:946-950.

34. Sadigursky, M., A. M. Acosta, and C. A. Santos-Buch. 1982. Muscle sarcoplasmic reticulum antigen shared by a Trypanosoma cruzi clone. Am. J. Trop. Med. Hyg. 31:934-941.

35. Sharma, S. D., F. G. Araujo, and J. S. Remington. 1984. Toxoplasma antigen isolated by affinity chromatography with monoclonal antibody protects mice against lethal infection with Toxoplasma gondii. J. Immunol. 133:2818-2820.

36. Snary, D., J. E. Flint, J. N. Wood, M. T. Scott, M. D. Chapman, J. Dodd, T. M. Jessell, and M. A. Miles. 1983. A monoclonal antibody with specificity for Trypanosoma cruzi central and peripheral neurones and glia. J. Exp. Immunol. 54:616-624.

37. Szarfman, A., P. M. Cossio, G. G. Schumis, and R. M. Arana. 1977. The EVI antibody in acute Chagas' disease. J.Parasit. 63:149-

38. Walter, M. F., and H. Biessmann. 1981. Cross-reaction of a monoclonal antibody with intermediate filament-like proteins from vertebrate and non-vertebrate organisms. J. Cell. Biol. 90: 236a (abstract).

39. Wood, J. N., L. Hudson, T. M. Jessell, and M. Yamaoto. 1982. A monoclonal antibody defining antigenic determinants on subpopulations of mammalian neurones and Trypanosoma cruzi parasites. Nature 296:34-38.

40. Yoshida, N., R. S. Nussenzweig, P. Potocnjak, V. Nussenzweig, and M. Aikawa. 1980. Hybridoma produces protective antibodies directed against the sporozoite stage of malaria parasite. Science 207:71-73.

Figure 1. Immunofluorescent micrographs demonstrating surface-flagellar reactivity of monoclonal antibody 85B-1E3 with (a) air-dired epimastigotes and (b) metacyclic trypomastigotes (arrows highlight undulating membrane) of T. cruzi. Magnification = 500 X.

Figure 2. Immunoelectronmicrograph of flagellar enriched fraction of T. cruzi epimastigotes. Magnification = 5800 X.

Figure 3. Western blot confirming the reactivity of monoclonal antibody 85B-1E3 with a 58 kd polypeptide in the flagella enriched fraction of T. cruzi epimastigotes (lane A) and purified rat brain tubulin (lane B).

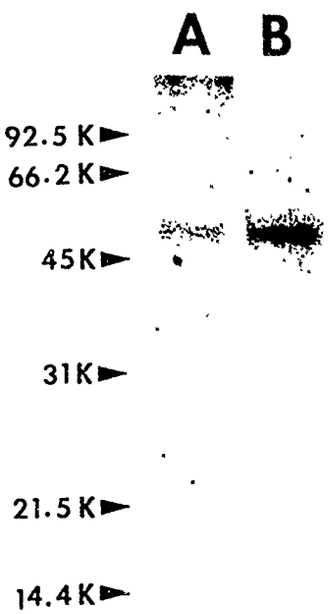


Figure 4. Immunofluorescent micrograph demonstrating the reactivity of monoclonal antibody 85B-1E3 with the cell matrix of a human epithelial cell. Magnification = 500 X.

Figure 5. Reactivity of monoclonal antibody 85B-1E3 with purified polypeptides of the vertebrate cytoskeleton.

Figure 6. Reactivity of human sera with the flagella enriched fraction of T. cruzi epimastigotes. Each point represents a mean value for three determinations. The difference in the distribution of the absorbance values for the Bolivian versus the Baltimore sera was significant ($p<0.001$).

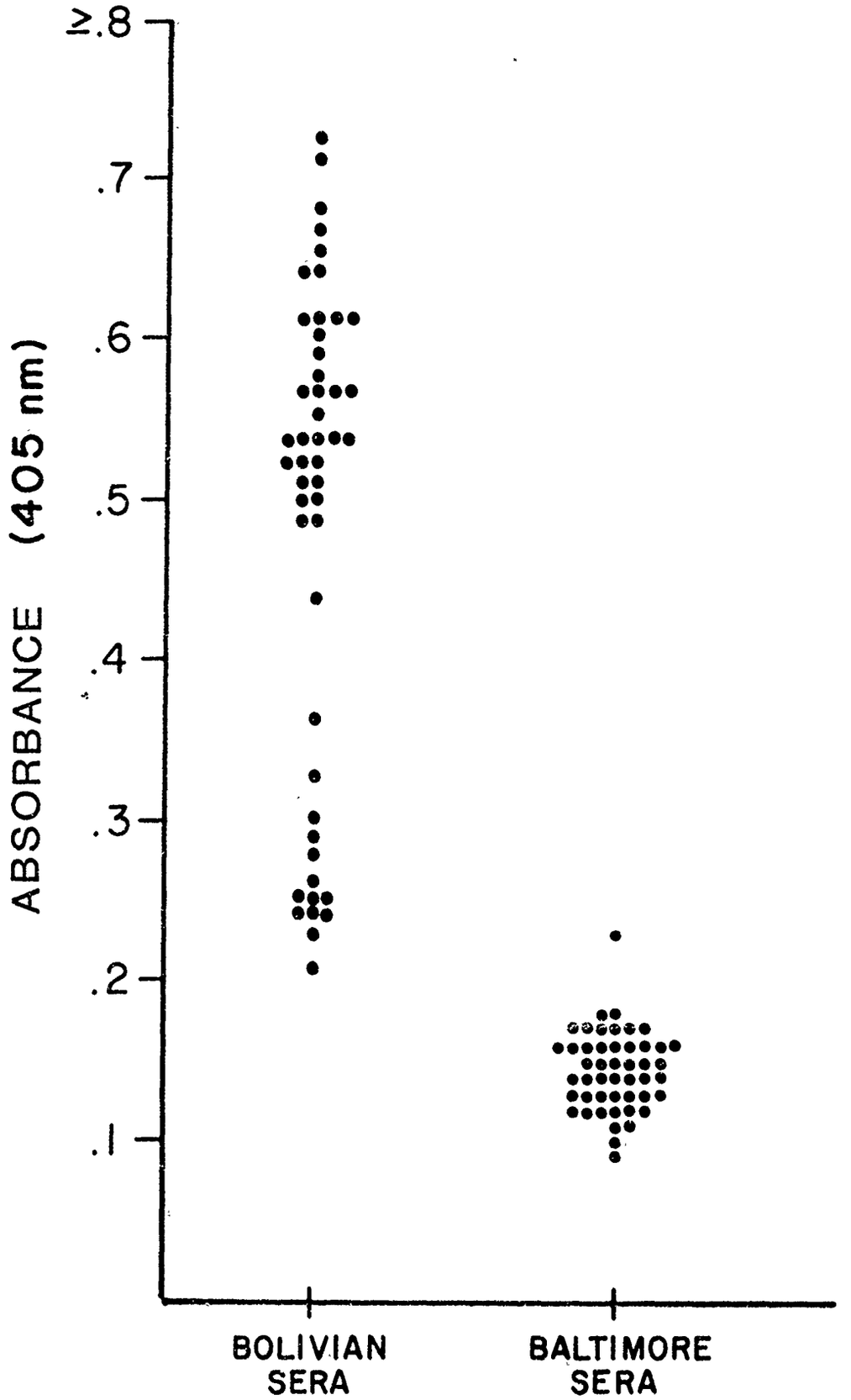
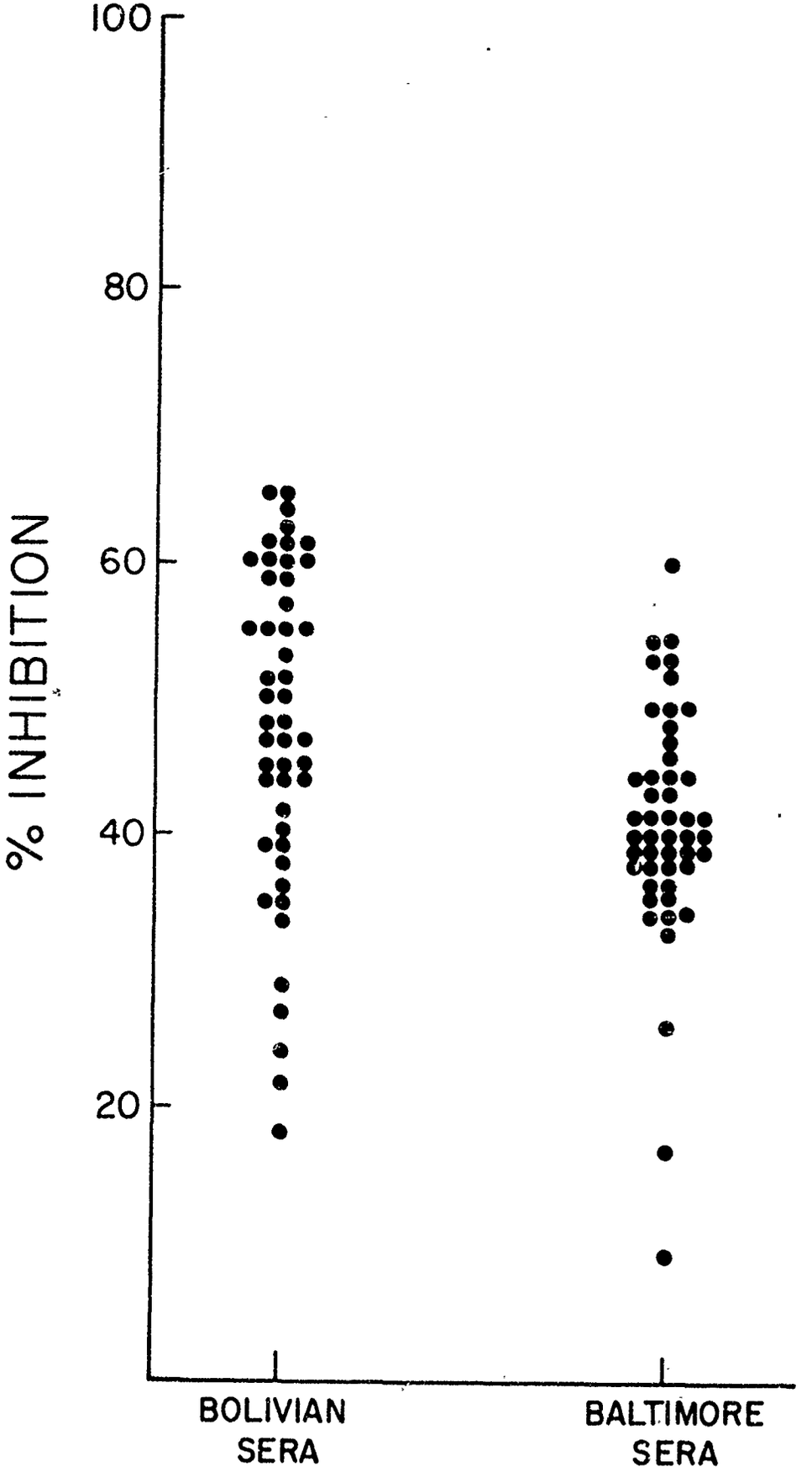

≥.8
.7
.6
.5
.4
.3
.2
.1
ABSORBANCE (405 nm)
BOLIVIAN SERA
BALTIMORE SERA

Figure 7. Capability of human sera to inhibit the binding of monoclonal antibody 85B-1E3 to epimastigotes of T. cruzi. Each point represents a mean value of three determinations. The difference in the distribution of values of percentage inhibition for the Bolivian versus the Baltimore sera was significant ($p<0.05$).


100
80
60
40
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RAPID IDENTIFICATION OF *LEISHMANIA* AMASTIGOTES IN INFECTED TISSUES USING STRAIN- AND SPECIES-SPECIFIC MONOCLONAL ANTIBODIES

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ABSTRACT

A panel of 18 monoclonal antibodies demonstrating specificity for membrane antigens of Leishmania promastigotes were evaluated for their reactivity with amastigotes of 8 New World isolates by indirect immunofluorescent microscopy. Amastigotes were generated in vitro by the infection of cultured peritoneal macrophages and in vivo by the footpad inoculation of susceptible (Balb/c) mice. Two promastigote-specific antigens were identified which appear as triplet membrane components when characterized by Western blot analysis. Several monoclonal antibodies could differentiate amastigotes of L. mexicana from those of L. braziliensis, and one antibody was found to be specific for a single isolate of L. braziliensis panamensis. The potential diagnostic use of these antibodies for the rapid speciation of Leishmania in cutaneous lesions is supported by the ease with which the amastigotes were visualized in tissue sections by indirect immunofluorescence microscopy.

INTRODUCTION

Species of Leishmania which cause human tegumentary leishmaniasis are responsible for a multiplicity of clinical syndromes. In the New World strains of the L. mexicana and L. braziliensis complexes are those most often isolated from dermal lesions. Identification of the causative organism is critical in terms of initial treatment and subsequent management of patients. While most skin lesions caused by species of the mexicana complex are relatively mild and heal spontaneously, infection with L. mexicana amazonensis or L. mexicana pifanoi in the immunocompromised host may progress to incurable diffuse cutaneous leishmaniasis (1, 2). Similarly, cutaneous lesions caused by species of the braziliensis complex are generally self-limiting. But because of its potential for metastatic spread to the mucocutaneous junctions of the face and oropharyngeal cavity, the isolation of L. braziliensis braziliensis from a lesion is an indication for the institution of immediate and rigorous therapy.

Accurate and simple serodiagnostic tests for the evaluation of leishmania infections are currently unavailable. Sera from patients with Chagas' disease (3), malaria, toxoplasmosis and amoebiasis (4) contain antibodies which cross-react with leishmania antigens. At this time, confirmation of leishmaniasis is contingent upon the isolation and culture of parasites from lesions with subsequent species identification using biological criteria or biochemical analyses. Unfortunately, direct culture from cutaneous lesions is complicated

by the fact that most ulcers are superinfected by bacteria or fungus. Furthermore, attempts to culture the parasites from mucocutaneous lesions are often unsuccessful because they are present in very small numbers. Biological methods, such as determination of promastigote growth characteristics in the gut of the sandfly vector (5, 6) or the appearance of lesions following parasite inoculation into hamsters (7), are time consuming and cannot differentiate at the sub-species level. Biochemical methods generally require large numbers of organisms leading to long delays when slowly growing strains are involved. Although kinetoplast DNA probe analyses has been used to differentiate small numbers of L. braziliensis from L. mexicana in touch blots of cutaneous lesions, there is significant sub-species kDNA homology within the braziliensis and mexicana complexes (8, 9).

Pathogenesis of the leishmania is attributed to the promastigote, or extracellular form, which establishes residence in the cells of the host reticuloendothelial system. There it differentiates into the intracellular form, or amastigote, which multiplies to the point of host cell rupture, and subsequently parasitizes adjacent macrophages. In impression smears or biopsy material from lesions containing large numbers of organisms, amastigotes are readily visualized by Giemsa or other histochemical stains. But in lesions of longer duration, or in cases of mucocutaneous involvement, parasites are often difficult to visualize (10). Furthermore, leishmania cannot be speciated on the basis of microscopic appearance. For this reason, we have produced monoclonal antibodies which exhibit species and strain specificity for amastigotes of 8 isolates of New World Leishmania. These antibodies

have been evaluated as potential diagnostic reagents by assessing their reactivity in indirect immunofluorescence assays with tissue sections from early murine infections produced by footpad inoculation of L. mexicana amazonensis promastigotes.

MATERIALS AND METHODS

Promastigotes. Human isolates of L. mexicana mexicana (WR 222), L. mexicana amazonensis (WR 303), L. braziliensis panamensis (GML 1), L. braziliensis guyanensis (WR 390), L. braziliensis braziliensis (WR 508, GML 18), L. species (Courtwright, WR 359) and a rodent isolate of L. mexicana amazonensis (GML 111) were obtained from the cryobanks at the Walter Reed Army Institute of Research and The Gorgas Memorial Laboratory. The origins and previous references to the strains have been detailed elsewhere (11). All leishmania were maintained as promastigotes at 26°C in liquid Medium-199 enriched with 20% fetal calf serum.

Production of Amastigotes In Vitro. Peritoneal exudate cells were isolated by lavage from Balb/c mice which had been stimulated 7 days previously with 2 ml of sterile 4% thioglycollate broth. Briefly, 5 ml of sterile Hanks' Balanced Salt Solution containing 100 units/ml of sodium heparin was injected into the peritoneal cavities of mice, and, following gentle massage, the fluid was aspirated using a 6 cc plastic syringe fitted with an 18-gauge needle. Cells were centrifuged at 500g for 5 min and resuspended in Eagle's Minimum Essential Medium with additives to a concentration of 10^6/ml. Sterile 8-well microscope slides were put into tissue culture dishes and 50 ul of the macrophage suspension was pipetted into each well. Macrophages were firmly attached after 2 hrs in 5% CO_2 humidified air at 37°C. At this time, the slides were flooded with 5 ml of fresh MEM containing 10^6 leishmania promastigotes/ml. After a 2 hr infection period, slides were washed vigorously and placed into fresh culture dishes.

Intracellular parasites were permitted to replicate at 35°C for 48 hrs, after which time slides were washed with phosphate-buffered saline, pH 7.2, air-dried for 10 min, and placed in a -70°C freezer until use.

Production of Amastigotes In Vivo. One ml of promastigotes from a stationary phase culture of L. mexicana amazonensis (GML 111) was washed extensively in Medium-199 without additives by centrifugation at 10,000 g for 2 min in 1 ml microfuge cups. This strain has been shown to retain its infectivity for mice even after long periods of culture (unpublished observations). Approximately 10^5 promastigotes in 0.1 ml of media were inoculated into the footpads of 8-week old Balb/c mice. One month later, all mice displayed minimal lesions with no visible evidence of cutaneous metastases. When the nodule had reached a 1 cc diameter but had not yet ulcerated, mice were sacrificed, and the footpad and draining inguinal lymph node were removed and cut into 2 mm cubes. Several cubes were placed into parasite culture media and the remainder were prepared for frozen sections. Cubed specimens were embedded in OCT Compound and snap frozen in liquid nitrogen. The biopsies were sectioned at 5-6 um thickness, and serial sections were placed in successive wells of 8-well microscope slides. Sections were fixed briefly in methanol and stored at -70°C.

Monoclonal Antibodies. Monoclonal antibodies were produced by the fusion of P3X63-Ag8 plasmacytoma cells with immune splenocytes from mice inoculated subcutaneously with promastigotes which had been repeatedly frozen (-70 C) and thawed (40 C), then disrupted by ultrasonication. Isolates used for immunization were WR 222 (Fusions H and T) and GML 1 (Fusion G). Additional fusions were performed using splenocytes from a mouse immunized to purified WR 222 promastigote

membranes isolated by the method of Dwyer (12) (Fusion U) and from a mouse inoculated with WR 303 promastigotes dissected from the gut of the sandfly vector. The fusions were carried out as previously described (13-15). Antibody secreting hybrids were screened by indirect immunofluorescence (IFA) or enzyme-linked immunosorbent assays (Elisa) using air-dried promastigotes bound to 8-well microscope slides or poly-L-lysine treated 96-well polyvinyl chloride microtitre plates (16). Positive cultures were expanded and cloned in soft agarose.

Amastigote Immunohistochemistry. Monoclonal antibodies with high-titred reactivity for surface membranes of homologous promastigotes by IFA or Elisa were tested for reactivity against leishmania amastigotes produced in vitro by the infection of isolated macrophages or in vivo by the inoculation of mouse footpads. Frozen sections and infected macrophages on 8-well slides were stained for indirect immunofluorescence as described and for light microscopic examination using a rapidly staining polychromatic dye (Stat Stain, VWR Scientific, Baltimore, Md.). Negative controls included infected cells and tissues stained with an irrelevant monoclonal antibody and uninfected cells and tissues stained with leishmania-specific antibodies.

Indirect Immunofluorescent Antibody Assay (IFA). Cultured promastigotes (500/well), amastigote-infected cultured macrophages and frozen sections of infected tissue on 8-well microscope slides served as the substrates for antibody detection. Twenty-five ul of undiluted hybridoma supernatant fluid was pipetted onto each well. After 30 min the slides were rinsed 3 times in PBS and a 1/50 dilution of affinity-purified FITC-labelled goat anti-mouse serum in 0.25% Evans Blue in PBS was added. After another 30 min the slides were washed, mounted with

buffered glycerol containing 0.1% (w/v) p-phenylenediamine to reduce quenching (17), and examined with a Zeiss Photomicroscope III adapted for epifluorescence. Fluorescent intensity was graded subjectively on a 0-4+ scale.

Western Blot Analysis. Promastigote-specific antigens were identified by electrophoretic separation of purified promastigote membranes by SDS-Page using a 12.5% acrylamide concentration and the discontinuous buffer system of Laemmli (18). Molecular weight standards were phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). Resolved proteins were electrophoretically transferred to strips of nitrocellulose paper using the buffer system of Towbin et al. (19). Strips were blocked for 30 min in 3% BSA in PBS, placed directly in undiluted hybridoma supernatant fluid and incubated for 2 hrs at room temperature with gentle rocking. Bound antibody was detected using an ^{125}I-labelled goat anti-mouse serum diluted to 30,000 cpm/ml in PBS containing 1% BSA. Between incubation steps, strips were washed 4 times in large volumes of PBS-0.05% Tween 20. Autoradiographs were developed for 3-5 days at -70°C using Kodak X-Omat AR film and a calcium tungstate intensifying screen.

RESULTS

Two monoclonal antibodies, 83T-5C6 and 83T-4D7, recognized antigens which were unique to the L. mexicana promastigote membrane. These antigens appeared as triplet bands on autoradiographs of immunostained nitrocellulose blots. Antigens recognized by 83T-5C6 were distinct bands of MW 48, 46 and 37,000, whereas those identified by 83T-4D7 appeared as 3 tightly migrating bands with molecular weights ranging from 38-42,000 (Fig. 1). The remaining monoclonal antibodies bound equally well, by indirect immunofluorescence, to surface membranes from both promastigotes and amastigotes, thus indicating a high degree of antigenic homology between the two life cycle forms.

Promastigotes representing all 8 isolates rapidly parasitized murine macrophages in vitro and, after a 48 hr. replication period, were present in large numbers as amastigotes within macrophage parasitophorous vacuoles. The specifities of the monoclonal antibodies which bound to amastigotes are summarized in Table I. 83H-2D6 and 84C-5B2 were positive on all amastigotes tested, with the exception of the Peruvian isolate of L. b. braziliensis, GML 18. Most of the remaining antibodies bound to amastigotes of all three L. mexicana isolates. Two antibodies, 83C-8B3 and 83T-3E7, were non-reactive with the L. mexicana rodent isolate, GML 111. 83G-8B10 is specific for the GML 1 isolate of L. b. panamensis. Seven of the monoclonal antibodies which were reactive toward L. mexicana also recognized antigens present on amastigote surface membranes of L. b. guyanensis, indicative of the significant level of antigenic cross-reactivity previously observed between these isolates (11). As demonstrated in Figs. 2A and 2B,

amastigotes generated by the in vitro infection of macrophages, which were readily visualized by routine histological stains, could also be speciated on the basis of their reactivity with monoclonal antibodies, as observed by indirect immunofluorescent microscopy.

Footpad sections of GML 111 infected mice showed the typical diffuse mononuclear infiltrate of histiocytes, epitheloid cells, plasma cells and lymphocytes into the dermis with some evidence of fibrosis (Figs. 3A-B). Histiocytes and epitheloid cells were the predominant cell type. Histiocytes were nonvacuolated, some heavily infected with amastigotes. A few extracellular amastigotes could be seen. No organisms were observed in the deep dermis or fat tissue. Inguinal lymph nodes were greatly enlarged, with evidence of germinal center activation and paracortical hypertrophy. Infected macrophages were generally located in the marginal or cortical sinuses, which were often enlarged (Fig 4A-B), but isolated parasite-laden macrophages were occasionally seen within the dense cortical tissue.

Indirect immunofluorescence proved to be an excellent method for the identification of amastigotes within tissue sections of biopsied material. The contrast of the brightly fluorescing organisms against the red background produced by the counterstain permitted easy identification, even when the number of parasites was extremely small. Minimal tissue autofluorescence was observed, and this was confined to the fibrous components of the dermal lesions. Air-dried cells or sections could be left at room temperature for up to a week, or stored at -70°C for up to one year with little observable loss of reactivity. In addition, the edges of stained and mounted slides could be sealed with nail polish and stored for up to 6 months in the dark at 4°C with

little quenching of fluorescence. Figs. 5A-B and 6A-B display positive immunofluorescent identification of amastigotes in tissue sections through a footpad nodule and a draining lymph node, respectively. On the basis of their reactivity with L. mexicana-specific monoclonal antibodies, no differences in antigenicity were observed between amastigotes produced in vitro and those from infected animals.

Although promastigotes were readily cultured from footpad specimens and could be visualized within a period of 24 hours, promastigotes were not observed in cultures of lymph node biopsies until the 10 days following their inoculation into media.

DISCUSSION

Accurate diagnosis of the Leishmaniases has been plagued by problems of antigenic cross-reactivity among the different species. A definitive diagnosis generally depends on culture of the organism from the suspect lesion followed by biochemical procedures such isoenzyme analysis (20, 21), characterization of kinetoplast DNA in terms of its buoyant density in a caesium chloride gradient (22), chromatographic analysis of total neutral and polar lipids (23), radiorespirometry (24), electrophoretic characterization of kinetoplast DNA restriction fragments (25, 26), or by the hybridization of radiolabelled kDNA probes with kDNA from unknown organisms (8, 9, 25). Although parasite identification is possible using any of these techniques, only the latter has eliminated the necessity for large numbers of parasites. Using touch blots of cutaneous lesions, or dot blots of cultured promastigotes, as few as 10^3 parasites can be detected. Unfortunately, the total incubation period, including the final autoradiographic development step, can be as long as 65 hours.

Monoclonal antibodies against leishmania promastigotes can be used in a variety of immunochemical techniques to differentiate the numerous species and sub-species (27-31). Although there is considerable antigenic homology between the extracellular and intracellular forms, the existence of stage-specific membrane components must be considered when using monoclonal antibodies generated against either stage. It has been suggested that promastigote-specific surface antigens may be involved in the attachment of the parasite to the gut wall of the

sandfly vector or in subsequent macrophage recognition and parasitization (32), but they are of no value in the immunohistochemical identification of parasites once they have converted to the amastigote form. Promastigote-specific antibodies were eliminated from our diagnostic panel by screening for reactivity against amastigotes produced by the in vitro infection of isolated macrophages. Two antibodies recognized promastigote-specific antigens which appeared as triplet bands when characterized by Western blot analysis.

Others have used polyvalent antisera to successfully localize leishmania amastigotes in biopsy specimens using indirect immunoperoxidase techniques (33, 34). We have utilized this procedure to identify amastigotes in tissue sections using species-specific monoclonal antibodies (not shown). However, this procedure is time consuming, it requires the use of a carcinogenic substrate, and subsequent interpretation is difficult. Immunoperoxidase protocols can be very useful, however, in cases where a permanent record is desired, in retrospective studies of paraffin-embedded tissues, or where fluorescent microscopy is unavailable. Because of ease in specimen handling (air-drying, brief fixation, or freezing), rapidity of performance (1 hour staining time) and ease of interpretation, we feel that indirect immunofluorescence using species-specific monoclonal antibodies in combination with Giemsa (or another modified Romanowsky-type stain) is the preferred method of parasite identification in tissue sections from suspected cases of cutaneous leishmaniasis. Such histological preparations should then be followed up by culture/biochemical methods where confirmation is desired.

REFERENCES

1. Zuckerman A: Parasitological review: Current status of the immunology of blood and tissue protozoa. I. Leishmania. Exp Parasitol 1975, 38:370-400

2. Convit J, Pinardi ME, Rondon AJ: Diffuse cutaneous leishmaniasis: A disease due to an immunological defect of the host. Trans Roy Soc Trop Med Hyg 1972, 66:603-610

3. Anthony RL, Christensen HA, Johnson CM: Micro enzyme-linked immunosorbent assay (Elisa) for the serodiagnosis of New World Leishmaniasis. Am J Trop Med Hyg 1980, 29:190-194

4. Pappas MG, McGreevy PB, Hajkowski R, Hendricks LD, Oster CN, Hockmeyer WT: Evaluation of promastigote and amastigote antigens in the indirect fluorescent antibody test for American Cutaneous Leishmaniasis. Am J Trop Med Hyg 1983, 32:1260-1267

5. Johnson PT, Hertig M: Behavior of Leishmania in Panamanian phlebotomine sandflies fed on infected animals. Exp Parasitol 1970, 27:281-300

6. Lainson R, Ward RD, Shaw JJ: Leishmania in phlebotomid sandflies: VI. Importance of hindgut development in distinguishing between parasites of the Leishmania mexicana and Leishmania braziliensis complexes. Proc Roy Soc Lond 1977, 199:309-320

7. Walton BC, Intermill RW, Hajduk ME: Differences in biological characteristics of three Leishmania isolates from patients with espundia. Am J Trop Med Hyg 1977, 26:850-855

8. Wirth DF, McMahon-Pratt, D: Rapid identification of Leishmania species by specific hybridization of kinetoplast DNA in cutaneous lesions. Proc Natl Acad Sci 1982, 79:6999-7003

9. Barker DC, Butcher J: The use of DNA probes in the identification of leishmanias: discrimination between isolates of the Leishmania mexicana and Leishmania braziliensis complexes. Trans Roy Soc Trop Med Hyg 1983, 77:285-297

10. Preston PM, Dumond DC: Immunology of clinical and experimental leishmaniasis, Immunology of parasitic infections. Edited by S Cohen, EH Sadum. Oxford, Blackwell Scientific Publications, 1976, pp 167-202

11. Anthony RL, Williams KM, Sacci JB, Rubin DC: Subcellular and taxonomic specificity of monoclonal antibodies to New World Leishmania: Am J Trop Med Hyg 1985, in press

12. Dwyer DM: Isolation and partial characterization of surface membranes from Leishmania donovani promastigotes. J Protozool 1980, 27:176-182

13. Kennett RH: Cell fusion, Methods in Enzymology, Vol 68, Edited by WR Jakoby, H Pastan. New York, Academic Press, New York 1979, pp 345-359

14. Kohler G, Milstein C: Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 1975, 256:495-497

15. Oi VT, Herzenberg LA: Immunoglobulin-producing hybric cell lines, Selected Methods in Cellular Immunology, Edited by BB Mishell, SM Shiigi, San Francisco, WH Freeman, 1980, pp 351-372

16. Kennett RH: Enzyme-linked antibody assay with cells attached to polyvinyl chloride plates. Monoclonal Antibodies. Hybridomas: A New Dimension in Biological Analysis, Edited by RH Kennett, TJ McKearn, KB Bechtol, New York, Plenum Press, 1980, pp 376-377

17. Johnson GD, de C Noguera Arujo GM: A simple method of reducing the fading of immunofluorescence during microscopy. J Immunol Methods 1981, 43:349-350

18. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970, 227:680-685

19. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979, 76:4350-4354

20. Kreutzer RD, Christensen HA: Characterization of Leishmania spp by multiple isozyme electrophoresis. Am J Trop Med Hyg 1980, 29:199-208

21. Kreutzer RD, Semko ME, Hendricks LD, Wright N: Identification of Leishmania spp by multiple isozyme analysis. Am J Trop Med Hyg 1983, 32:703-715

22. Barker DC, Arnot, DE: Biochemical identification of cutaneous leishmanias by analysis of kinetoplast DNA. I. Ultrastructure and buoyant density analysis. Mol Biochem Parasitol 1981, 3:33-46

23. Beach DH, Holz GG Jr, Anekwe GE: Lipids of Leishmania promastigotes. J Parasitol 1979, 65:203-216

24. Decker JE, Schrot JR, Levin GV: Identification of Leishmania spp by radiorespirometry. J Protozool 1977, 24:463-479

25. Arnot DE, Barker DC: Biochemical identification of cutaneous leishmaniasis by analysis of kinetoplast DNA. II. Sequence homologies in Leishmania kDNA. Mol Biochem Parasitol 1981, 3:47-56

26. Jackson PR, Wahlhieter JA, Jackson JE, Sayles P,Diggs, CL, Hockmeyer WT: Restriction endonuclease analysis of Leishmania kinetoplast DNA characterizes parasites responsible for visceral and cutaneous disease. Am J Trop Med Hyg 1984, 33:808-819

27. Handman E, Hocking RE: Stage-specific, strain-specific, and cross-reactive antigens of Leishmania species identified by monoclonal antibodies. Infect Immun 1982, 37:28-33

28. Jaffe CL, McMahon-Pratt D: Monoclonal antibodies specific for Leishmania tropica. I. Characterization of antigens associated with stage- and species-specific determinants. J Immunol 1983, 131: 1987-1993

29. McMahon-Pratt D, Bennett E, Grimaldi G, Jaffe CL: Subspecies- and species-specific antigens of Leishmania mexicana characterized by monoclonal antibodies. J Immunol 1985, 134:1935-1940

30. McMahon-Pratt D, Bennett E, David JR: Monoclonal antibodies that distinguish subspecies of Leishmania braziliensis. J Immunol 1982, 129:926-927

31. McMahon-Pratt D, David JR: Monoclonal antibodies that distinguish between New World species of Leishmania. Nature 1981, 291:581-583

32. Dwyer DM, Gottlieb M: The surface membrane chemistry of Leishmania: Its possible role in parasite sequestration and survival. J Cell Biochem 1983, 23:35-45

33. Sells PG, Burton M: Identification of Leishmania amastigotes and their antigens in formalin fixed tissue by immunoperoxidase staining. Trans Roy Soc Trop Med Hyg 1981, 75:461-468

34. Livni N, Abramowitz A, Londner M, Okon E, Morag A: Immunoperoxidase method of identification of Leishmania in routinely prepared histological sections. Virchows Arch [Path Anat] 1983, 401: 147-151

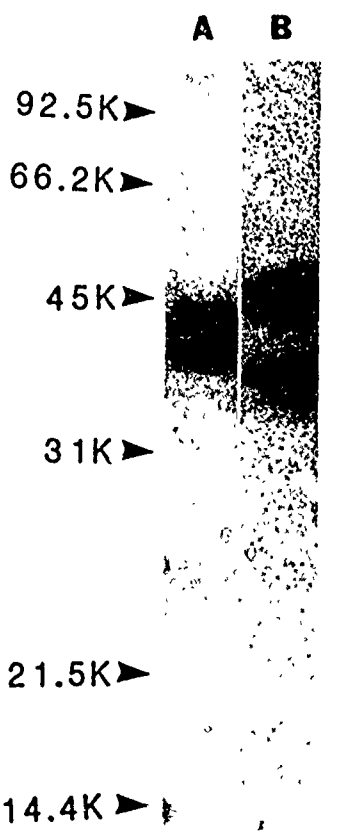
TABLE I

REACTIVITY OF MONOCLONAL ANTIBODIES WITH AMASTIGOTES OF NEW WORLD LEISHMANIA SPECIES

MONOCLONAL ANTIBODY	*L. m. mexicana* (WR 222)	*L. m. amazonensis* (WR 303)	*L. m. amazonensis* (GML 111)	*L. species* (WR 359)	*L. b. guyanensis* (WR 390)	*L. b. panamensis* (GML 1)	*L. b. braziliensis* (WR 508)	*L. b. braziliensis* (GML 18)
83H-2D6	4+	4+	4+	4+	4+	4+	4+	-
83L-2D3	4+	4+	4+	4+	4+	-	4+	-
83L-5G9	4+	4+	4+	-	4+	-	-	-
83T-3E7	4+	4+	-	-	-	-	-	-
83T-3E9	4+	4+	4+	-	4+	-	-	-
83T-4D7*	-	-	-	-	-	-	-	-
83T-5C6*	-	-	-	-	-	-	-	-
83T-9D3	4+	4+	4+	-	-	-	-	-
83T-10E4	4+	4+	4+	-	4+	-	-	-
83U-2F11	4+	4+	4+	-	4+	-	-	-
83U-5F2	2+	2+	2+	-	-	-	-	-
83U-7D5	4+	4+	4+	-	-	-	-	-
83U-9B3	4+	4+	4+	-	-	-	-	-
84C-4F4	4+	4+	4+	-	4+	-	-	-
84C-5B2	4+	4+	4+	4+	4+	4+	4+	-
84C-8B3	4+	4+	-	-	-	-	-	-
84C-8C7	4+	4+	4+	-	4+	-	-	-
84G-8B10	-	-	-	-	-	4+	-	-

* Specific for L. mexicana promastigote membrane.

Fig. 1: Western blot identification of L. mexicana mexicana promastigote-specific antigens. Monoclonal antibodies recognizing these determinants were 83T-4D7 (Lane A) and 83T-5C6 (Lane B).



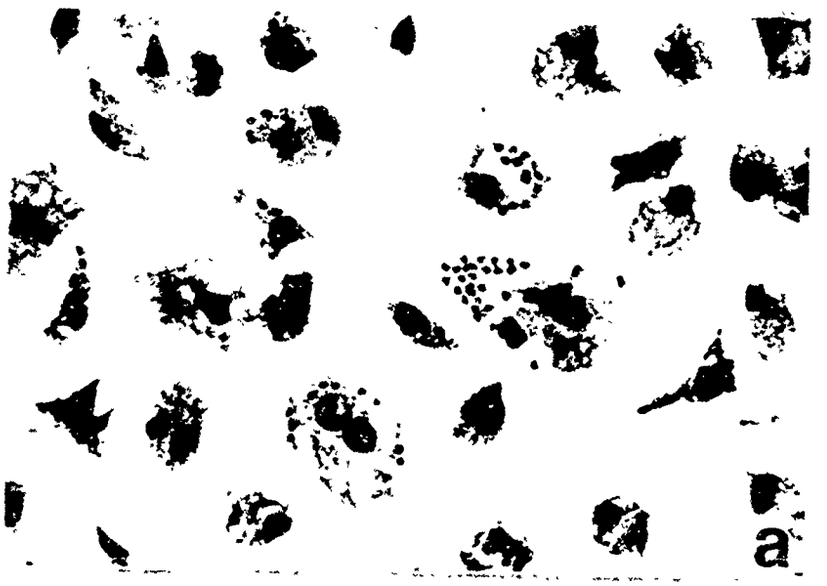


Fig. 2: Visualization of amastigotes within cultured peritoneal macrophages 48 hrs after in vitro infection with Leishmania promastigotes. Numerous amastigotes are seen in histological preparations (A), which could then be identified by indirect immunofluorescence using species-specific monoclonal antibodies (B). Magnification = 800x.

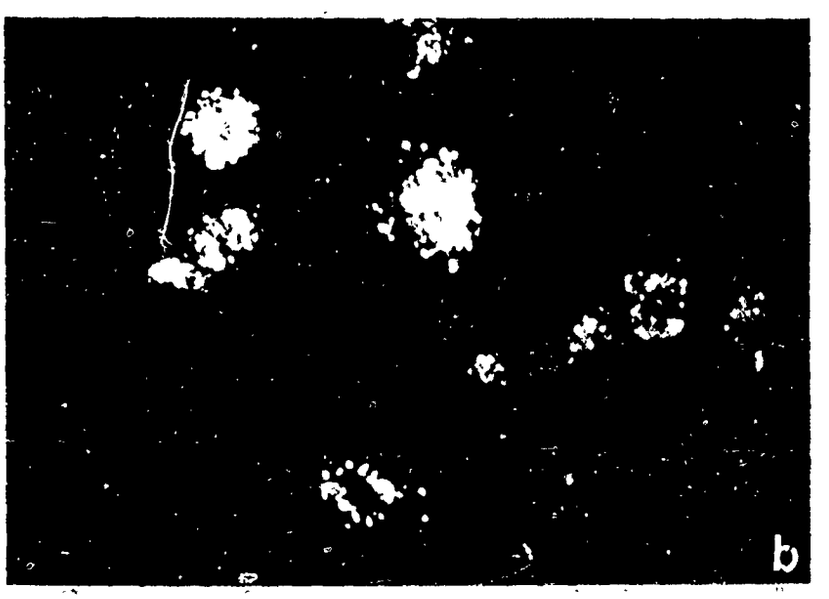
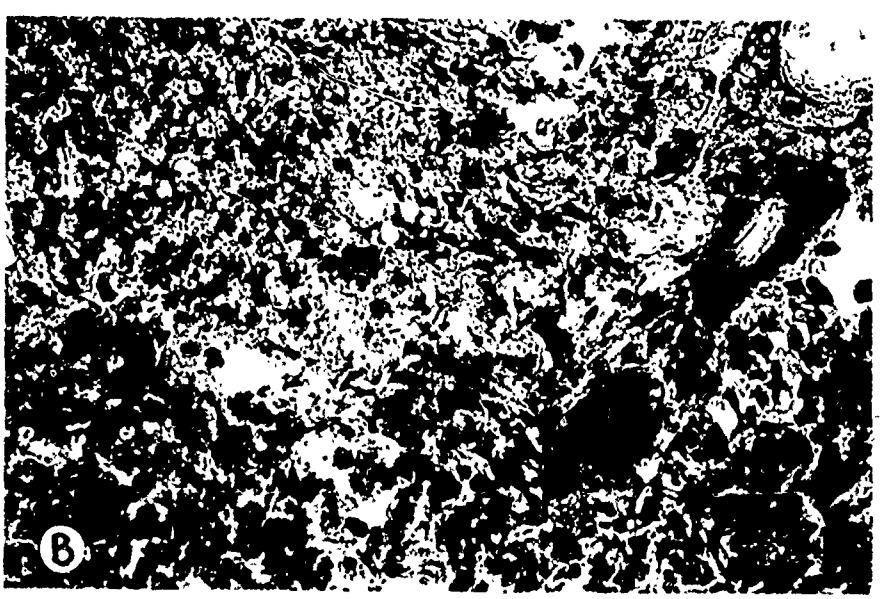


Fig. 3: One-month old footpad lesion in L. m. amazonensis-infected Balb/c mouse. Heavily parasitized macrophages are non-vacuolated and localized to the superficial dermis (A). Some extracellular amastigotes are also observed (B). Magnification = 600x.



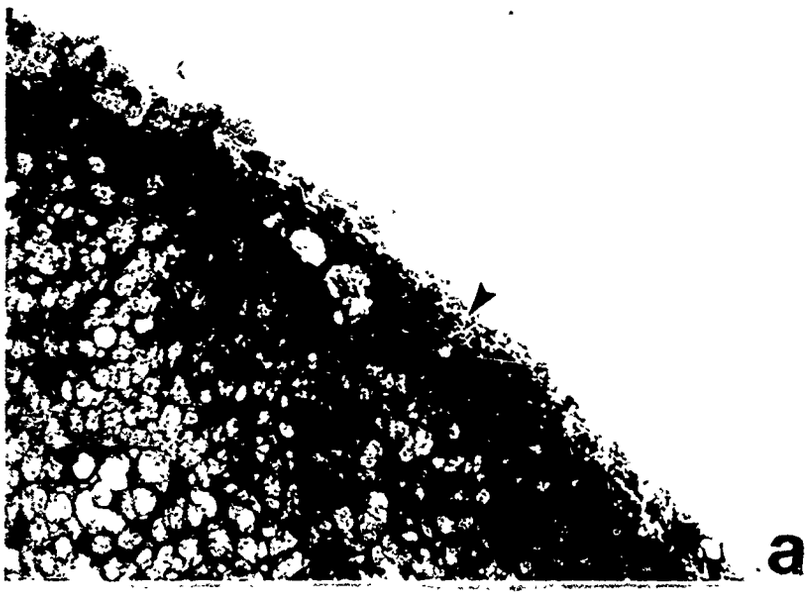
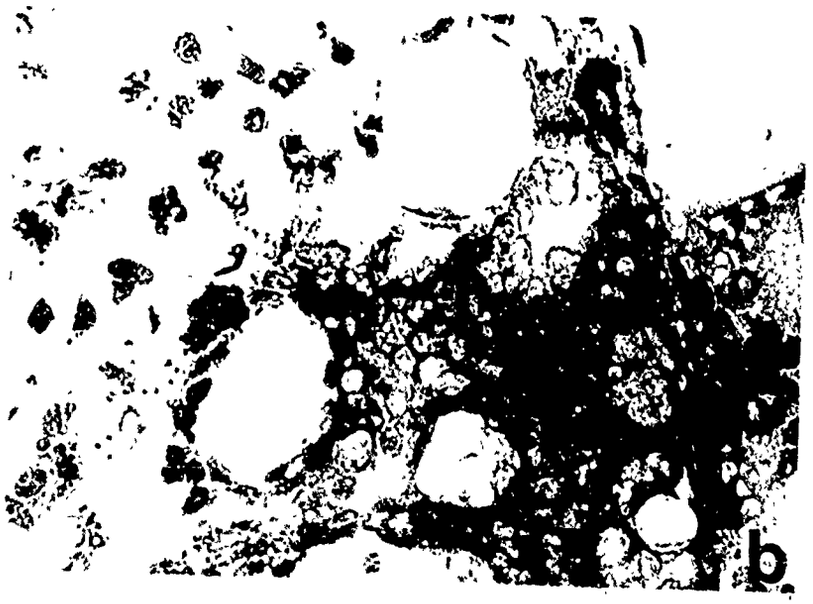


Fig. 4: Popliteal lymph node of L. m. amazonensis infected Balb/c mouse, 1 month post infection. Heavily parasitized macrophages are seen in the cortical and paracortical sinuses (A-B), which were often greatly enlarged. Magnification = 600x.



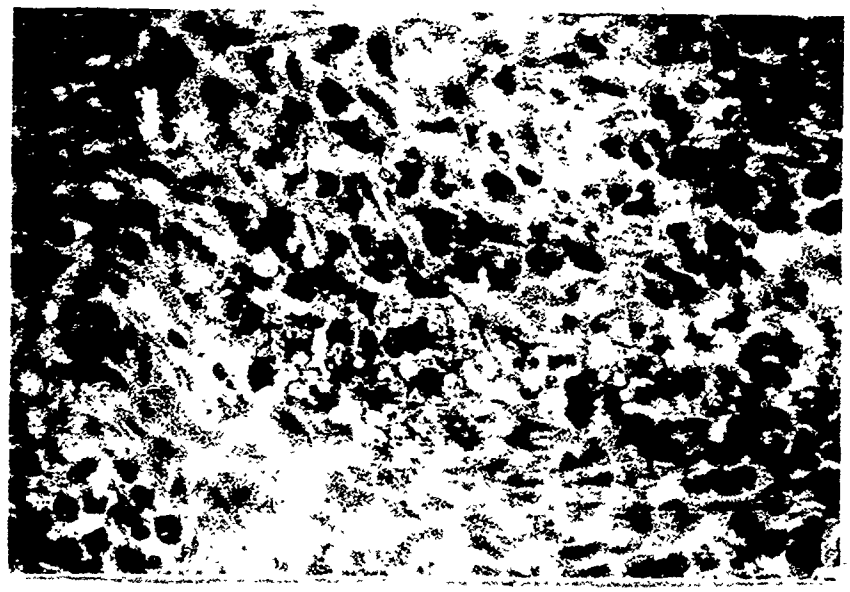
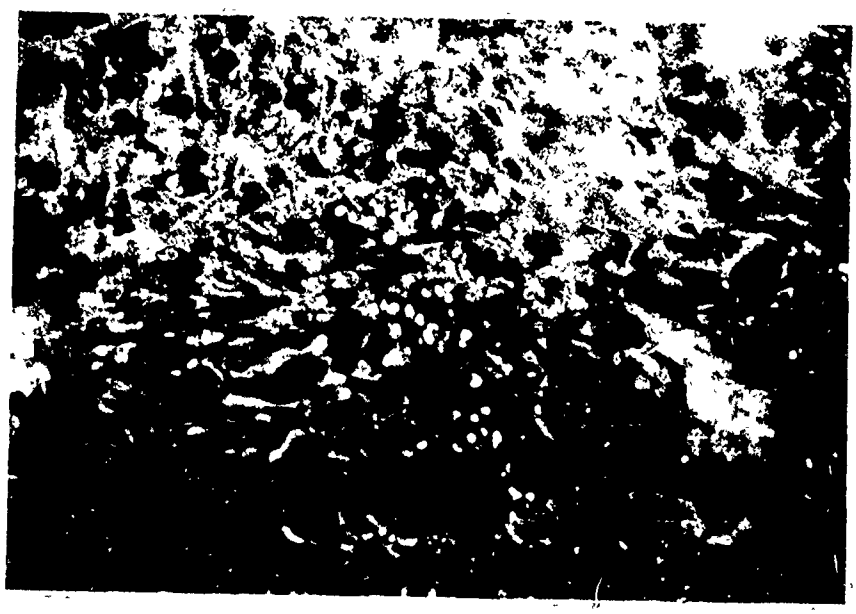


Fig. 5: Immunofluorescent identification of amastigotes in the footpad nodule of a Balb/c mouse 1 month after inoculation with L. m. amazonensis promastigotes. Frozen sections stained with L. mexicana-specific monoclonal antibody 83U-7D5 as described in Materials and Methods. Most amastigotes are localized to dermal macrophages, but extracellular amastigotes are often seen (A-B). Magnification = 900x.



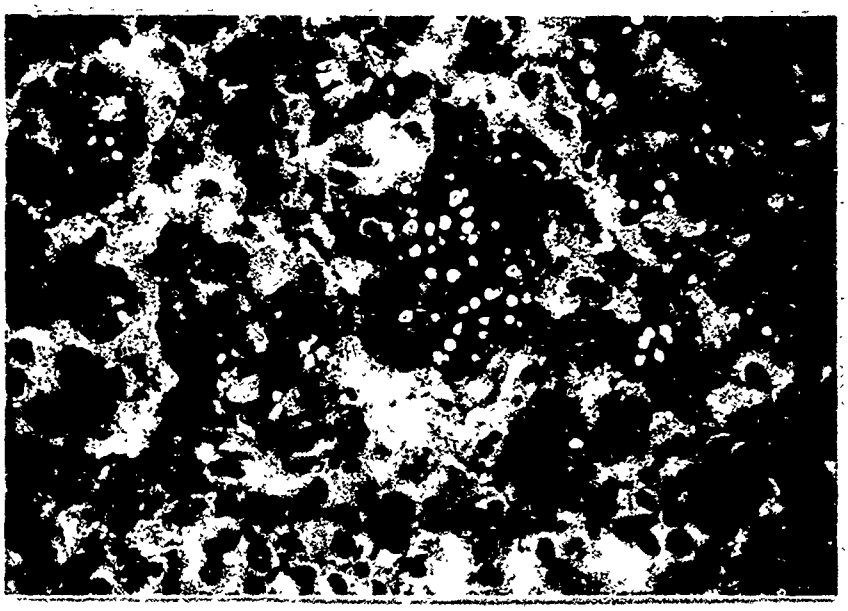
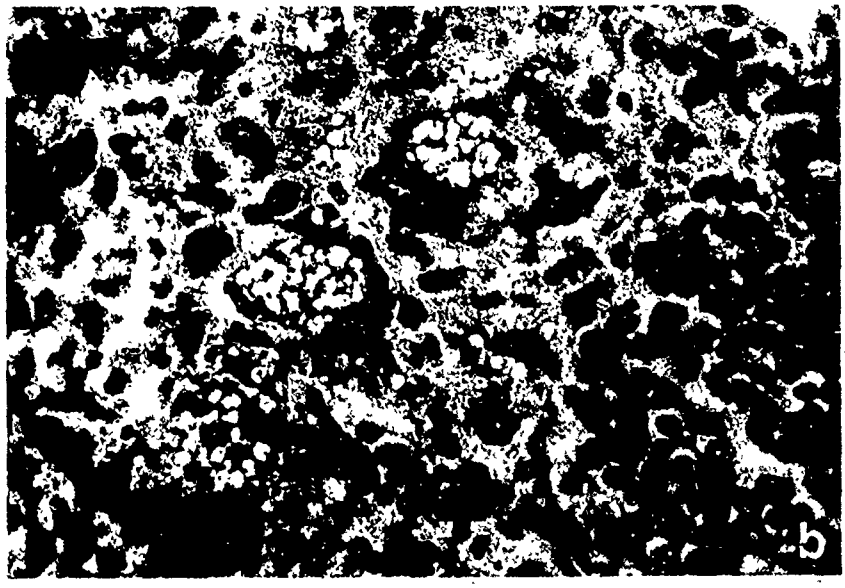


Fig. 6: Immunofluorescent localization of amastigotes in the popliteal lymph node of a Balb/c mouse 1 month after inoculation with L. m. amazonensis promastigotes. Frozen sections stained with monoclonal antibody 84C-4F4 as described in materials and methods. Numerous amastigotes within macrophages are easily visualized (A-B). Magnification = 900x.



Serodiagnosis of Leishmaniasis

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

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Trypanosoma cruzi and the Leishmania, L. braziliens
.. mexicana , possess several common surface antigens whic
cross-react serologically (1-3). Moreover, both of these c
American trypanosomiasis (Chagas' disease) and New World
Leishmaniasis, are indigeneous to many of the same villages
and Central America. Consequently, results of seroepidemic
surveys, derived from the reactivity of serum antibodies wi
parasites or crude extracts, must be interpreted with cauti

The advent of hybridoma-monoclonal antibody technology
possible to identify both genus-specific and species-specif
among the parasites of the Family Trypanosomatidae (4-6).
the use of these antibodies as ligands in immunoaffinity
chromatography now permits the purification of the reactive
which are prerequisites for the development of the species-
assays for the serodiagnosis of human diseases (7-8). As p
continued efforts to dissect the complex antigenic profiles
trypanosomatids, we have generated several leishmania-speci
monoclonal antibodies. We now report on the use of one suc
for the recovery of a leishmania-specific surface antigen w
used in an enzyme linked immunosorbent assay (ELISA), is hi
reactive with sera from human leishmaniases but non-reactiv
% of thesera from Chagas' disease.

Materials and Methods:

Parasites:

Promastigotes of Leishmania braziliensis panamensis (WRAIR-470) and epimastigotes of Trypanosoma cruzi (Tulahuen strain) were maintained at 26C in Medium 199 containing 12 mM HEPES, 20 mM L-glutamine, 20 % heat inactivated fetal bovine serum, 50 units penicillin/ml, 50 units streptomycin/ml and 50 ug gentamycin/ml. Parasites were collected in their stationary phase and washed, three times with phosphate buffered saline (PBS), by centrifugation.

Methods for the extraction of the parasite antigens in CHAPS have been outlined elsewhere (9).

Sera:

The serum collection was comprised of specimens of individuals from whom leishmania parasites had been isolated but whom had not yet begun chemotherapy. The Chagasic sera were from persons whose disease had been confirmed by xenodiagnosis and /or clinical impression. The sera from cases of Toxoplasmosis were identified by indirect fluorescent microscopy using a T. gondii substrate and, as with the disease-free specimens, were obtained from persons without any history of exposure to the trypanosomatids.

Monoclonal antibodies:

Immunization schedules, procedures for cells fusion and methods of screening hybridomas for production of monoclonal antibodies

(Fusion L) have be described in previous reports (9-10).

One hundred thousand hybridoma cells from clone 83L-5G9 were introduced into the peritoneal cavity of a Balb/c mouse which had been primed with Pristane (11). After two weeks, the resultant ascitic fluid was collected and the immunoglobulin was purified on an Affi-gel Blue column (Biorad Laboratories, Richmond, California) acccording to the protocol developed by Bruck (12). The immunoglobulin fraction was dialysed against 0.02 M Tris-HCl buffer, pH 7.2, and lyophilized. The protein content was determined by the Bio-Rad 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 (Biorad Laboratories, Richmond, California), at 4C for 18 hours. All remaining active ester sites were then blocked by the addition of 200 microliters of 0.1 M glycine ethyl ester. The gel was packed into a 0.7 x 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 equilibrated with the pH 7.2 glycine buffer.

The CHAPS extract of the WRAIR 470 promastigotes (1mg/ml) was applied to the columnn 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 4C. Unbound proteins were then eliminated by elution in the 0.05 M glycine budfer 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 buffer.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE):

The purity and the molecular weight of the 83L-5G9 leishmanial antigen were verified by SDS-PAGE (13), using a 12.5 % slab gel (1.5 mm x 80 mm x 60 mm).

Immunoblotting:

Proteins, resolved by SDS-PAGE, were electrophoretically transferred to a sheet of nitrocellulose. Precise procedures have been outlined by Towbin (14). After electroblotting for 2 hrs at 1 amp, the sheet was treated with 5% bovine serum albumin (BSA) for 30 min. This treatment prevents the non-specific binding of proteins which were not reactive with the resolved antigen.
The sheet was next placed in medium supporting the 83L-5G9 hybridoma for 18 hrs at 4C. After extensive washing in phosphate buffered saline (PBS), pH 7.2, containing 0.05% Tween 20, the sheet was submerged, for 2 hr at room temperature, in a 1:1000 diultion 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 (ELISA):

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, Virginia). After an overnight incubation at 4 C, the

wells were emptied by inversion and 100 microliters of human serum, diluted 1:20 in PBS-Tween, was added. Incubation continued for 2hr at room temperature. Unreactive serum was eliminated by 3 washes in PBS-Tween 20, and 100 microliters of a 1:500 dilution of goat anti-human immunoglobulin serum labeled with alkaline phoshastase was added. Incubation continued for 2 hrs 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 adddition of 50 micoliters of 3 M NaOH and the resultant color change was measured, spectrophotometrically, at 405 nm in the Titertek Multiscan.

Results:

Figures 1a and 1b are immunofluorescent micrographs confirming the reactivity of monoclonal antibody 83L-5G9 (IgG,1) with a dominant antigen on the surface membrane of promastigotes and amastigotes of Leishmania braziliensis panamensis (WRAIR isolate 470). The genus-specificity of this antibody,—as measured by reactivity in the ELISA, is presented in Table 1.

Sequential analyses of the crude CHAPS extract of WRAIR 470 promastigotes by SDS-PAGE, immunoblot and immunoautoradiography verified that the epitope recognized by 83L-5G9 was associated with both a 58 kd and a 31 kd polypeptide (Fig. 2). When this same extract was eluted through the 83L-5G9 affinity column, these reactive polypeptides were recovered in a small but compact peak of the pH 2.5 elution profile (Fig.3). The immunoblot of this fraction, using 83L-5G9 monoclonal antibody as the probe, is presented in Figure 4.

The capability of the purified 83L-5G9 antigen to distinguish the antibody reponse in New World Leishmaniasis from that of American Trypanosomiasis is presented in Figure 5. The distribution in absorbance values between the two set of sera was significant by the F-test ($p < 0.005$). As expected, the purified leishmanial antigen was also non-reactive with all sera from healthy controls as well as with sera which were reactive with Toxoplasma gondii in an indirect immunofluorescent antibody assay.

Discussion:

The controlling element of specificity in all serologic assays designed to detect an specific antibody response is the quality of the antigen. Thus, it is not suprising that that the usefulness of assays which use whole parsites or crude extracts for the diagnosis of the human leishmaniases is hindered by a high frequency of false positive results (1,3). These false positive assays are due to a large array of cross-reactive antigens which are shared by both the pathogenic and non-pathogenic species of the major genera comprising the Family Trypanosomatidae. Moreover, many of the immunogeneic epitopes of the crude extract have been highly conserved throughout phylogeny and thus will react with antibodies elicited in any number of different infectious diseases and/or autoimmune processes (4, 15).

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 these antibodies can now been used to identifiy species-specific epitopes and, perhaps most importantly, to purify the respective polypeptide from complex extracts (7-9). As others have used such polypeptides as antigens in the development of serologic assays for the diagnosis of American trypanosomiasis, we now report on the recovery of a leishmanial specific antigen from the surface membrane of leishmania promastigotes and its use, in enzyme linked immunosorbent assays, for the diagnosis of human disease.

Although the epitope recognized by 83L-5G9 was common to isolates representative of the three species of New World Leishmania, the L.

braziliensis braziliensis (WRAIR - 508) isolate from a mucocutaneous case of disease was non-reactive (Table 1). This very apparent difference in the antigenic composition of isolates which cause cutaneous versus mucocutaneous disease has been noted in a previous report (4). Noteworthy, of course, is the obvious lack of reactivity of 83L-569 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.

The serologic data clearly established that an ELISA, using the affinity purified 83L-563 antigen, had the capacity to identify leishmania-specific antibodies in human sera (Table 5). Although the distribution in the absorbance values of the leishmania sera was significantly different from that of the Chagasic sera, several specimens from this latter group did fall within the positive range (absorbance > .200) Unfortunately, we cannot rule out the possibility that these sera were from individuals who also had a history of exposure to Lesihmania species . It is also most likely that the large 58 kd polypeptide displaying the genus-specific antigen also expresses some non-specific determinants which bind to anti-trypanosomal antibodies.

In summary, the need for a genus-specific serologic assay for the diagnosis of New World Leishmaniasis is very obvious. It now appears that such an assay can now be developed by using genus-specific antigens identified by monoclonal antibodies. The subsequent use of these antibodies, in conjunction with recombinant DNA technology, to recover adequate amounts of sepcies-specific antigens for large scale sero-epidemiologic surveys remains as a top priority goal of our

References:

1. Badaro, R., Reed, S.G., and E.M. Carvalho. 1983. Immunofluorescent antibody test in American visceral leishmaniasis: Sensitivity and specificity of different morphological forms of two Leishmania species. Am. J. Trop. Med. Hyg. 32:480-484.

2. Carmargo, M.E., and C. Rebonato. 1969. Cross-reactivity in immunofluorescence test for Trypanosoma and Leishmania antibodies. A simple inhibition procedure to ensure specific-results. Am.J.Trop.Med.Hyg. 18:500-505.

3. Anthony, R.L., Christensen. H.A. and C.M. Johnson. 1980. Micro enzyme linked immunosorbent assay (ELISA) for the serodiagnosis of New World Leishmaniasis. Am. J. Trop.Med. Hyg. 29:190-194.

4. Anthony, R.L., Williams, K.M., Sacci,J.B., and D.C.Rubin. 1985. Subcellular and taxonomic specificity of monoclonal antibodies to New World Leishmania. Am.J. Trop.Med. Hyg. 34:1085-1094.

5. McMahon-Pratt, d., and J. R. David. 1981. Monoclonal antibodies that distinguish between New World Species of Leishmania . Nature, 291:581-583.

6. Handman, E., and R.E. Hocking. 1982. Stage-specific, strain-specific and cross reactive antigens of Leishmania species identified by monoclonal antibodies2. Infect. Immun. 37: 28-33.

7. Sharma. S.D., Araujo, F.G., and J. S. Remington. 1984. Toxoplasma antigen isolated by affinity chromatography with monoclonal antibody protects mice against lethal infections with Toxoplasma gondii . J. Immunol. 133:2818-2820.

8. Kasper, L.H., M.S. Brad.ey, and E.R. Pfefferkorn. 1981. Identification of stage-specific sporozoite antigens of Toxoplasma gondii by monoclonal antibodies. J. Immunol. 132: 443-449.

9. Williams, K.M., Sacci,J.B., and R.L. Anthony. 1986. Flow cytometric analyses of the effects exerted by monoclonal antibodies on binding and uptake of Leishmania mexicana mexicana promastigotes by murine peritoneal macrophages. Infect. In. (in press).

10. Constantine, N.T., and R.L. Anthony. 1983. Antigenic differentiation of the Kinetoplastids Leishmania braziliensis and Trypanosoma cruzi by means of monoclonal antibodies. J. Protozool. 30:346-350.

11. Oi, V.T., and L.A. Herzenberg. 1980. Immunoglobulin producing hybrid cell lines. Pages 351-372 in B B. Mischell and S.M. Shiigi, eds., Selective Methods in Cellular Immunology . W.H. Freeman Co., San Francisco.

12. Bruck, C., Portelle, D., Glineur, C., and A. Bollen. 1982. One-step purification of mouse monoclonal antibodies from ascitic fluid by DEAE Affigel Blue chromatography. J. Immunol. Meth. 53:313-319.

13. Laemmli, U.K. 1979. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227:660-685.

14. Towbin, H., Staehelin, T., and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets:procedures and some appications. Proc.Natl. Acad. Sci. USA. 76:4350-4354.

15. Boreham, P.F.L. and C.A. Facer. 1974. Autoimmunity in Trypanosome infections. II. Anti-fibrin/fibrinogen (anti-F) autoantibody in Trypanosoma (Trymanozoon) brucei infections in the rabbit. Internat. J. Parasitol. 4:601-607.

TABLE 1

Reactivity of monoclonal antibody 63L-569 with New World Trypanosomatids

Isolate	Absorbance (405 nm)
L. b. panamensis (WRAIR - 470)*	0.345
L. m. mexicana (WRAIR - 222B)	0.413
L. m. amazonensis (WRAIR - 303)	0.340
L. b. peruivana (WRAIR - 140)	0.445
L. d. chagasi (WRAIR - 484)	0.418
L. b. braziliensis (WRAIR - 508)	0.000
Endotrypanum schaudinni	0.000
Trypanosoma cruzi	0.000

FIGURE 1: INDIRECT IMMUNOFLUORESCENCE STAINING OF *L. BRAZILIENSIS PANAMENSIS* PROMASTIGOTE USING MONOCLONAL ANTIBODY 83L-569. B = AMASTIGOTES IN MOUSE PERITONEAL EXUDATE CELLS.

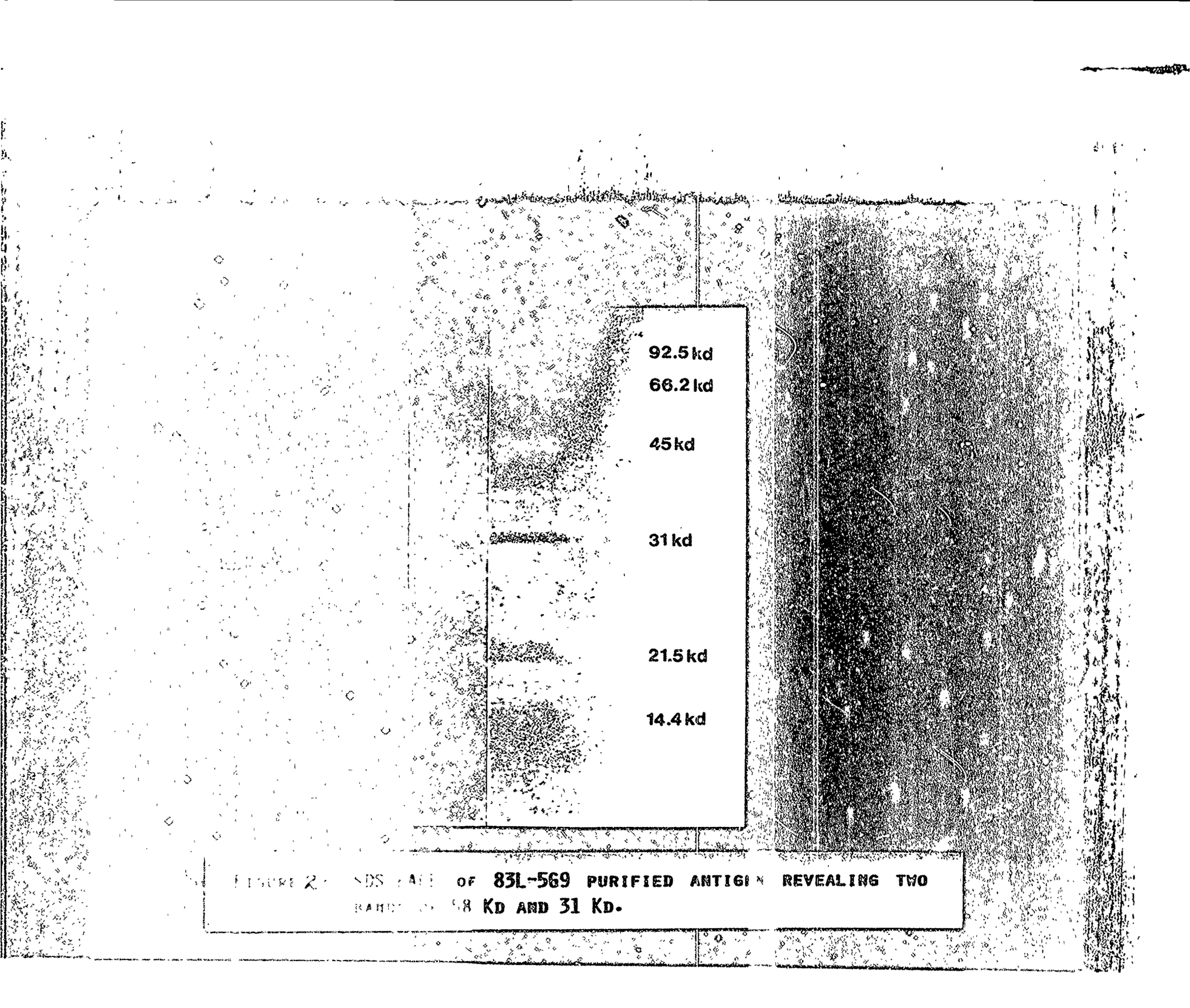
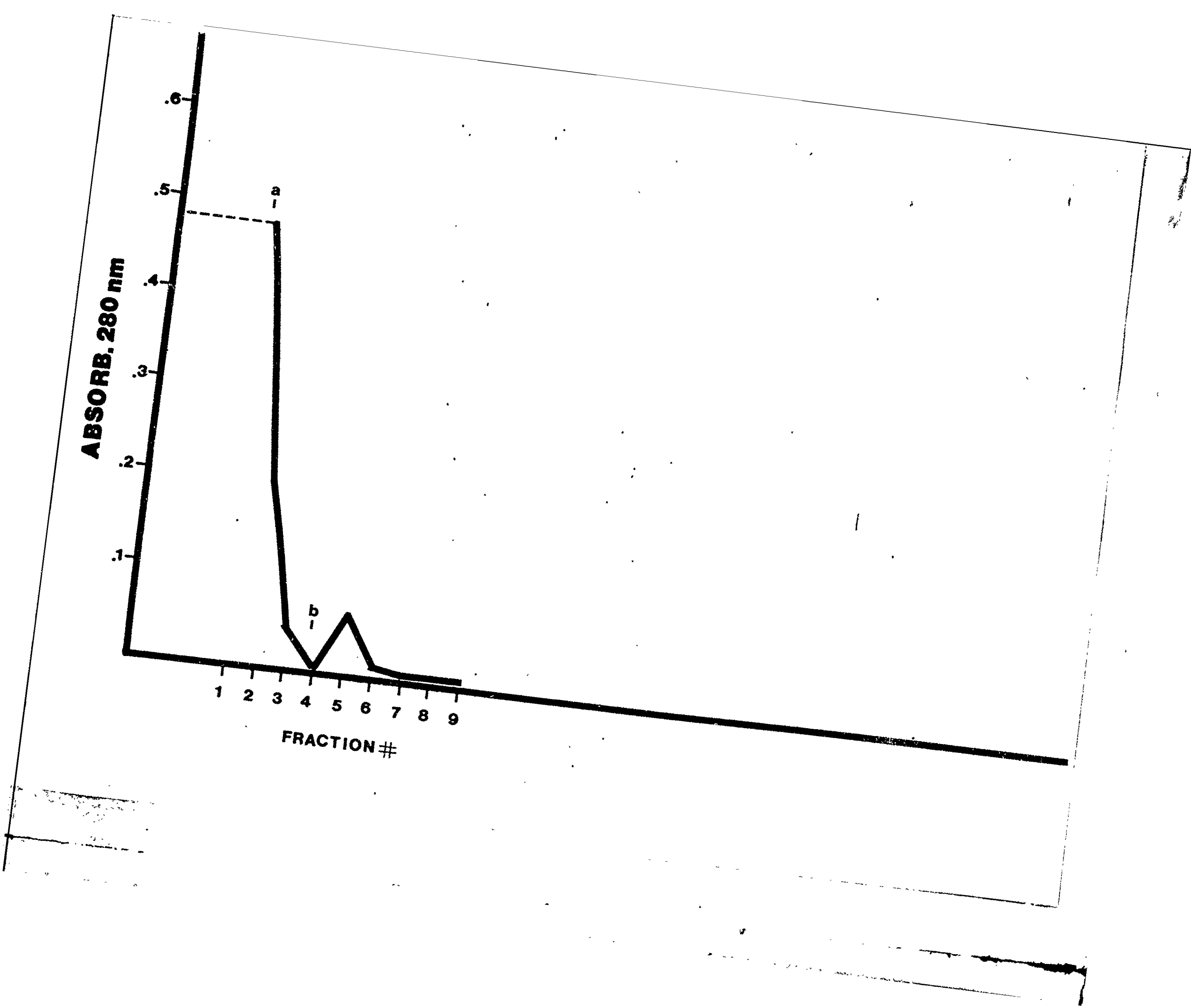
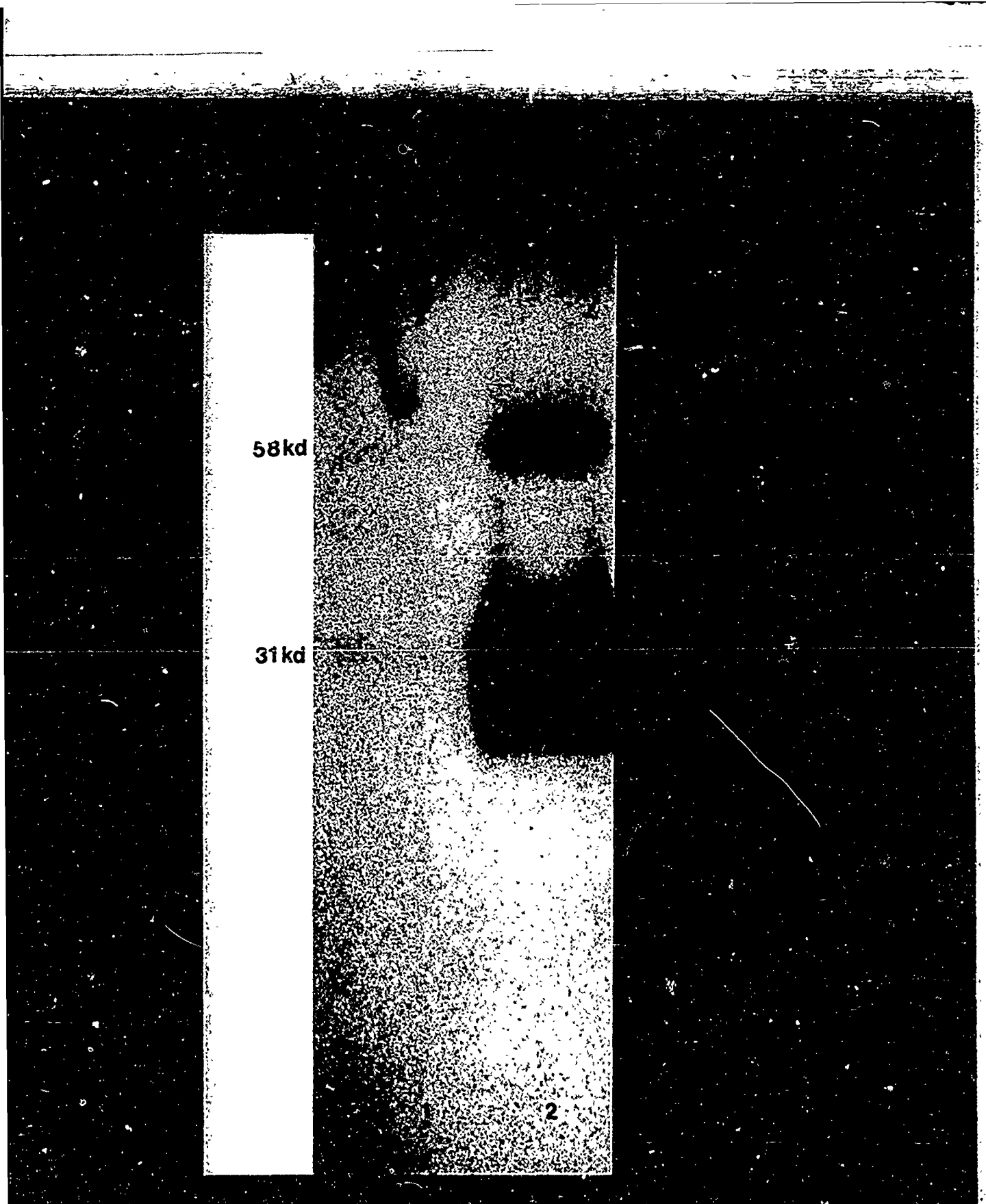


FIGURE 2: SDS PAGE OF 83L-569 PURIFIED ANTIGEN REVEALING TWO BANDS AT 68 KD AND 31 KD.


ABSORB. 280 nm
.6
.5
.4
.3
.2
.1
a
b
1 2 3 4 5 6 7 8 9
FRACTION #



IGURE 4: WESTERN BLOT CHARACTERIZATION OF 83L-569 PURIFIED ANITGEN (LANE 1) AND CRUDE MEMBRANE EXTRACT (LANE 2).

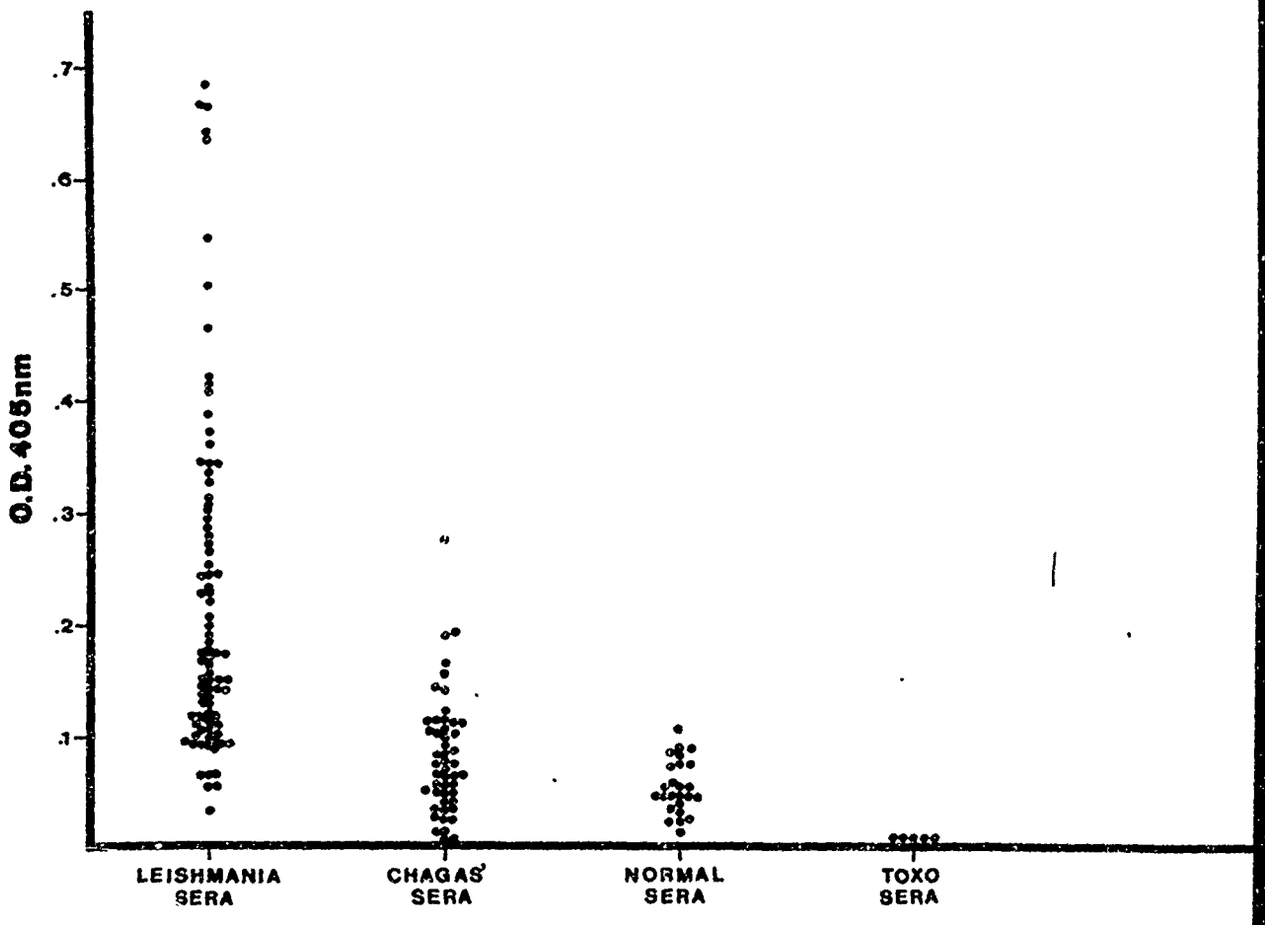


FIGURE 5: ENZYME LINKED IMMUNOSORBENT ASSAY RESULTS OF HUMAN SERA WHEN PURIFIED 83L-569 ANTIGEN IS USED TO COAT THE MICROTITRE PLATE.

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