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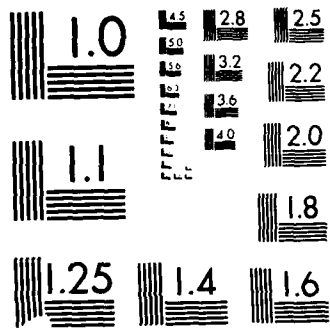
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In vitro and in vivo Studies for  
Development of a Leishmaniasis Vaccine

FINAL REPORT

David J. Wyler, M.D.

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## SUMMARY

The purpose of the work supported by this Contract is to identify antigens of Leishmania that could serve as candidates for an antileishmanial vaccine. To accomplish this goal, monoclonal antibodies against Leishmania major promastigotes have been developed as analytic and preparative reagents. In the second year of the Contract (covered by this report) we prepared 11 additional monoclonal antibodies (all IgG<sub>3</sub>) and expanded 4 in ascites. We used immunoprecipitation of metabolically labeled promastigote antigens and Western blot analysis to begin our assessment of the epitopes recognized by some of these monoclonal antibodies. We developed a radioimmunoassay to assess parasite antigens expressed on the surface of infected macrophage-like cells (P388D<sub>1</sub>) as a means of screening for monoclonal antibodies that recognize such antigens. These studies are being conducted in anticipation of employing the monoclonal antibodies for immunoaffinity purification of antigens that can be tested in vivo for their potential as vaccines.

FOREWORD

In conducting the research described in this report, 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 (DHEW Publication No. (NIH) 86-23, Revised 1985).

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REPORT

The long-range goal of the research effort covered by this contract is development of a safe and effective vaccine for the prevention of leishmaniasis in military personnel deployed to endemic areas. Current methods of immunoprophylaxis against cutaneous disease that are employed in the USSR, in some areas of the Middle East, and by the Israel Defense Force utilize live parasites. Although generally effective, these are "vaccines" occasionally associated with clinical complications. Killed parasite vaccines have proven generally ineffective but are undergoing trials in Brazil. Accordingly, it is now deemed desirable to develop a subunit vaccine that employs defined antigens. To this end, we have developed monoclonal antibodies as a state-of-the-art approach to provide reagents for identification and isolation of Leishmania antigens, specifically those expressed on infected macrophage surfaces. Antigens isolated by immunoaffinity chromatography will be used to vaccinate mice that will be subsequently challenged and the rate of spontaneous disease resolution will be measured. Furthermore, their immunological responses to the vaccine(s), including the presence of effector cells that activate antileishmanial effects in infected macrophages will be assessed. Ultimately, studies will include challenging mice with heterologous parasite species. To facilitate the earliest development of a widely-applicable vaccine for human use, emphasis in these studies is placed on identifying "conserved antigens," that is, ones present in different Leishmania species. It is reasoned that the successful identification of protective conserved antigens might prevent disease due to several different Leishmania species.

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The TECHNICAL OBJECTIVES for the second year of the Contract were to produce IgG monoclonal antibodies against L. major, to begin carrying out certain characterization studies and to improve methods for screening monoclonal antibodies for their ability to detect parasite antigens expressed on the surface of infected host cells.

Monoclonal antibodies were prepared by fusion of spleen cells of BALB/c mice that were immunized with gluteraldehyde-fixed L. major promastigotes using published protocols<sup>1</sup>; each mouse received i.p.  $2 \times 10^8$  promastigotes emulsified in complete Freund's adjuvant on two consecutive weeks. Two weeks later an i.v. challenge of  $10^7$  fixed parasites was administered 5 days prior to sacrifice. Spleen cells were fused with the myeloma line P3X63 Ag8 and resulting hybridomas were cultured, cloned, and subcloned by published methods<sup>2</sup>.

Identification of clones producing antileishmanial antibody was established by assaying culture supernatants in an ELISA assay.  $10^6$  promastigotes (gluteraldehyde-fixed) were bound to each well of Immulon II plates with poly-L-lysine. Hybridoma supernatant (100  $\mu$ l) was added to each well and incubated for 2h. Plates were extensively washed and incubated with 100  $\mu$ l goat anti-mouse Ig (GAM or G) followed by rabbit anti-goat Ig conjugated to alkaline phosphatase for 2h. After washing, the phosphatase substrate was added for approximately 45 min and the reaction stopped before reading E409 in an ELISA reader. To assess the possibility that the monoclonal antibodies so identified might actually have specificity for proteins adsorbed to the parasite surface during cultivation in fetal bovine serum-containing medium,

(rather than to intrinsic parasite antigens) we also measured their reactivity to fetal bovine serum, bovine serum albumin (BSA), and plasma fibronectin. These "antigens" were bound to ELISA plates with Voller's carbonate buffer in the following concentrations: fetal bovine serum, 10%; BSA, 200 mg/ml; fibronectin, 800 ng/ml. The ELISA assay was then carried out as noted, above. The clones reacting with these non-leishmanial antigens were not further analyzed.

Monoclonal antibodies were isotyped by ELISA. Hybridoma supernatants were bound to ELISA plates with Voller's carbonate buffer, followed by addition of isotype-specific goat anti-mouse antibody conjugated with alkaline phosphatase.

We consider leishmanial antigens displayed on the surface of infected macrophages as the most interesting candidate targets of protective immune responses. There is little doubt that the major antileishmanial effector mechanism(s) involve bi-directional interactions of specifically-sensitized  $Lyl^{+}2^{-}$ , L3T4 lymphocytes with infected macrophages. Lymphocytes are triggered by the interaction with the infected cells in a genetically-restricted (I-A) manner<sup>3</sup>, and in turn, activate antimicrobial effects in the infected macrophages, through lymphokine<sup>4</sup> or possibly membrane-bound<sup>5</sup> molecules. Polyclonal (rabbit) antibodies to leishmanial antigens have been used to demonstrate that such antigens are present on the surface of infected cells<sup>6</sup>, and a monoclonal antibody has been produced that identifies one of these antigens<sup>7</sup>. In our experience, employing indirect immunofluorescence methods, non-specific binding to uninfected macrophages

occurs to a substantial degree even when efforts are made to block macrophage Fc receptors (with high concentrations of goat IgG). Accordingly, we have attempted to enhance the sensitivity and specificity of the screening methods by developing a radioimmunoassay for detection of surface antigens.

The RESULTS of these efforts during the second year of this Contract are the generation of 11 hybrid clones that produced antibody that reacted with fixed promastigotes in an ELISA assay (O.D.<sub>409</sub>:0.447-0.594). From these, four were selected for two cycles of subcloning (Table 1) on the basis of maximum OD<sub>409</sub> values, for screening hybridoma supernatants in the radioimmunoassay, and for ascites production. Ascites were used as probes in the Western blot immunoprecipitation, and radioimmunoassay (see below). The results of these experiments indicated that purification of IgG<sub>3</sub> from the ascites would be desirable for further experiments. Accordingly, we attempted to separate IgG from albumin and transferrin using AbX ion exchange (Baker Scientific) (see below). By the end of year 2 we were focusing our efforts on large-scale production of ascites and IgG fractionation methods, essential prerequisites for attaining our stated goals for the Contract period.

The RADIOIMMUNOASSAY (RIA) for antigens expressed on the surface of infected P388D<sub>1</sub> cells (murine macrophage-like line) was developed using rabbit polyclonal anti L. major antiserum (Figure 1). Briefly, the methods used were as follows: Cultured P388D<sub>1</sub> cells were infected in suspension (RPMI 1640 with 10% fetal bovine serum and antibiotics) at a 1:1 ratio with L. major amastigotes freshly harvested from the footpads of BALB/c mice. The infection was allowed to continue for 24 hours at 37°C. The infected cells

were then washed three times with large volumes of fresh media.  $5 \times 10^4$  to  $1 \times 10^6$  cells were added (in 100 $\mu$ l of media) to each well of a sterile polyvinyl 96-well plate. The cells were incubated for an additional 24 hr at 37°C. Non- or weakly-adherent cells were washed from the 96-well plate by gentle addition of 200 $\mu$ l/ well of fresh medium and drained by inverting the plate. This was repeated three times. Cells were then fixed for 30 minutes at room temperature with 1% paraformaldehyde in RPMI 1640. Fixed cells were washed three times with PBS. Samples for assay were added to the prepared wells (100 $\mu$ l/well) and allowed to incubate at 37°C x 2 hr. After three washes with PBS + 1% normal goat serum (NGS) 100 $\mu$ l/well of rabbit anti-mouse IgG antibody diluted 1/100 in PBS + 1% NGS was added and incubated at 37°C for 1 hr. The wells were washed three times with PBS + 2% BSA and 100K CPM of staphylococcal protein A labeled with  $I^{125}$  (diluted in PBS + 2% BSA) was added. The plates were incubated at 37°C for 1 hr. After three washes with PBS + 2% BSA the plates were cut up and each well was counted separately in a gamma counter for 1-10 minutes.

When fixed L. major-infected P388D<sub>1</sub> cells were incubated with polyclonal antiserum or pre-immune serum at different concentrations, a clear distinction between nonspecific antibody binding (pre-immune serum) and specific binding (immune serum) was discernible (Figure 1). When cells infected for different periods were examined, this distinction was also apparent (Figure 2). However, the level of antibody binding to infected cells (expressed as CPM) remained relatively constant overtime. To analyze this further, amastigotes were incubated with live, gluteraldehyde-fixed, or cytochalasin E-treated P388D<sub>1</sub> cells for 30 min, after which cells were

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washed and incubated for various times (up to a total of 4 h) before the RIA was performed (Figure 3). The early appearance of antigen could be ascribed to attachment of amastigotes to the surface of P388D<sub>1</sub> cells. The subsequent decrease in binding was due to interiorization of amastigotes since blocking entry (cell fixation or cytochalasin treatment<sup>a</sup>) resulted in persistence of antigen. Culture supernatants of fourteen subclones were assayed, and binding (CPM) to uninfected and infected cells was compared (Figure 4). Two sub-clones (1D1 and 1D4) were apparently positive in the assay. However, the high level of binding of Mabs to uninfected P388D<sub>1</sub> cells even in the presence of high concentrations of normal goat IgG (to block FcR) indicated that methods for increasing signal-to-noise ratio would be needed. In this regard, we consider monoclonal antibody purification from ascites as a critical step toward this goal.

Ascites were produced by intraperitoneal injection of hybridomas into BALB/c mice. A summary of ascites produced is provided in Table 2. Unfractionated ascites prepared with representative hybridomas was used for WESTERN BLOT (Table 3) and IMMUNOPRECIPITATION (Table 4) analysis of L. major antigens. (Additional assays will be performed on other Mabs as sufficient ascites is obtained.)

Finally, we began efforts to fractionate IgG from ascites using an AbX ion exchange column, reportedly excellent for this purpose. (IgG<sub>3</sub>, the isolate of all our Mabs, does not bind staphylococcal protein A.) To facilitate monitoring of fractionation, we tested fractions for their reactivity with

promastigotes in the ELISA (Figure 5). Our finding indicated that this was not an adequate fractionation procedure since too much contamination with transferrin was present in the IgG fractions.

The PROGRESS during this Contract year can therefore be summarized as follows: We have supplemented our bank of IgM monoclonal antibodies with IgG<sub>3</sub> monoclonals, the latter generally being easier to use as analytic and preparative reagents and possibly of different specificities than the IgM Mabs. We have begun to produce these in ascites and are attempting to fractionate the IgG. We have adopted Western blot and immunoprecipitation methods for analysis of our Mabs and have preliminary results that indicate IA1 and ID4 (from subclones of the same hybrid clone) recognize proteins with Mr 69, 55, and 46 kd while polyclonal rabbit antibody recognizes proteins of Mr 65 (69), (?55), 46, 36, and 14 kd. Since the 46 and 14 kd proteins bind non-specifically to normal mouse IgG, the specific recognition is to proteins with Mr 69 and 55 kd and 65 and 36 kd respectively. (This specificity appears to be distinct from our IgM Mab.) We have developed an RIA that we will use to screen purified Mab for recognition of parasite antigen expressed on infected P388D<sub>1</sub> cells.

The PROBLEMS we have encountered are the nonspecific binding of Mab-containing ascites to uninfected P388D<sub>1</sub> cells (which we believe is due to transferrin contamination), and non-specific binding of some parasite proteins for IgG. The former we anticipate will be resolved by purification of IgG from ascites. The latter we hope to circumvent by preadsorption of promastigote lysate with Sepharose-bound normal mouse IgG prior to

Immunoprecipitation. In addition, substantial time and effort had to be invested in building up an adequate bank of ascites to carry out purification steps. Our immediate GOALS for the next year are to purify the IgG Mabs; prepare F(ab')<sub>2</sub> fractions for iodination to carry out direct RIA on infected P388D<sub>1</sub> cells; further analyze the Mabs by immunoprecipitation of metabolically-labeled parasite material and for cross-reactivity; and, prepare parasite antigens by immunoaffinity chromatography for vaccine trial.

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Table 1: Results of anti-promastigote ELISA using culture supernatants from cloned hybridomas prepared from spleen cells of L. major immunized mice.

OD<sub>409</sub>

Alkaline phosphatase-conjugate

<u>Clone</u>	<u>anti-IgG</u>	<u>anti-IgG, A, M</u>
5C3	0.578	1.006
30 D-1	0.902	1.896
37 D-2	0.389	-
42 A-1	1.018	0.806
43 A-2	0.427	0.393
44 A-3	0.412	0.770
54 B-1	0.406	1.712
55 B-2	0.327	0.816
56 B-3	0.326	1.148
57 B-4	0.671	1.082
66 C-1	0.906	1.537

Figure 1: Reactivity of rabbit anti L.major polyclonal antibody with fixed promastigotes (A) or fixed P338D1 cells infected with L.major amastigotes (B) in RIA.

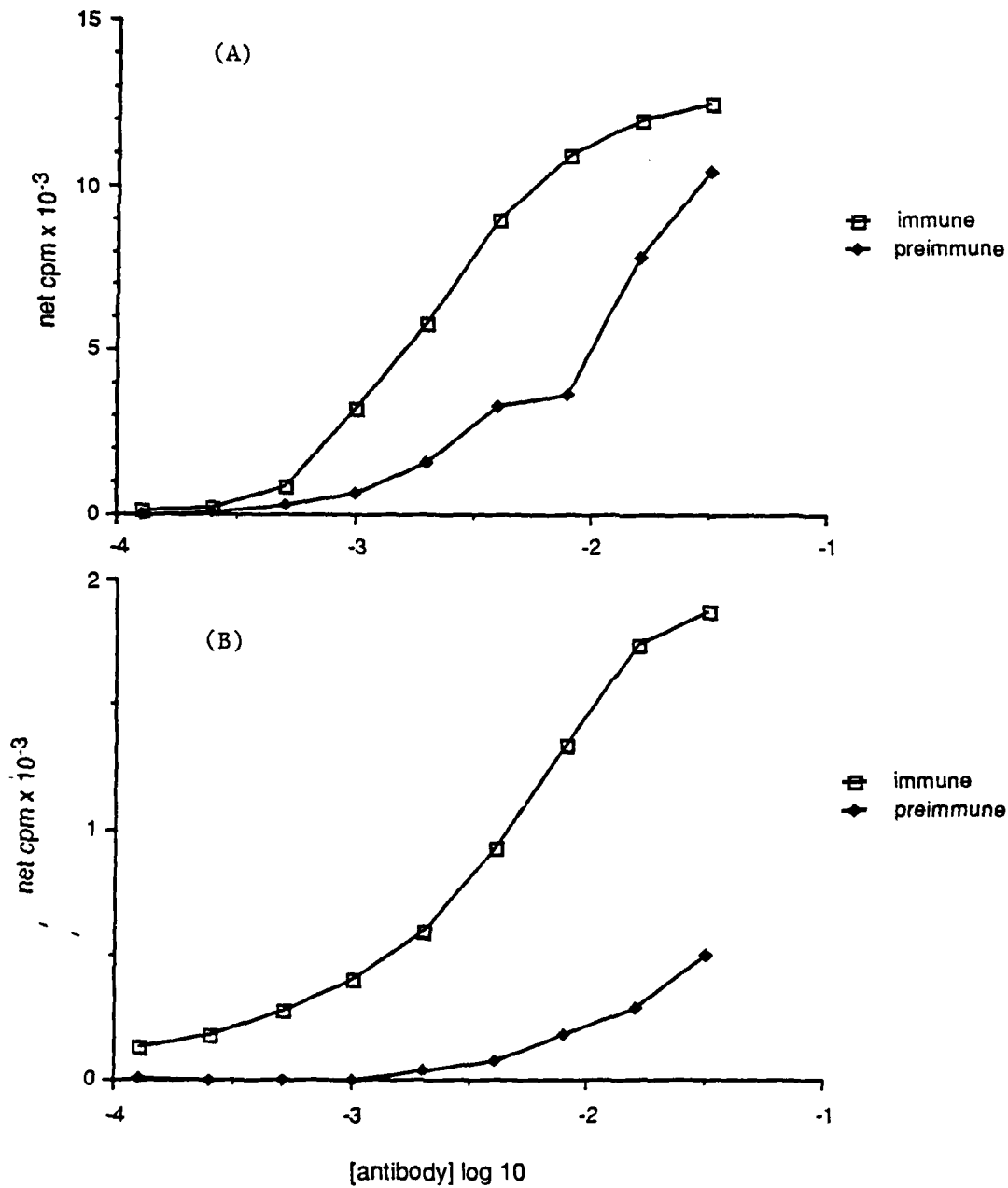
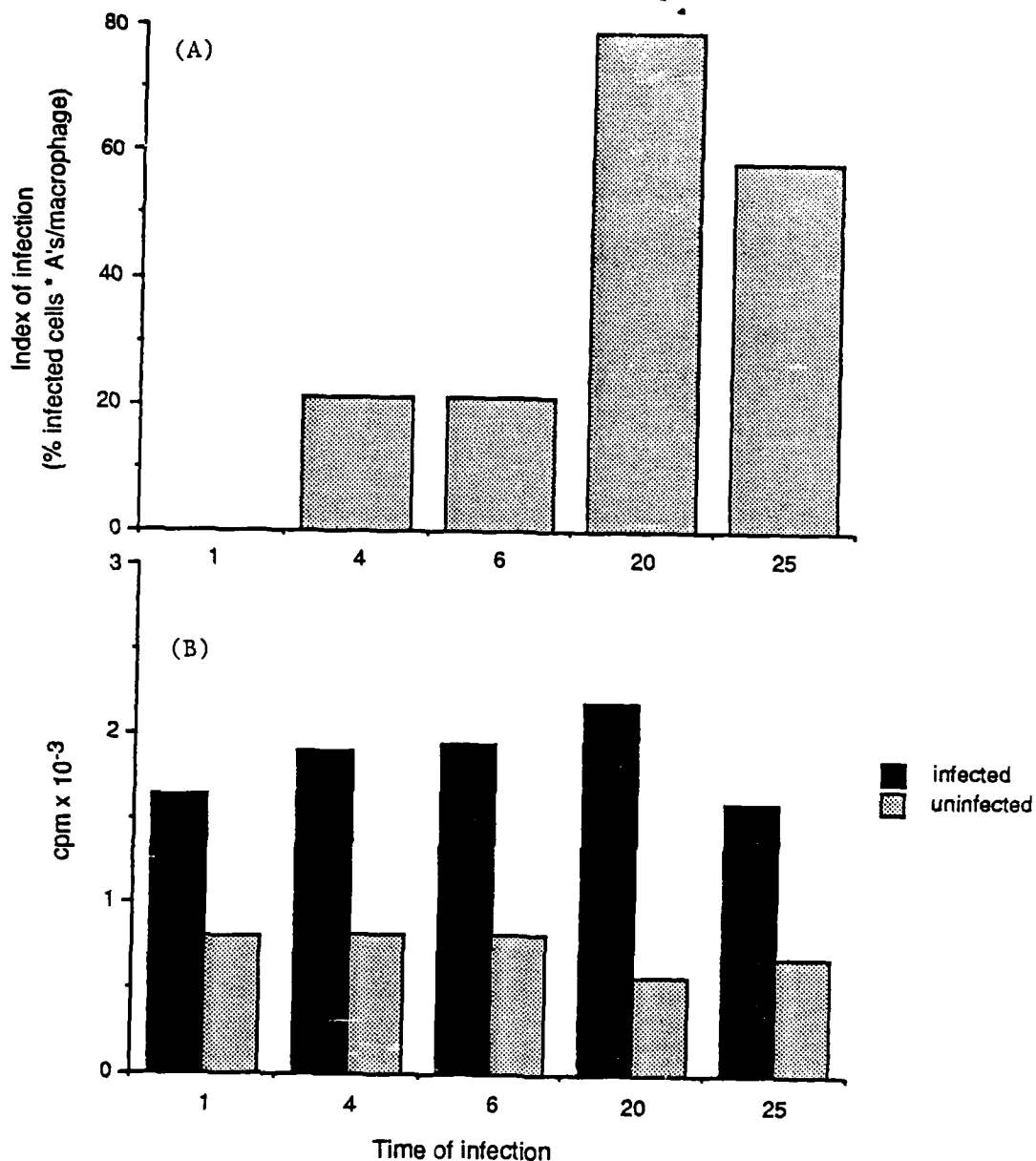
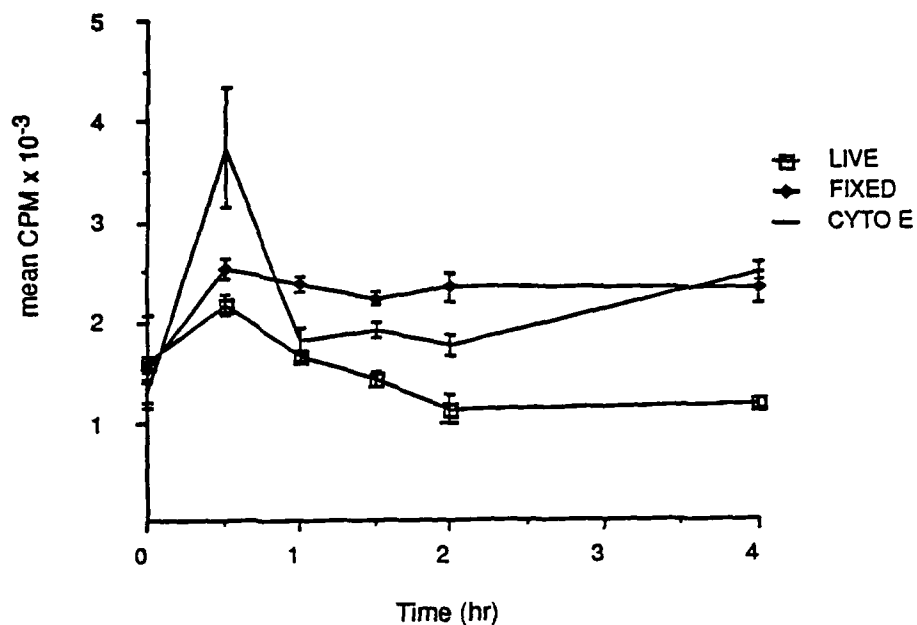


Figure 2: Kinetics of binding of rabbit antileishmanial antibody to *L.major*-infected P388D1 cells, assessed in RIA.



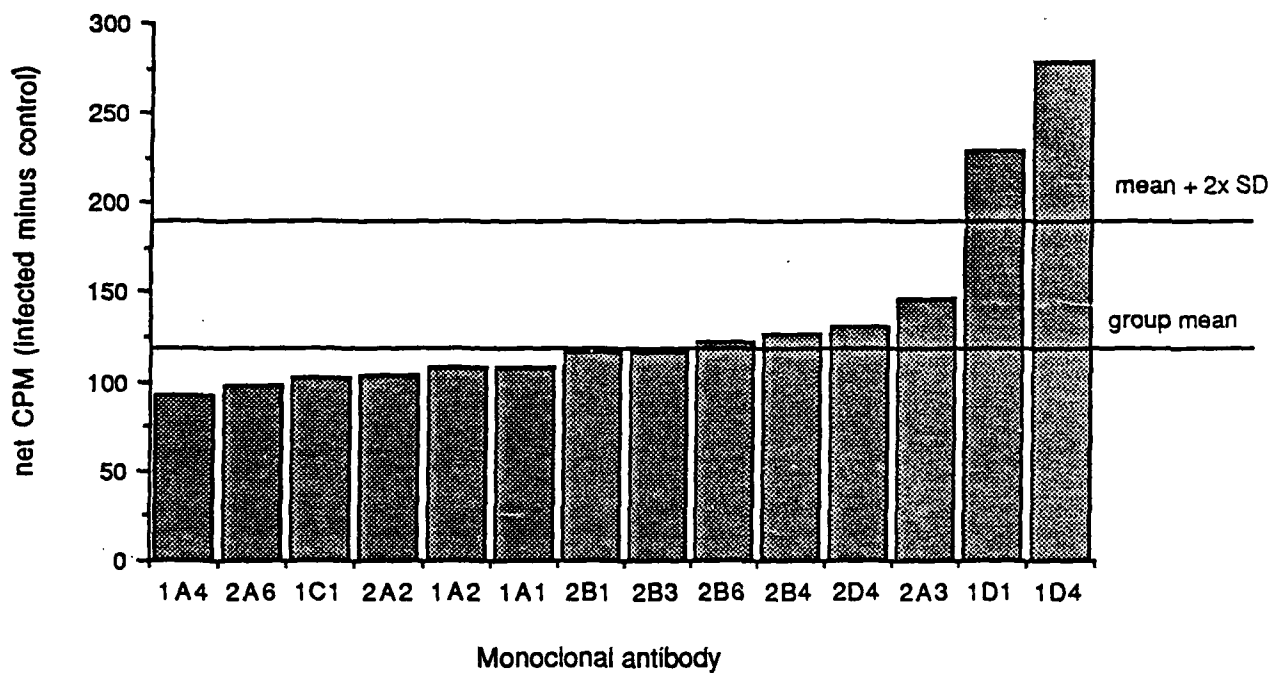
Cells were infected with amastigotes (see text) and aliquots harvested at intervals from 1-25h later. Infection was assessed microscopically (A) (Index of Infection represents amastigotes per 100 macrophages) and RIA was performed using infected (or uninfected) P388D1 cells incubated for the same period of time (B)

Figure 3: Early kinetics of RIA using live, fixed or cytochalasin E treated P388D1 cells.



P388D1 cells were untreated, fixed, or treated with cytochalasin E (to block endocytosis) prior to exposure to live *L. major* amastigotes, after which they were washed, fixed, and subjected to RIA. Following amastigote binding, parasites internalize into live cells with progressive decrease in binding of rabbit antibodies to cells; amastigotes remain on the surface of fixed or cytochalasin-treated cells.

Figure 4: Antileishmanial monoclonal antibodies screened in RIA using L.major-infected P388D1 cells.



**Table 2: Summary of IgG<sub>s</sub> Mab against L. major.**

<u>Clone</u>	<u>Sub-Clone</u>	<u>Ascites volume</u> <u>(ml)</u>	<u>[IgG]</u> <u>mg/ml</u>	<u>Amastigote</u> <u>binding (RIA)</u>	<u>Promastigote</u> <u>binding(EIA)</u>
5					
	1A13B	3.25	2	1/400	1/200
	1A16N	12	0.7	1/400	
	1A17	10	0.6	1/50	
30					
	301'	1.5	0.9	1/200	
	1D1	-	0.025		1/25
	1C4	-			1/1600
	1C12R	2	1.7	1/200	1/800
	1C18	30	0.005	<1/50	
37					
	376R	25	0.5	1/100	
	379	10	0.8	<1/50	
	2B15L	1.5	0.4		1/200
	2B4A	1.5	5.3	1/400	1/100
	2B44T	2	0.6	1/400	
42					
	2D45T	2	0.4	1/50	1/100
66					
	66R	2	0.06	1/100	1/200
	66L	1.75	1.75	<1/50	1/50

Table 3: Summary of Western blot analysis with monoclonal antibodies produced by two subclones of an anti L. major hybridoma.<sup>1</sup>

<u>Antibody</u>	<u>Mr of protein (Kd)</u>
Rabbit polyclonal	>100, 69, 55, 34, 17
Mab	
IA1	69, 55
ID4	69, 55

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<sup>1</sup>Method according to Huyh, T.V., Young, R.A. and Davis, R.W. 1985. in: DNA Cloning: A Practical Approach, vol. 2 (D.M. Glover, ed) pp. 49-78, IRL Press, Oxford.

Promastigotes were lysed by three freeze (-80°)-thawed (37°) cycles and subjected to SDS-PAGE (10% acrylamide). Promega goat anti-mouse Ig antibody and rabbit anti-goat Ig antibody conjugated to alkaline phosphatase were used as probes for Mab binding following electro-transfer. BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and NBT (nitroblue tetrazolium) were used as chromogenic substrates.

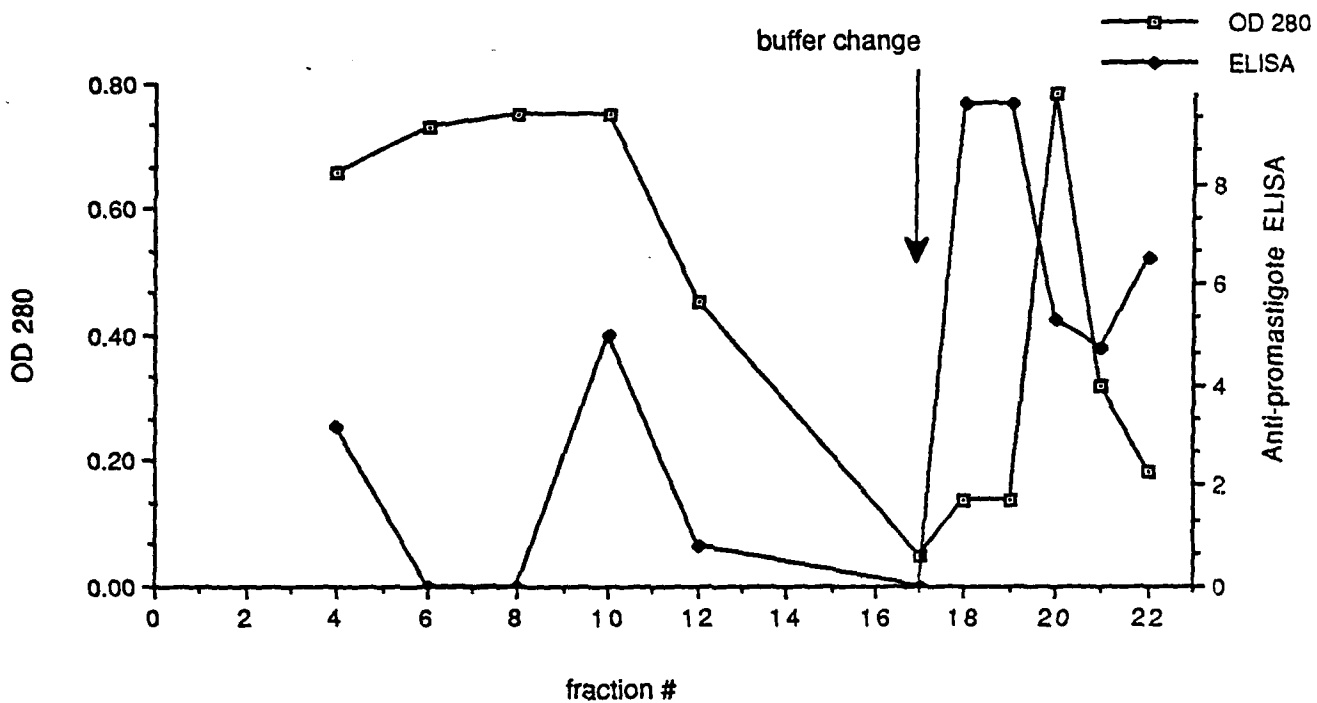
**Table 4:** Summary of immunoprecipitation of lysate from  $^{35}\text{S}$ -methionine-labeled L. major promastigotes.<sup>1</sup>

<u>Antibody</u>	<u>Mr of protein (kd)</u>			
Rabbit polyclonal	>100,	69, 52, 48,	38	
IA1		69, 55, 46, 42,	25.7	
ID4		69, 55, 46		
IIB4		65, 46,	36,	14
66		46,	36	
NMIgG	>100,	46,	14	
IB1 (IgM)		95, 88, 68,	43	

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<sup>1</sup>Performed as described in: Goding, J.W. 1984. Molecular and Chemical Characterization of Membrane Receptors, pp. 31-60, Alan R. Liss, Inc., NY.

Figure 5: Chromatogram of monoclonal antibody-containing ascites following ion exchange fractionation on AbX (Baker) column.



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