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

ANNUAL REPORT

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The goal of this contract is to identify, characterize, and test antigens of Leishmania that could serve as candidate vaccines against cutaneous leishmaniasis. Monoclonal antibodies were raised against promastigotes and tested for their reactivity with leishmanial antigens using immunoprecipitation and Western blot analysis. A radioimmunoassay was developed to detect the presence of leishmanial antigens on the surface of infected P388D1 cells, and was employed to screen monoclonal antibodies for their ability to detect such antigens.

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SUMMARY

The goal of this work is to identify antigens of Leishmania that can impart protection against cutaneous leishmaniasis. We previously prepared hybidomas from splenocytes of mice immunized with L.major promastigotes and selected ones that produced antibody to promastigotes as determined in an ELISA assay using whole, fixed parasites. During the contract period covered by this report, we subcloned several clones and determined that these all secreted antileishmanial antibody with IgG_3 isotype. We expanded the clones in vivo and harvested monoclonal antibody-containing ascites. Ascites were used to immunoprecipitate metabolically-labeled promastigote antigens and as probes in Western blot analysis. We also continued efforts to develop a radioimmunoassay to detect leishmanial antigens expressed on the surface of infected $P388D_1$ cells, and used this method to screen monoclonal antibodies for their ability to detect such expressed antigens. Finally, we began efforts to purify monoclonal antibodies from ascites.

FOREWORD

In conducting the research described in this report, the investigators 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).

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REPORT

The long-range goal of the research effort is development of a subunit vaccine for the prevention of cutaneous leishmaniasis. The strategy we are employing is to prepare monoclonal antibodies against Leishmania major and utilize these in the immunoaffinity purification of parasite antigens. We are particularly interested in identifying those antigens that are expressed on the surface of infected macrophages, since we believe these to be the likely targets of lymphocytes that mediate defense in this disease.

The FIRST TECHNICAL OBJECTIVE for the period covered by this report (February 1, 1987 through January 31, 1988) was to subclone hybridomas we previously prepared from splenocytes of mice immunized with L. major using published methods (1). Cloning and subcloning was carried out by limiting dilution methods. Identification of clones and subclones producing antileishmanial antibody was established by assaying culture supernatants in an ELISA assay. One million gluteraldehyde-fixed promastigotes of L.major (obtained from stationary-phase cultures) were bound to each well of 96-well Immulon II plates using poly-L-lysine. One hundred microliters of hybridoma supernatant was added to each well and followed by incubation at 37° C for two hours. Plates were extensively washed and incubated for two hours with 100 µL goat anti-mouse Ig followed by rabbit antigoat Ig conjugated to alkaline phosphatase.This was followed by an additional wash and the addition of a phosphatase substrate for approximately 45 min. The reaction was stopped and plates were read in an ELISA reader at E_{409}. As a control for the possibility that some of the monoclonal antibodies might have specificity for proteins in the fetal calf serum present in promastigote cultures (and adsorbed onto the parasites used for vaccination), we also tested the hybridoma culture supernatants against fetal bovine serum, bovine serum albumin, 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. Antibody from a few few clones reacted with these control antigens and were discarded.

The RESULTS of two cycles of subcloning of 7 clones originally selected on the basis of specific antileishmanial reactivity in the ELISA assay was the subsequent selection of 11 subclones (Appendix 1). The monoclonal antibody present in the hybridoma supernatants was isotyped by an ELISA method, whereby the supernatants were bound to

Immulon II plates with Voller's carbonate buffer and rabbit antibody to individual isotypes of mouse Ig was reacted with the samples; enzyme conjugated anti rabbit Ig was added as the penultimate reagent to detect reactivity. All monoclonal antibodies were determined to be IgG_3. Selected hybridomas were expanded in culture flasks and were injected ip into pristane-primed BALB/c mice according to published methods (2), for the production of monoclonal antibody-containing ascites.

Several months were required to obtain sufficient quantities of ascites to carry out antibody purification and undertake efforts to characterize the antigens recognized by the monoclonal antibodies. We encountered a common problem in monoclonal antibody production; some of the hybridomas produced little ascites when injected into mice. In an effort to circumvent this problem, we tried injecting different numbers of cells or cells from cultures of different densities into mice to ascertain whether this might improve the antibody yields. We found that the rate and volume of ascites production was unpredictable and could not be controlled by these manipulations. Obtaining suitable quantities of ascites was a time and animal-supply intensive undertaking.

Antileishmanial activity in the ascites was assessed by titration in the ELISA assay using intact fixed promastigotes and tissue-derived amastigotes (prepared according to ref 3). The concentration of IgG in the ascites was determined in an ELISA assay in which ascites was bound to Immulon II plates and reacted with rabbit antibody against mouse IgG, followed by enzyme-conjugated goat anti-rabbit Ig. As summarized in Appendix 2, reciprocal titers of antileishmanial antibody ranged from <50 to 1600; IgG concentrations ranged from 5 µg/ml to 5.3 mg/ml in different pooled ascites preparations. Unfortunately, the larger volumes of ascites produced tended to have lower IgG concentration. As we subsequently found, much of the protein in the ascites was host rather than hybridoma derived..

In related efforts, we prepared polyclonal mouse antipromastigote antibody by immunizing BALB/c mice with gluteraldehyde-fixed promastigotes (10^8) in Freund's complete adjuvant, followed by boosting with promastigotes emulsified in Freund's incomplete adjuvant. Serum pooled from three mice immunized in this manner served as a "positive control" in our subsequent studies to characterize the antigens recognized by the monoclonal antibodies.

The SECOND TECHNICAL OBJECTIVE was to identify specific antigens recognized by selected monoclonal antibodies. We employed two

approaches: 1) immunoprecipitation of antigens from metabolically-labelled promastigotes; and, 2) Western blot analysis of protein antigens. Certain differences have been observed between plasma membrane components of promastigotes in log and at stationary phases of in vitro growth (4). Accordingly, we first compared the SDS-PAGE autoradiograms of ^{35}S-methionone labelled promastigotes from log and stationary phase cultures. We detected no discernible differences in chromatogram pattern of the two parasite populations (data not shown).

For these and subsequent experiments, we employed the following preparative protocol. Promastigotes grown to stationary phase in liqid medium (5) were washed and transferred (10^7/ ml) to MEM culture medium that was specifically deficient in methionine (Selectamine, GIBCO) and supplemented with antibiotics as well as extensively dialysed fetal bovine serum (10%). ^{35}S-methionine was added for a final activity of 10mCi/ml and incubation was carried out at 20°C for 4h. Promastigotes were then subjected to three cycles of freeze-thaw followed by brief sonication in the presence of phosphate (0.02 M) buffered saline (0.15M NaCl) supplemented with 2mM EDTA, 2mM PMSF, 1U apotinin, and 2mM iodoacetamide (sonication buffer). Subsequent centrifugation (40,000g x 30 min) partitioned the preparation into the sonicate supernatant (referred to as sonicate) and the membrane-containing pellet. The pellet was treated with 0.5% Triton X100 in sonication buffer with incubation at 4°C for 1h. The supernatant from this preparation was retrieved after centrifugation (40,000g x 30 min) and was used for immunoprecipitation experiments.

Early in our immunoprecipitation efforts, we did not carry out any pre-adsorption steps. However, the initial chromatograms suggested that non-specific adsorption of promastigote lysate proteins to mouse immunoglobulin might be occurring (appendix 3). We subsequently introduced a preadsorption step meant to remove proteins that non-specifically bound to mouse Ig. For this, we bound to staphylococcal protein A, rabbit antibody against mouse Ig. This conjugate was incubated with normal mouse Ig (NMIg), creating the complex: NMIg-rabbit anti NMIg -protein A-Sepharose. The labelled lysate was incubated with this complex overnight. The non-bound material was retrieved after centrifugation and incubated overnight with ascites containing monoclonal antibody. This was then mixed with protein A-Sepharose-bound rabbit anti mouse Ig and incubated over night. The beads were washed extensively and then boiled in Laemmli buffer (6) under reducing conditions (SDS/DTT). The supernatant of the treated material was then subjected to SDS-polyacylamide gel electroporesis

(PAGE; acrylamide gradient 10-15%), and autoradiographs were prepared.

The RESULTS of these experiments are summarized in Appendix 3 and 4 . Two compelling conclusions emerged from these results. First, non-specific adsorption of promastigote proteins to NMIg-rabbit antibody-protein A Sepharose conjugate is extensive. Second, all the monoclonal antibodies tested (including one with IgM isotype) as well as a rabbit polyclonal antibody raised against the promastigotes recognized a protein with
Mr≅ 60 kD. This suggests that this protein is likely to be an immunodominant antigen, and most likely represents gp63 that has been previously identified (7).

Western blot analysis was carried out by standard methods (8). Promastigotes were processed without prior labelling by the methods described above and the lysates were subjected to SDS-PAGE in 10% acrylamide followed by electrotransfer to nitrocellulose. The electroblot was washed with PBS containing 0.3% Tween 80 and blocked with bovine serum albumin treatment. Individual strips were then incubated with monoclonal (ascites) or polyclonal antibody, washed, and reacted with alkaline phosphatase-conjugated anti mouse or anti rabbit Ig. Reaction with the substrate finally identified the antigen. Molecular weight standards (Rainbow™ Protein Molecular Weight Markers [Amersham]) were run in the same gels.

The RESULTS are shown in Appendix 5. They indicate that whereas polyclonal rabbit antibody identified a vast array of antigens (in excess of 25 bands) and normal mouse Ig did not identify any bands by this method, monoclonal antibodies identified two major antigens that apparently corresponded to those identified by immunoprecipitation. Two monoclonal antibodies (#30 and II B-1) failed to detect any antigens.

The THIRD TECHNICAL OBJECTIVE was to begin purification of monoclonal antibodies from crude ascites. A major motivation was our growing appreciation that contaminating proteins present in crude ascites could potentially interfer with the utility of the monoclonal antibodies both in antigen identification and ultimately purification. Not only might parasite proteins bind to non Ig proteins but the non Ig proteins in the ascites might interfer with parasite-antibody association nonspecifically. In addition, our attempts to assess whether the monoclonal antibodies could detect leishmanial antigens on the

surface of infected macrophages using a sandwich radioimmunoassay (RIA) suggested that contaminants in the ascites bound to macrophages and were then recognized by the iodinated rabbit anti mouse antibody. Considering that the manufacturer of the rabbit antibody may not immunize with totally pure mouse Ig, the chance that there are in the preparation antibodies against (for example) transferrin seems likely. Thus, we began to recognize the need to attempt careful purification.

Our approach to purification was to utilize ion exchange chromatography to separate Ig from the bulk of other proteins in the ascites (most notably, albumin and transferrin). We employed an AbX (Baker) ion exchange column and monitored reactivity of the eluate by OD_{280} and reactivity in the antipromastigote ELISA. This ion exchange resin, newly introduced commercially, was selected because it is reportedly particularly suited for purification of Ig. Initial experiments followed methods recommended by the manufacturer for single buffer change elution, but results were unsatisfactory; SDS-PAGE of eluates indicated contaminating proteins in reactive fractions. Accordingly, we proceeded to carry out gradient elution. A representative experiment is shown in Appendix 6 in which ascites containing monoclonal I D-1 was eluted with a pH/ionic strength gradient of sodium acetate and the eluate was analysed by SDS-PAGE. Of note is that the yield of Ig was insignificant and that persistant albumin contamination remained.

We next investigated the utility of DEAE Affi Gel Blue (BioRad) because of its known affinity for albumin. As shown by results of a representative experiment in Appendix 7, using a salt gradient elution, the Ig-containing fraction was contaminated with transferrin. We carried out a large number of affinity purification runs under different conditions but without adequate success to feel assurred that our Ig was suitably purified.

The FOURTH TECHNICAL OBJECTIVE was to develop a means of detecting the presence of leishmanial antibody on the surface of infected macrophages. We infected $P388D_1$ cells (murine macrophage-like sarcoma) with tissue derived amastigotes of L. major and used a rabbit polyclonal antibody raised against promastigotes of this species to develop the basis for a solid-phase radioimmunoassay (RIA) that we then intended to use to screen monoclonal antibodies. The basic protocol we employed was to react the antibody at different dilutions with the infected $P388D_1$ cells. After washing, iodinated staphylococcal protein A was added; fopllowing incubation, cells were washed and counted.

The RESULTS of these studies are as follows. As shown in Appendix 8-- that summarizes a representative experiment-- antibody bound to infected cells to a significantly greater extent than did the Ig present in pre-immune serum. Furthermore, binding of immune serum to uninfected cells was minimal. Interestingly, although the magnitude of antibody binding to fixed promastigotes was nearly an order of magnitude greater than to infected cells, non-specific binding of Ig in preimmune serum ("natural antibodies") was greater to promastigotes than to infected cells.

We carried out a series of experiments in an effort to more clearly define the RIA for antigens appearing on the surface of infected cells. As shown in Appendix 9, antibody binding to infected cells increased with time after addition of amastigotes to $P388D_1$ cell cultures, but only minimally so, and not in parallel with infection rate of cells as assessed morphologically (9). This suggested that the assay might be incapable of distinguishing amastigote antigens attached to the surface and those expressed following parasite interiorization and export from the phagolysosome to the plasma membrane surface. We therefore compared antigen expression on fixed and live $P388D_1$ cells following the addition of amastigotes. Fixed cells cannot ingest amastigotes, and therefore retain the attached parasites on their surfaces. As shown in Appendix 10, "antigen expression" as detected in the RIA remained relatively constant after 30 min co-incubation of fixed $P388D_1$cells and amastigotes, whereas this decreased when live $P388D_1$ cells and amastigotes were incubated together. The decrease between 30 min and 4 h occurred in association with parasite endocytosis. In related studies with RIA and by our earlier immunofluorescence experiments, we determined that antigens were subsequently expressed at later times following endocytosis of live amastigotes.

Several additional experiments were carried out with polyclonal antibodies in this assay in an effort to enhance sensitivity and specificity as well as to further confirm that the antigens detected represented ones transported from the phagolysosomes to the host cell surface. The results of these experiments failed to improve on the basic assay that we have described. We therefore proceeded to screen supernatants of hybridoma cultures in the RIA. For these studies, we incubated culture supernatants at high concentration with infected $P388D_1$ cells, washed the cells, and incubated the cells with a rabbit anti mouse Ig, followed by iodinated staphylococcocal protein A. As summarized in Appendix 11, wherein the net binding (in CPM) is shown (ie: CPM with infected cells minus CPM with uninfected cells treated

with antibodies), monoclonals ID-1 and ID-4 were the only ones that seemed efficacious. On this basis we payed particular attention to these monoclonals in our subsequent studies.

In SUMMARY, the work carried out during the contract period covered by this report entailed efforts to: 1) sub-clone and expand hybridomas and develop monoclonal antibody-containing ascites; 2) analyse the ascites for their IgG content and relative antileishmanial antibody activity; 3) begin analysis of antigens recognized by the monoclonal antibodies using immunoprecipitation and Western blot analysis; 4) begin purification of monoclonal antibodies from ascites; and, 5) develop a radioimmunoassay that would permit the identification of those monoclonal antibodies that recognized antigens displayed on the surface of infected macrophages, antigens we consider the important targets of defense.

The TECHNICAL DIFFICULTIES we encountered included: 1) failure of some of the hybidomas to produce quantities of ascites in mice that would permit high levels of retrieval of purified monoclonal antibody; 2) non-specific binding of promastigote antigens to the normal mouse Ig or to rabbit antibody-staphylococcal protein A-Sepharose conjugates needed for immunoprecipitation; and, 3) difficulties in purifying the monoclonal antibodies that, by virtue of their IgG_3 isotype could not be purified on protein A columns.

The GOALS for the forth-coming 6 month period remaining in the contract are to circumvent these obstacles. Specifically, we will attempt to purify monoclonal antibodies by alternative methods and continue our antigen identification efforts, proceed with the radioimmunoassay for antigens expressed on infected macrophage surfaces, and begin antigen isolation by affinity chromatography.

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

OD_{409}

Alkaline phosphatase-conjugated antibody vs. mouse[1]

Clone	IgG	IgG,A,M
5C3	0.578	1.006
30 D-1	0.902	1.896
37 D-2	0.389	N D[2]
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

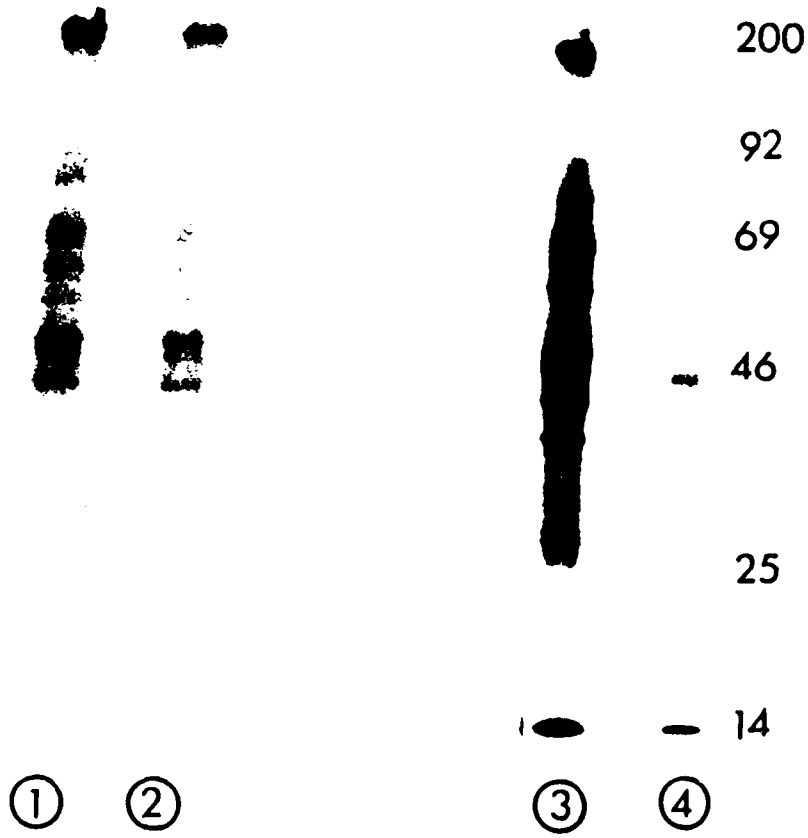
1. Conjugated antibody had specificity for mouse IgG or IgG, IgA, and IgM.
2. Not determined

Appendix 2: Summary of ascites harvested which contained antileishmanial monoclonal antibodies.

Hybridoma		Ascites volume	[IgG]	anti-promastigote
Clone	Sub-clone	(ml)	(mg/ml)	titer (EIA)[1]
5				
	IA13B	3.25	2.0	1/200
	IA16N	12.0	0.7	1/200
	IA17	10.0	0.6	1/200
30				
	301L	1.5	0.9	1/100
	ID1		0.025	1/25
	IC4			1/1600
	IC12R	2.0	1.7	1/800
	IC18	30.0	0.005	<1/50
37				
	376R	25.0	0.5	1/50
	379	10.0	0.8	<1/50
	2B15L	1.5	0.4	1/200
	2B4A	1.5	5.3	1/100
	2B44T	2.0	0.6	1/100
42				
	2D45T	2.0	0.4	1/100
66				
	66R	2.0	0.06	1/200
	66L	1.75	1.75	1/50

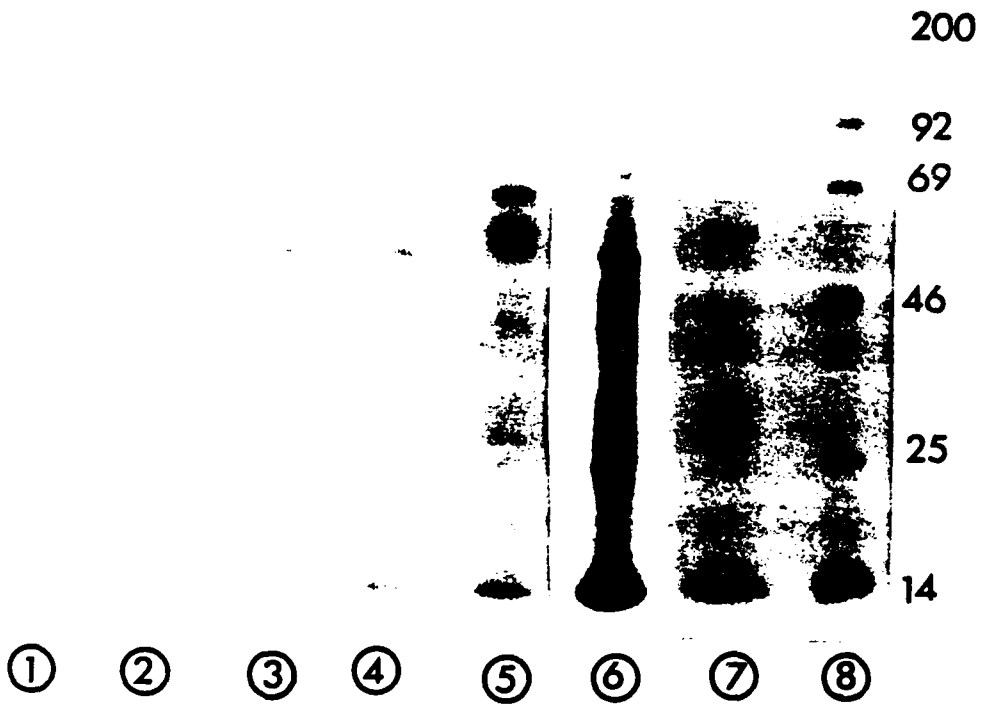
1. Positive reaction in ELISA for determining end-point in titration was defined as OD_{409} 2 X SD above mean negative control values.

Appendix 3: Autoradiograph of SDS-PAGE of lysate of L.major promastigotes metabolically labelled with ^{35}S-methionine and subjected to immunoprecipitation without preadsorotion.



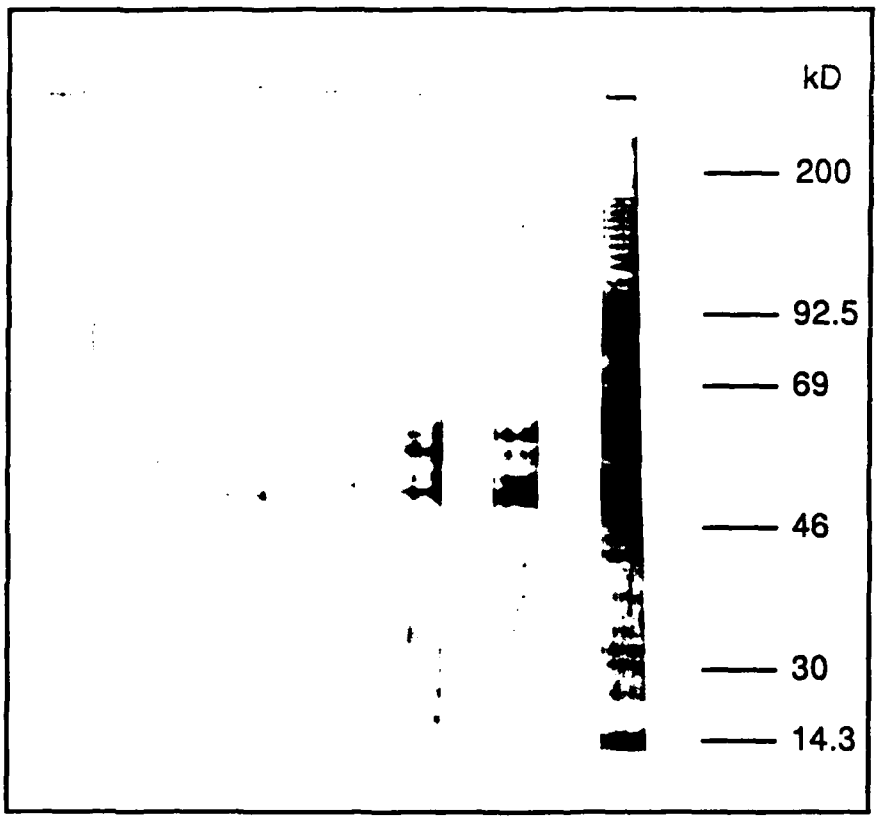
Lysates were reacted with ascites containing monoclonal antibody #56 (lane 1), #66 (lane 2), or normal (non-immune) mouse IgG (lane 3). Molecular weight standards (lane 4) include myosin (200kD), phosphorylase b (92.5 kD), bovine serum albumin (69kD), ovalbumin (46kD), α chymotrypsin (25.7kD) and lysozyme (14.3kD). The approximate Mr is denoted in the right column adjacent to lane 4. In preparing the print of the autoradiograph, lane 3 was "dodged" to enhance resolution.

Appendix 4: Autoradiograph of SDS-PAGE of lysate of L. major promastigotes metabolically labelled with ^{35}S-methionine and subjected to immunoprecipitation.



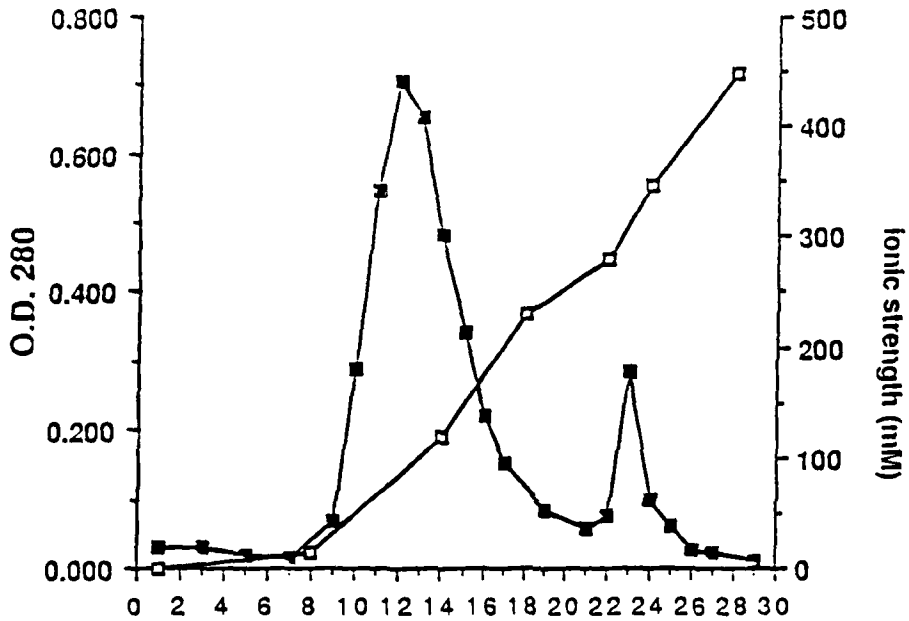
Lysates were first adsorbed with normal mouse Ig and then subsequently subjected to immunoprecipitation with ascites containing monoclonal antibody ID-4 (lane 1), #56 (lane 2), #66 (lane 3), II B-4 (an IgM; lane 4); or rabbit polyclonal antileishmania antibody (lane 5). The profile of proteins that adsorbed to normal mouse Ig is shown in lane 6, and the profile of proteins remaining after adsorption is shown in lane 7. Molecular weight standards (lane 8) include myosin (200kD), phosphorylase b (92.5 kD), bovine serum albumin (69kD), ovalbumin (46kD),α chymotrypsin (25.7kD) and lysozyme (14.3kD). The approximate Mr is denoted in the right column adjacent to lane 8.

Appendix 5: Western blot analysis of L. major promastigote lysate antigens recognized by polyclonal rabbit antileishmanial antibodies and murine monoclonal antibodies.

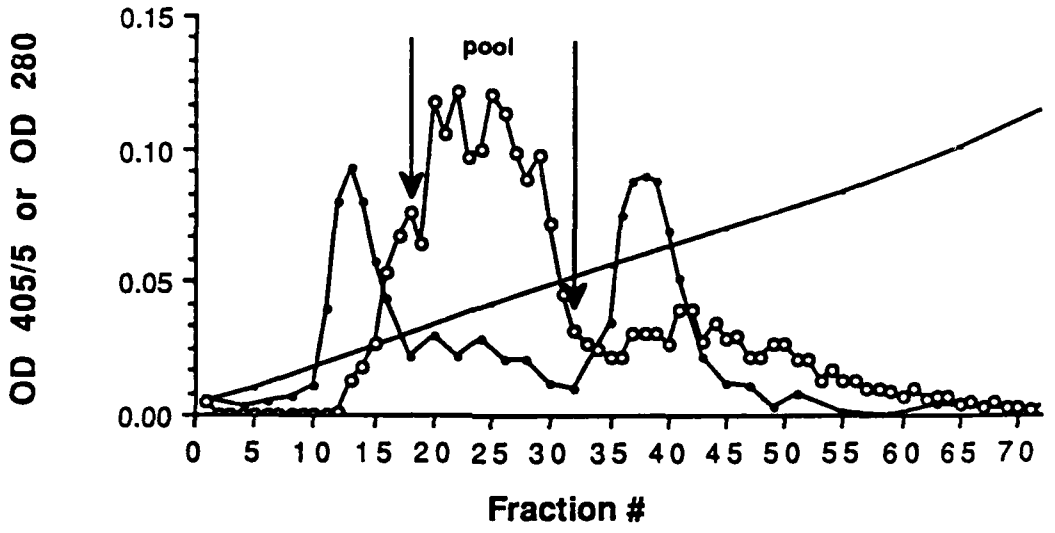


Polyclonal rabbit antileishmanial antibody (lane 7) or various ascites containing monoclonal antibodies (#30 [lane 1]; IIB-1 [lane 2]; IID-4 [lane 3]; 66R [lane 4]; IC-1 [lane 5]; IA-1 [lane 6]) were used to probe electroblots of SDS-PAGE (reducing conditions) of promastigote lysates.

Appendix 6: Gradient elution of ascites (containing monoclonal antibody ID-1) from AbX ion exchange affinity column.

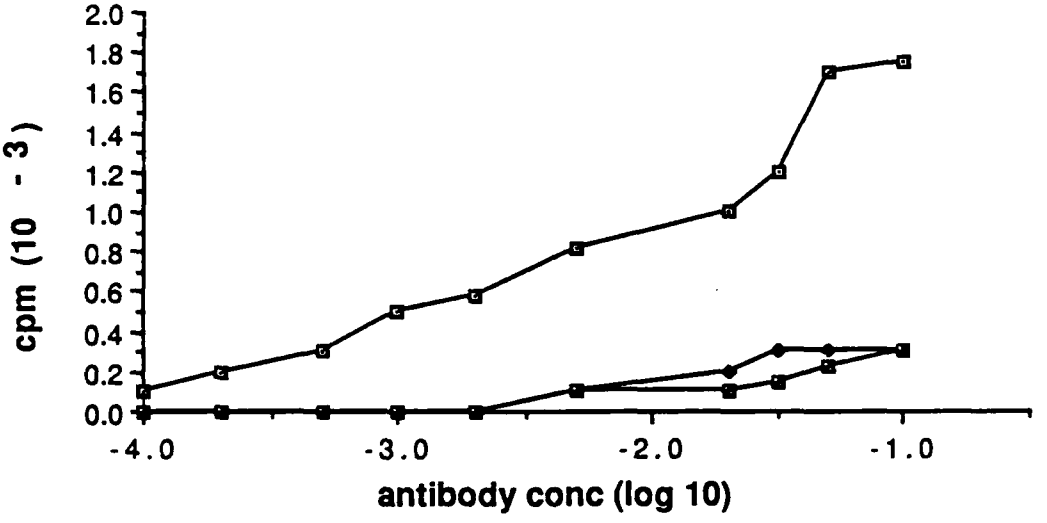


Appendix 7: Representative elution profile of double-purified ascites containing monoclonal antibody ID-4 on CM Affi-Blue.



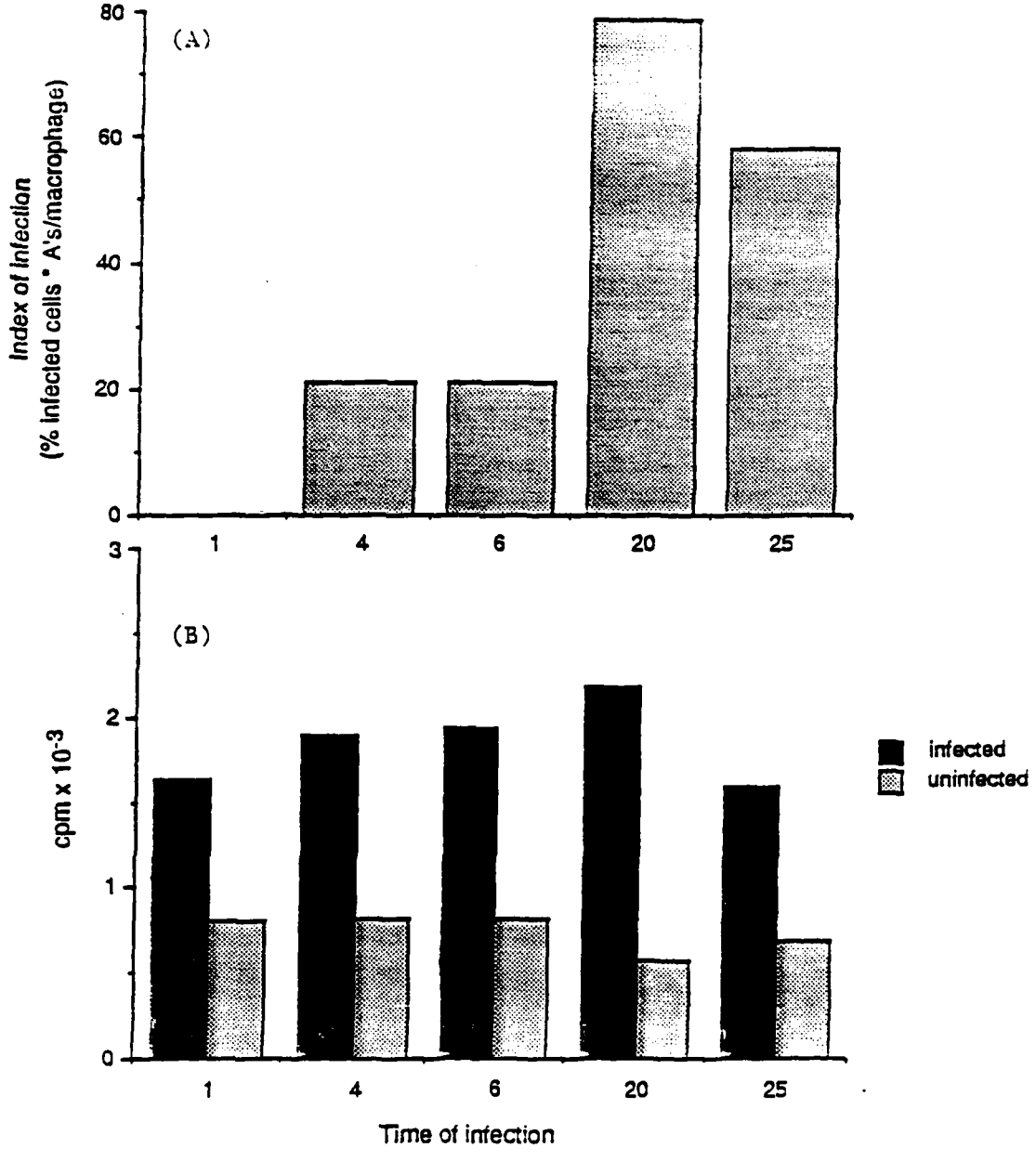
Ascites ID-4 was first subjected to DEAE Affi Blue and antitransferrin scrubber column purification. The IgG-containing fractions were then subjected to CM-Affi-Blue chromatography. Elution was carried out with sodium acetate (conductivity shown by straight line). Fractions were assessed for OD_{280} (closed dots) and for reactivity to promastigotes in ELISA (open dots).

Appendix 8: Radioimmunoassay of binding of rabbit polyclonal antileishmanial antibody to leishmania-infected P388D1 cells.



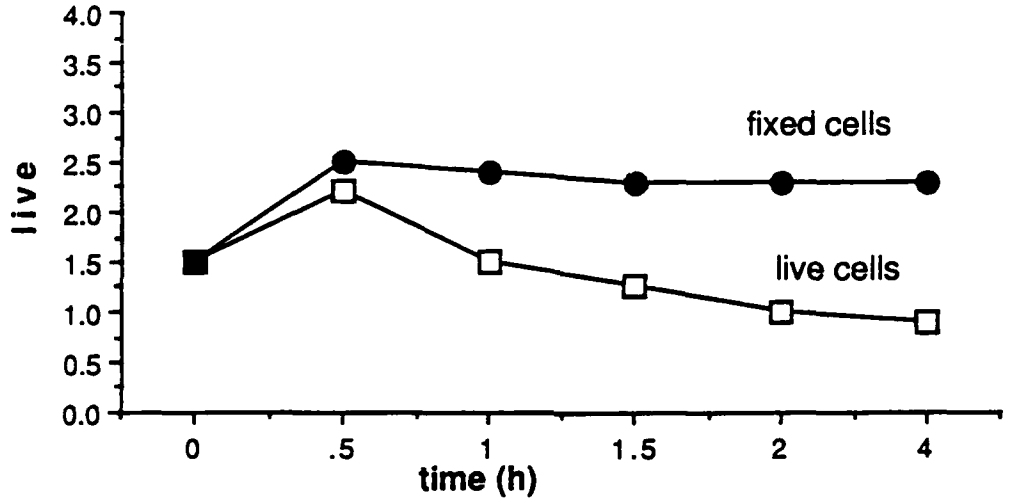
Infected cells were fixed with paraformaldehyde and treated with polyclonal antileishmanial antiserum (open boxes) or pre-immune serum (solid boxes). Binding of immune serum and pre-immune serum (diamonds) to uninfected cells was indistinguishable.

Appendix 9: Kinetics of binding of rabbit antileishmanial antibody to L. major-infected P388D$_1$ cells assessed by radioimmunoassay.



Cells were infected with tissue-derived amastigotes and aliquots of cell suspensions were harvested at intervals from1-25h. Infection was assessed microscopically (A; Index of Infection represents the product of percent infected macrophages and mean number of amastigotes per infected macrophage, or number of amastigotes per 100 total macrophages) and RIA was performed using infected (or uninfected) P388D$_1$cells incubated for the same time.

Appendix 10: Early kinetics of leishmanial antigen detection (by radioimmunoassay) on live and fixed $P388D_1$ cells following addition of amastigotes to culture.



$P388D_1$ cells were untreated or fixed in gluteraldehyde prior to adding live amastigotes. At various intervals (time shown), cells were washed, fixed with paraformaldehyde, and subjected to RIA. Following amastigote binding, parasites are internalized by live but not fixed $P388D_1$ cells, with progressive decrease in parasite "antigen expression"; amastigotes remain on the surface of fixed cells. (SEM $\leq$ 10% mean in all cases, so that differences in groups at 1h and thereafter are statistically significant at $p < 0.001$).

Appendix 11: Antileishmanial monoclonal antibodies (hybridoma supernatant) screened in radioimmunoassay with leishmania-infected $P388D_1$ cells.