Membrane Glycoprotein M-2 Protects against
Leishmania amazonensis Infection

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Received 2 May 1988/Accepted 11 September 1988

Previous passive antibody transfer experiments have indicated that immunity to a 46-kilodalton membrane
glycoprotein (M-2) of Leishmania amazonensis may protect against infection with this parasite. In the studies
described in this paper, we investigated the ability of the purified M-2 molecule to elicit a protective immune
response in conjunction with Freund incomplete and complete adjuvants, saponin, and Corynebacterium
parvum. Both relatively susceptible (BALB/c and CBA) and resistant (C57BL/6) strains of mice were examined.
C. parvum appeared to be the most effective adjuvant in the three mouse strains tested. The level of protection
varied with the mouse strain, although all animals received identical preparations of antigen and adjuvant.
Immunization of CBA mice with the M-2 glycoprotein and C. parvum resulted in complete protection against
a challenge infection of 10^6 and 10^7 late log-phase promastigotes of L. amazonensis. In the BALB/c strain,
complete protection was observed in some of the immunized animals (28% to 50%); in the rest of the mice the
onset of infection was significantly delayed. Protective immunity for C57BL/6 mice was observed only at the low
infecting dose (10^4 L. amazonensis organisms). The level of protection observed is reflected by increased
antibody response (immunoglobulins G1 and G2) developed to the M-2 molecule. The relationship of pure
T-cell (nonantibody) immunity to this protection remains to be elucidated.

Leishmaniasis is a zoonotic parasitic disease caused by members of the protozoan genus Leishmania. The leish-
manias are associated with a broad spectrum of disease (5), ranging from simple cutaneous to visceral leishmaniasis.
Estimates of the incidence of leishmaniasis vary (49) but suggest that annually several million new cases occur world
wide.

The Leishmania parasite has a relatively simple life cycle (5). The flagellated promastigotes multiply within the aliment-
ary tract of the sandfly vector and subsequently migrate anteriorly. The promastigotes are then transmitted by the
bite of the sandfly to a mammalian host, where they enter macrophages and transform into amastigotes
within the phagolysosomal vacuole.

Species of the Leishmania mexicana complex have a wide geographic distribution, including South and Central
America, the West Indies and (to a limited extent) the southern United States (7, 11, 23, 25). Members of the L. mexicana
complex are associated with both simple and diffuse forms of cutaneous leishmaniasis. Diffuse cutaneous leishmaniasis,
caused primarily by L. amazonensis and L. pifanoi in a fraction of infected individuals (30), is characterized by
disseminated large histiocytomalike nodules containing abundant parasites and by deficient cell-mediated immunity
(7, 43) that may be associated with immunological suppression (35). In general, these cases are resistant to chemother-
apy (7).

Vaccination, at least for the cutaneous forms of leishmaniasis, is possible. For centuries, vaccination with living
organisms that produce a lesion has been practiced by the inhabitants of the Middle East. Such vaccination by infec-
tion generally is followed by lifelong immunity and has been the basis for vaccination programs in both Israel and the
USSR (10). Complications that occur with the live vaccine (10) indicate the need for an attenuated or defined vaccine

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for cutaneous leishmaniasis. Recent studies in animal model systems (18, 26, 33, 40) and humans (1, 24, 27) with irradi-
ated or killed Leishmania organisms suggest that a nonlive vaccine is possible. In addition, recent studies of purified
leishmanial antigenic components have resulted in significant (14, 37) protection in murine model systems.

Our laboratory has been involved in the identification of potentially protective Leishmania antigens by using mono-
clonal antibodies specific for membrane components (28, 30). With L. amazonensis, monoclonal antibodies specific
for a 46-kilodalton (kDa) (9, 25, 37) membrane glycoprotein (M-2) specific for the promastigote stage of the parasite (2,
20; P. J. Langer and D. McMahon-Pratt, unpublished data) completely protects in passive antibody transfer experi-
ments against an infective challenge of 10^6 or 10^7 promastigotes. The protection observed in the passive-immunity
experiments did not appear to be dependent on complement (2). In the studies described here, the effectiveness of the
M-2 glycoprotein in eliciting protection was evaluated in direct-immunization studies. The data presented suggest
that, with T-cell help, a mature immune response to this antigen is generated which results in protection.

MATERIALS AND METHODS

Mice. Female mice and C57BL/6 mice 8 to 12 weeks old
were purchased from Charles River Breeding Laboratories,
Inc., Kingston, N.Y. Female CBA and BALB/cByJ mice 8
to 12 weeks old were obtained from Jackson Laboratory,
Bar Harbor, Maine.

Leishmania. L. amazonensis (formerly L. mexicana ama-
zonensis) LTB0016 was kindly provided by Philip Marsden
of the University of Brasilia, Brasilia, Brazil. The strain was
characterized as L. amazonensis by both isoenzyme analysis
(profiles) and species-specific monoclonal antibodies. Para-
sites were isolated from infected mouse tissue and kept in
culture at 22 to 24°C in Schneider Drosophila medium

3272
supplemented with 10% fetal bovine serum (lot 27N0361; Gibco, Grand Island, N.Y.).

Antigen. M-2, a 46-kDa promastigote-specific glycoprotein, was isolated as previously described (20). The protein was further purified by removal of detergent with an anion-exchange column (DE52; Whatman Ltd., Kent, England) as follows. Protein was equilibrated with the resin for 30 min at 4°C, and the resin was then washed three times with 0.02 M Tris (pH 7.0) and then eluted with 1 M NaCl-0.02 M Tris (pH 7.0) containing 0.1% Brij 97. M-2 was then dialyzed against phosphate-buffered saline (PBS; 0.01 M phosphate–0.15 M NaCl [pH 7.2]) before use. The M-2 epitope was 70 to 90% antigenically active as assessed by a competitive radioimmunoassay which used mildly glutaraldehyde-fixed promastigotes as the competing antigen (20). The purity of the protein preparation was homogeneous as assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and staining for protein with Coomassie brilliant blue. The amount of protein used for immunization was quantitated by amino acid analysis based on a molecular mass of 42 kDa (20) at the Yale University School of Medicine protein laboratory facility.

Adjuvants. Killed Corynebacterium parvum was obtained from H. M. McGuire, Wellcome Research Laboratories, Research Triangle Park, N.C. (CN6134; batch CA776/2; 7 mg/ml). Saponin was kindly donated by David Snary of Wellcome Research Laboratories, Beckenham, England. Incomplete and complete Freund adjuvants were obtained from Difco Laboratories, Detroit, Mich.

Immunization of animals. (i) Experiment 1. Incomplete Freund adjuvant was emulsified with M-2 glycoprotein at a final concentration of 0.03 mg/ml. BALB/c mice were injected intraperitoneally, with each mouse receiving 3 μg of the antigen. C. parvum and the M-2 glycoprotein were diluted in PBS at final concentrations of C. parvum and M-2 of 1 and 0.03 mg/ml, respectively. BALB/c mice received 100 μl of this solution intraperitoneally. Saponin (stock solution, 10 mg/ml in PBS) and M-2 were diluted in PBS to final concentrations of 0.25 mg of saponin per ml and 0.05 mg of M-2 per ml. BALB/c mice were given 100 μl subcutaneously. Control groups consisted of untreated animals. The animals in each group (immunized and control) were immunized three times at weekly intervals.

(ii) Experiment 2. BALB/c, CBA, and C57BL/6 mice were immunized intraperitoneally with M-2 and C. parvum as in experiment 1, except that the amount of C. parvum used in immunizations 2 and 3 was reduced to 0.05 mg per immunization (50% of that used in experiment 1). BALB/c mice that received M-2 alone or M-2 together with saponin were immunized intravenously. Since saponin was found to be toxic for CBA and C57BL/6 mice when administered intravenously, CBA mice were not immunized with saponin and C57BL/6 mice were immunized intraperitoneally with 100 μl of a PBS solution containing 0.25 mg of saponin per ml and 0.03 mg of M-2 per ml. Control groups of animals consisted of untreated animals or animals that received either C. parvum or saponin alone. In addition, a group of animals was given M-2 alone intravenously. Animals were then challenged with either 10^5 (BALB/c mice) or 10^6 (CBA and C57BL/6 mice) late-log-phase promastigotes at 3 weeks after the final immunization. Animals that received the higher challenge were dosed intraperitoneally, i.e., 10^5 (BALB/c mice) and 10^6 (CBA and C57BL/6 mice), were inoculated at 4 weeks after the final immunization. Six mice in each group were evaluated for lesion development. Additional groups of immunized and control BALB/c mice were used for experiments that evaluated infection by quantitation of the parasite burden (described below).

(iii) Experiment 3. Groups of eight BALB/c mice received subcutaneously 100 μl of a PBS solution containing 0.25 mg of saponin per ml and 0.03 mg of M-2 per ml. Control groups of eight animals consisted of untreated BALB/c mice and BALB/c mice which received subcutaneously 100 μl of either a 0.25-mg/ml saponin solution or a 0.03-mg/ml M-2 solution. Mice were immunized as in previous experiments, three times at weekly intervals.

Infection of animals. Animals were rested for 2 to 4 weeks after final immunization and challenged in the right hindfoot with late-log-phase promastigotes. Parasites used for infection were passaged a maximum of four times. Challenge doses of 10^5, 10^6, 10^7, and 10^8 were used. In addition, animals from experiment 2 were tested bleb at week 3 postimmunization.

Evaluation of infection. Each group consisted of a minimum of six animals. Lesion measurements were made biweekly with a micrometer (B.C. Ames Co., Waltham, Mass.) as previously described (2).

In addition, parasite burden levels were assessed by using a limited-dilution assay (46) modified for L. amazonensis in our laboratory as described below. All experiments were done in triplicate. In brief, the infected tissue was excised steriley and homogenized in Ten Broeck tissue grinders. Homogenate was suspended in a total final volume of 1 ml of Schneider medium supplemented with 10% fetal bovine serum, gentamicin (final concentration, 0.1 mg/ml), penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 10 μg/ml). Serial 10-fold dilutions of the homogenate were made in Schneider medium and plated (100 μl/well) on blood agar slants in 96-well flat-bottom tissue culture plates (Falcon; Becton Dickinson Labware, Oxnard, Calif.). Plates were stored in a humidified 24°C incubator, and wells were visually scored for growth with a Leitz-Orthoplan inverted-phase microscope. Initial experiments with known concentrations of L. amazonensis amastigotes purified from infected tissue by differential centrifugation and percoll gradients (4) and counted with a hemacytometer showed a plating efficiency of 200%. These results were consistent with those previously reported (plating efficiencies of 80 to 200%) (16, 17, 46) and most likely reflect an error in counting the clumped amastigotes. The plates were scored at day 10, since preliminary experiments showed that this was the optimal time required to detect growth of the cloned amastigotes.

Indirect radioimmunoassay. Parasite membranes were sonicated and incubated overnight at 4°C on U-bottom polyvinyl chloride microtiter plates (Falcon 3911). The microtiter plates were washed with PBS containing 5% fetal bovine serum and 0.02% NaN₃. Mouse sera were serially diluted in PBS and incubated with the antigen-coated plates. The plates were washed with PBS-fetal bovine serum, and affinity purified 125I-labeled rabbit F(ab'₂) anti-mouse immunoglobulin (5 to 10 ng per well; 10⁶ cpm) was added to the wells and incubated for 1 h at 0°C. Microtiter plates were washed and air dried, and the amount of bound radioactivity per well was determined with an AutoGamma counter (Packard Instrument Co., Inc., Rockville, Md.) as previously described (19, 30).

Western blot (immunoblot) analysis. Western blots were performed with crude promastigote glycoprotein preparations resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophotochemically transferred to nitrocellulose paper (Schleicher & Schuell, Inc., Keene, N.H.) as
described by Towbin et al. (47). Mouse antisera were diluted to 1:75 in PBS containing 3% powdered nonfat milk (Carnation) and incubated overnight at 4°C with the nitrocellulose strips. The nitrocellulose strips were washed four times in wash buffer (PBS with 3% Carnation milk) and then incubated with 125I-labeled, affinity-purified rabbit F(ab')2 anti-mouse immunoglobulin (2 x 10^6 cpm/μg; 10^6 cpm/ml) for 1 h. Excess radioiodinated probe was removed by extensive rinsing with wash buffer. The strips were air dried and exposed for autoradiography with Kodak X-Omat AR film (XAR-5) to detect antigens.

**Gel electrophoresis.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (22), with 12% polyacrylamide gels.

**Determination of levels of specific immunoglobulin isotypes.** The antibody titers of immunized and nonimmunized mice were determined. Pooled mouse sera (from experiment 2, 3 weeks after the final immunization) were used with peroxidase-conjugated subclass-specific goat antisera (Litton Bionetics, Kensington, Md.) as recommended by the manufacturer. Thus, both quantitative (titer) and qualitative evaluations were made. Briefly, sonicated *L. amazonensis* membrane preparations were diluted in PBS (50 to 100 μg/ml), and 50 μl was placed in wells of flat-bottom polystyrene microtiter plates (Falcon 3912; Microtest III; Becton Dickinson) overnight at 4°C. The plates were washed extensively with PBS-fetal bovine serum and then incubated with twofold serial dilutions of mouse serum overnight at 4°C. The plates were then washed and incubated for 1 h at room temperature with diluted peroxidase-conjugated mouse isotype-specific antisera. The plates were washed again and then incubated for 15 to 30 min with 2,2’azino-di-(3-ethyl-benzthiazoline) sulfonate in 0.03% H2O2. The plates were read at 414 nm with a Titertek Multiscan. Titers were determined in duplicate at 50% maximal binding.

**RESULTS**

**Role of adjuvant in protective immune response.** In a preliminary experiment to determine the efficacy of the M-2 glycoprotein as a protective antigen, BALB/c mice were vaccinated intraperitoneally three times at weekly intervals with complete or incomplete Freund adjuvant together with 5 to 8 μg of M-2 glycoprotein. The mice were challenged a month after the final immunization with either 10^4 or 10^5 late-log-stage promastigotes of *L. amazonensis*. Partial protection was observed, resulting in a delay in the onset of disease of 6 to 8 weeks in comparison with those that received incomplete or complete Freund adjuvant alone; however, no significant difference was detected between the group of animals that received the M-2 glycoprotein and complete versus incomplete Freund adjuvant (data not shown).

In the next set of protective immunization studies, experiment 1, the adjuvant used in conjunction with the M-2 glycoprotein was varied in an attempt to maximize the level of protection afforded by the M-2 glycoprotein in BALB/c mice. Saponin and *C. parvum* were chosen and tested against incomplete Freund adjuvant to determine which elicited an optimal immune response. The adjuvants have been successfully used in vaccine studies in conjunction with antigens in other protozoan parasite systems (28, 33, 34, 42). The details for the immunization schedules are given in Materials and Methods. Mice were challenged with either 10^5 or 10^3 late-log phase *L. amazonensis* promastigotes. Consecutive measurements of lesion size (Fig. 1) showed significant protection in the groups that received the M-2 glycoprotein and adjuvant, but better protection was observed when *C. parvum* and saponin were used as adjuvants in comparison with incomplete Freund adjuvant in animals challenged with 10^3 *L. amazonensis*. The immunized mice were tested for long-term immunity; all eventually succumbed to disease, except the *C. parvum*-M-2 glycoprotein group, in which 28% (3 of 11) of the mice infected with 10^3 *L. amazonensis* remained disease free for 9 months postchallenge.

**Immunization of susceptible and resistant mouse strains.** In experiment 2, *C. parvum* and saponin were used as adjuvants to test the effect of immunization of relatively resistant (C57BL/6) (3, 29, 41) and susceptible (CBA and BALB/c) (9) strains against an infective challenge of *L. amazonensis*. The CBA strain of mice, although resistant to infection with *L. major*, has been observed to be susceptible to infection with *L. mexicana* and *L. amazonensis* (9; D. McMahon-Pratt, unpublished data), resulting in progressively growing lesions. However, the course of infection appears to differ from that observed in BALB/c mice in that in the initial phase of infection the parasite burden (based on limiting-dilution analysis) appeared to be contained for a period of time (months), dependent on the inoculating dose; subsequently, the nodules began to grow in an apparently unrestrained manner, similar to that observed in BALB/c mice. In addition, the threshold inoculation dose for infection was about 10-fold higher for CBA mice than for BALB/c mice when *L. amazonensis* LTB0016 was used. C57BL/6 mice maintained a chronic but contained infection for up to 6 months postinoculation with either 10^3 or 10^5 LTB0016 strain promastigotes.

On the basis of lesion size, immunization with *C. parvum* and the M-2 glycoprotein showed the best protection from disease in the three strains of mice studied (Fig. 2). Of the BALB/c mice, 50% of those that received both 10^5 and 10^3 *L. amazonensis* promastigotes (3 of 6 in each group) showed no lesion development (signs of infection) at 5 months postin-
FIG. 2. Results of immunization experiment 2 with BALB/c, CBA, and C57BL/6 mice with the M-2 glycoprotein alone (○), M-2 and saponin (△), and M-2 and C. parvum (□). Control groups of animals received C. parvum (●) or no treatment (■). Standard errors are indicated. Development of lesion size with time is shown. The course of infection found in mice that received saponin alone was identical to that of normal (untreated) mice.

Infection. The rest of the mice in these groups developed lesions smaller than those of the mice in the control groups; the visible onset of disease in the protected group was delayed from that observed in the control groups. Interestingly, intravenous immunization with the M-2 glycoprotein and saponin gave no apparent protection on the basis of lesion measurements.

Protection of BALB/c mice against infection was also
TABLE 1. Results of limiting-dilution analysis of parasite burden of BALB/c mice immunized with M-2 glycoprotein

<table>
<thead>
<tr>
<th>Immunization group</th>
<th>Log$_{10}$ viable L. amazonensis organisms recovered postinfection with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^5$ promastigotes</td>
</tr>
<tr>
<td>Control</td>
<td>6.75</td>
</tr>
<tr>
<td>C. parvum</td>
<td>6.56</td>
</tr>
<tr>
<td>Saponin</td>
<td>6.45</td>
</tr>
<tr>
<td>M-2</td>
<td>6.40</td>
</tr>
<tr>
<td>M-2-C. parvum</td>
<td>1.62</td>
</tr>
<tr>
<td>M-2-saponin*</td>
<td>4.83</td>
</tr>
</tbody>
</table>

*Mice that received $10^5$ promastigotes were analyzed at 8 weeks postinfection, and mice that received $10^3$ promastigotes were analyzed at 9 weeks postinfection, before obvious lesion development (Fig. 2C and D). Determinations were done in duplicate, as previously described (44), by using two mice from each group.

* Intravenous immunization.

quantitated as a function of parasite burden by using a limited-dilution assay (46). The results of these experiments (Table 1) essentially confirm the results of lesion size measurements. This technique is more sensitive than lesion size measurement (16, 17, 46) and allows for examination of protection in an earlier phase of infection. A 2 to 5 order of magnitude difference in the levels of viable parasites recovered was found between the groups immunized with M-2 and C. parvum and the control groups. In addition, the limiting-dilution analysis showed a low level of protection in mice that received M-2 glycoprotein together with saponin intravenously, although this effect was not evident by lesion size. The reason for the quantitative difference in the level of protection observed between the two infected groups ($10^5$ and $10^3$) is unclear but parallels the differential found by lesion measurement (Fig. 2). Since the groups that received $10^3$ versus $10^5$ L. amazonensis were infected at different times, a difference in the infectivities of the parasites could account for this variation.

The CBA mice were immunized by a protocol identical (antigen preparation, adjuvant preparation, and schedule) to that used for BALB/c mice. Complete protection was observed for mice that received M-2 together with C. parvum. No protection was observed for the groups that received C. parvum or M-2 alone. Surprisingly, the group immunized with the M-2 glycoprotein alone and infected with $10^6$ L. amazonensis showed exacerbation of the disease when compared with those in the nontreated control group or those that received C. parvum alone. This phenomenon was not apparent in M-2-immunized animals that received the lower infective challenge dose of L. amazonensis ($10^5$), in which, although lesions were slightly larger than those of the control group, the difference was not significant.

When immunized with the M-2 glycoprotein, C57BL/6 mice showed no measurable protection at the $10^6$ challenge dose of parasites. At the lower infective dose ($10^5$), partial protection was observed in the group that had received M-2 and C. parvum but not in those that had received M-2 and saponin (Fig. 2). Fifty percent of the animals (3 of 6) that received the lower inoculating dose were completely protected, showing no lesion development through the 6 months of observation postinfection.

Immunization of BALB/c mice subcutaneously with saponin and M-2. Since Howard and co-workers have described variation in protective immunity with the routes of vaccination (18, 26) and since the results with saponin and M-2 in experiment 1 (subcutaneous immunization) differed from those of experiment 2 (intravenous immunization), the immunological protection elicited by subcutaneous immunization of glycoprotein and saponin was reevaluated in BALB/c mice (experiment 3). The results of this experiment are shown in Fig. 3. BALB/c mice were infected with $10^5$ late-log-phase promastigotes of L. amazonensis (●). Standard errors are indicated. Development of lesion size with time is shown.

Infect. Immun.

FIG. 3. Results of immunization experiment 3 with BALB/c mice immunized with the M-2 glycoprotein and saponin subcutaneously (●) or control groups of mice challenged with $10^5$ late-log-phase promastigotes of L. amazonensis (○). Standard errors are indicated. Development of lesion size with time is shown.

Antibody response to immunization. The antibody response to vaccination was examined in the sera of immunized mice (CBA, BALB/c, and C57BL/6; experiment 2). The sera were collected 3 weeks after the final immunization—1 week before infection with L. amazonensis. The results from these experiments are given in Table 2. In general, the highest antibody response levels were seen in protectively immunized animals. Elevated antibody levels were observed in the immunoglobulin M (IgM), IgG1, and IgG2 subclasses in mice that received M-2 and C. parvum. The antibody responses of CBA and BALB/c mice were significantly greater than those of C57BL/6 mice. This difference in immune response as measured by antibody titer and isotype appears to reflect the response of the individual strain to the M-2 glycoprotein in the context of the adjuvants used, since the response of C57BL/6 mice to the M-2 glycoprotein alone was comparable to that of CBA mice. This suggests a role for T cells in this protection, since they influence the quality and quantity of the antibody response. It should be noted that the three strains of mice were immunized at the same time with identical antigen and adjuvant preparations.

Specificity of immune response to M-2. The specificity of
TABLE 2. Antibody response by subclass in mice immunized with M-2 glycoprotein

<table>
<thead>
<tr>
<th>Antibody subclass</th>
<th>BALB/c mice</th>
<th>CBA mice</th>
<th>C57BL/6 mice</th>
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</thead>
<tbody>
<tr>
<td>IgM</td>
<td>23</td>
<td>70</td>
<td>226</td>
</tr>
<tr>
<td>IgG1</td>
<td>&lt;10</td>
<td>452</td>
<td>6,860</td>
</tr>
<tr>
<td>IgG2a</td>
<td>&lt;10</td>
<td>40</td>
<td>2,263</td>
</tr>
<tr>
<td>IgG2b</td>
<td>&lt;10</td>
<td>40</td>
<td>2,770</td>
</tr>
<tr>
<td>IgG3</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>40</td>
</tr>
</tbody>
</table>

* Results of antibody determinations with isolated promastigote membranes as antigens. Nonimmunized control mice had titers identical to those of mice that received C. parvum or saponin alone in a preliminary radioimmunoassay to determine total antibody levels.

the response of the immunized animals to the M-2 glycoprotein was assessed by Western blot analysis with membrane preparations of L. amazonensis as the antigen. Since M-2 is a glycoconjugate with one N-linked carbohydrate side chain of 2 kDa (32; unpublished data), it was possible that the protection observed was a result of additional cross-reaction with other carbohydrate-containing components. In addition, preparations of M-2 made subsequent to this study showed a minor (≈1%) contaminant at 65 kDa, visible only on silver-stained sodium dodecyl sulfate-polyacrylamide gels. The immunized mice appeared to recognize primarily a 46-kDa (M-2) component (Fig. 4). On longer exposures, weaker bands were apparent at higher molecular masses, as well as with components of 30 and 24 kDa, consistent with the proteolytic breakdown products of the M-2 molecule (20) observed to occur at a low level during membrane preparation.

DISCUSSION

In these studies we investigated the potential of the 46-kDa M-2 glycoprotein to elicit a protective immune response against an infective challenge of L. amazonensis promastigotes. The M-2 glycoprotein is molecularly distinct from (i) the lipid-containing glycoconjugate of L. major (13, 14) that has been demonstrated to confer protection and (ii) GP-10/20, the glycoconjugates of L. amazonensis (36) that have been shown to induce cellular responses in infected and immunized mice. Protein structural studies (32) and sequence data have indicated that the M-2 glycoprotein and the GP63 protein are molecularly distinct. The M-2 glycoprotein appears to be specific for the promastigote developmental stage of the parasite and is found on infective promastigotes developing within the insect vector, the phlebotomine sandfly (30, 31). The effect of promastigote stage specificity of the M-2 molecule on the protection elicited is not clear. An effect on the early phase of infection, resulting in either complete protection or delay of onset of disease, is consistent with what might be expected for a promastigote stage-specific molecule. However, a similar pattern of protection was observed in BALB/c mice immunized with C. parvum and the macroglycolipid antigen (excreted factor) (14) which is present on both developmental stages of the parasite. Consequently, other factors (adjuvant, mouse strain) may also determine the extent of protection.

Experiments with C. parvum, saponin, and incomplete Freund adjuvant as adjuvants consistently indicated that C. parvum was the most effective adjuvant in eliciting a protective immune response. Saponin has been used successfully as an adjuvant in protective immunity studies of other parasitic protozoa (28, 29, 42) in which, in certain cases, C. parvum was ineffective; however, saponin was not as effective an adjuvant as C. parvum in these studies. C. parvum has been shown to be an effective adjuvant in eliciting a protective response against infection with L. major when used together with whole parasites or a purified lipid-containing glycoconjugate (14, 33). In addition, a recent study has shown that simultaneous injection of C. parvum with an infective inoculum of L. major led to modulation of disease (15); treatment with Mycobacterium bovis BCG has given similar effects in human patients (6). C. parvum has been reported to enhance the T helper cell-antibody response to erythrocytes (IgG2a> IgM> IgG1) (48). Enhancement of the antibody response was observed in this study; a significant effect was observed in the IgG1 subclass, with the levels of antibody as follows: IgG1> IgG2a> IgM> IgG3. C. parvum has also been reported to enhance the level of natural killer (NK) and macrophage cell activity. NK cell activity has been indicated to affect the course of leishmanial infection (21). Since the NK activity stimulated by C. parvum may last for several weeks (45), NK cell activity may be involved in or facilitate the protective response observed here. Further work is required to determine this. The macrophage activation by C. parvum appears, however, to be
short-lived (a period of days) (44), and C. parvum-elicited macrophages have been reported to be tumoricidal but not leishmanicidal (12). In addition, no apparent nonspecific adjuvant effects were observed in the control groups of mice that received C. parvum alone. Therefore, the action of C. parvum on macrophages in this study most likely results in the enhanced immune response to M-2 observed (T-cell-B-cell activation) rather than a direct leishmanicidal macrophage effect. With C. parvum, the level of protection observed in the three groups of mice studied varied from apparently complete protection, as observed in the susceptible CBA strain, to protection at only the 10^4 challenge infecting dose, as observed in the relatively resistant C57BL/6 strain. In the susceptible BALB/c strain and the C57BL/6 strain (10^4 inoculum), some of the animals (3 of 6 or 50%) immunized with C. parvum together with M-2 were completely protected while the rest were partially protected, showing a delay in onset of the disease. The reason for the differences observed in level of protection elicited in the three strains of mice are unclear, although the best protection obviously occurred in the strains of mice most susceptible to infection with L. amazonensis. Differences have also been observed in the immune responses of these mouse strains to infection with L. amazonensis. Immunocytochemical studies of leishmanial lesion development suggest that, in contrast to C57BL/6 mice, there is in BALB/c mice a defect in T-cell (both L3T4+ and Lyt-2+) influx into the infected area (29). Moreover, lymphokine-activated C57BL/6 macrophages have been shown to fail to destroy L. amazonensis (41). Since these two mouse strains apparently differ in their immunological responses to the infecting leishmanial parasite, the immune effector mechanisms that must be activated by immunization to elicit a protective response may differ as well.

In addition, there is a difference in the immune response levels observed in the three mouse strains, as indicated by the levels of antibodies specific for the M-2 glycoprotein. The overall response level was greater in BALB/c and CBA mice than in C57BL/6 mice. Within the immunized groups of CBA and BALB/c mice, the level of protection correlated with the level of the antibody to M-2. The role of the antibody itself in protection against leishmaniasis is controversial. In transfer experiments, T cells but not the antibody from BALB/c mice protectively immunized with irradiated promastigotes of L. major gave significant protection, although antibody levels of the immunized donor mice showed a correlation with protection. Consequently, although the antibody level was indicative of the immune status of the animals, it did not appear to directly affect the protection observed (18, 26). However, studies of suppressed mice (38, 39) suggest a role for B-cell-modulated effector T cells. Moreover, passive transfer of monoclonal antibodies to specific leishmanial components (2, 8, 14) can protect against an infective challenge. The role of antibodies in the protection observed in this study is unclear. Antibody levels may parallel the activation of specific effector mechanisms required for a protective response. The roles of T cells (subsets), B cells, and immune effector mechanisms (lymphokines) involved in the protection induced by the M-2 molecule are currently under investigation.

Previous studies with animal models and humans (1, 24) indicated that cross-protection occurs between Leishmania species that cause the cutaneous form of the disease. Recent experiments with heteroserum to the M-2 molecule (McMahon-Pratt, unpublished data), as well as recent Southern blot analyses (32) with the cloned M-2 gene suggest that the M-2 protein is not restricted to L. amazonensis, although the epitopes recognized by various monoclonal antibodies are species specific. Work is in progress to verify this. If the M-2 molecule or a member of a related family is present on various Leishmania species, the effect of M-2 in eliciting a protective immune response against other Leishmania species would be of interest. Recent efforts to develop a vaccine for leishmaniasis have focused on delineating defined antigenic components that produce an effective protective response, either alone or in combination with other defined antigenic molecules. This study suggests that the M-2 glycoprotein would be useful for the production of a defined vaccine against leishmaniasis.

ACKNOWLEDGMENTS

We thank Carmel Bierwirth for help in preparation of the manuscript and Vivian Kim for technical assistance.

The work was supported by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, a Public Health Service grant from the National Institutes of Health (AI-23004) and a grant from the MacArthur Foundation. J. C. J. was supported by medical student research fellowships from the American Heart Association (85-501) and the National Heart, Lung, and Blood Institute (T-35HL7479).

LITERATURE CITED


