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Evidence for T-Cell Recognition in Mice of a Purified Lipophosphoglycan from *Leishmania major*

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We have previously reported that a *Leishmania major* lipophosphoglycan (LPG), given with killed *Corynebacterium parvum* as an adjuvant, can vaccinate mice against cutaneous leishmaniasis. In order to analyze whether T cells are able to recognize this important parasite antigen, we have studied both humoral and cellular immune responses to *L. major* LPG that had been isolated from promastigotes by sequential solvent extraction and hydrophobic chromatography. The data show that immunization of mice with highly purified LPG induced an increase in frequency of *L. major*-reactive T cells and the production of immunoglobulin G antibodies to LPG. Furthermore, genetically resistant mice infected with *L. major* were able to develop a specific delayed-type hypersensitivity response in the ear to *L. major* LPG. These findings strongly suggest that T cells can recognize and respond to glycolipid antigens, in this case a host-protective *Leishmania* LPG, even though such antigens appear not to be potent T-cell stimulators in mice.

Leishmania spp. are digenetic protozoan parasites alternating between the promastigote form in the sand fly vector and the obligatory intracellular amastigote form that resides in phagolysosomes of mammalian macrophages. Two parasite molecules have been implicated in the process of invasion of host macrophages, a glycoprotein (gp63) that binds to the complement receptor CR 3 on macrophages (2) and a lipophosphoglycan (LPG) (14). Both gp63 and LPG have a similar mode of anchoring into the parasite plasma membrane via glycosyl inositol phospholipids (2, 25, 35) which resemble the membrane anchors of several eucaryotic proteins (see reference 24 for a review), including the *Trypanosoma brucei* variant surface glycoproteins (3, 8).

Leishmania major is the cause of cutaneous leishmaniasis, and the spectrum of disease patterns seen in humans can be reproduced in mice. Mice of genetically resistant inbred strains (e.g., C57BL/6) can control the infection, whereas genetically susceptible mice (e.g., BALB/c) develop progressive disease. A considerable body of experimental evidence demonstrates the importance of T-cell-dependent immunity in both resistance and susceptibility to disease (18, 23). Resistance to cutaneous leishmaniasis can be attributed to lymphokines with macrophage-activating properties produced by specifically sensitized T cells. There is evidence that these resistance-promoting T cells belong largely, if not exclusively, to the L3T4$^+$ subpopulation (21, 28, 31). Various *L. major* parasite antigen preparations have been shown to induce host protection in mice (9, 17, 39, 42), and there is considerable effort to develop a molecularly defined vaccine for immunoprophylaxis or immunotherapy of cutaneous leishmaniasis in humans. The two best-defined vaccine candidates are the parasite surface antigens gp63 and LPG, epitopes of at least the latter being detected on the surfaces of infected macrophages by using monoclonal antibodies (15).

Studies in our laboratory have focused on the biochemical and immunological characterization of *L. major* LPG. We have shown that intraperitoneal administration of the purified antigen with *Corynebacterium parvum* as an adjuvant can vaccinate genetically susceptible mice against cutaneous leishmaniasis (17, 25). In contrast, immunization with the hydrophilic carbohydrate (CHO) moiety of LPG, which is released into the culture supernatant of promastigotes, is nonprotective and, when given with Freund complete adjuvant, can exacerbate lesion development (29). In order to implicate T cells in protection, we have studied both humoral and cellular immune responses to highly purified LPG from *L. major*. Here we present evidence that T cells recognize and are activated by LPG. Our data show that LPG-immunized mice contain an increased frequency of *L. major*-reactive T cells and produce T-cell-dependent immunoglobulin G (IgG) antibodies to LPG. Furthermore, mice infected with *L. major* parasites develop specific delayed-type hypersensitivity (DTH) to *L. major* LPG.

MATERIALS AND METHODS

Mice. Female mice of the inbred strains BALB/c, BALB/c.H-2^k, and C57BL/6 were used at an age of 7 to 12 weeks. The mice were bred under specific pathogen-free conditions at the Walter and Eliza Hall Institute but during experimentation were maintained under conventional conditions in an isolation facility. For some experiments, mice were purchased from Charles River Breeding Laboratories, Inc., Sulzfeld, Federal Republic of Germany.

Parasites and preparation of antigens. The cloned virulent *L. major* parasite line V121 was produced from human isolate LRC-L137 (16) that was initially obtained from the World Health Organization Reference Center for Leishmaniasis, Jerusalem, Israel. For infection of mice, promastigotes were grown in blood-agar cultures (13). Stationary-phase promastigotes were washed, and 2×10^6 organisms were injected in a volume of 50 μl intradermally on the dorsum of the mouse close to the base of the tail. For preparation of antigen, promastigotes were grown in Schneider *Drosophila* medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum (Flow Laboratories, North Ryder, N.S.W., Australia). Antigen for DTH experiments and for in vitro stimulation of lympho-

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cytes was generated by UV irradiation (254 nm) of *L. major* promastigotes for 20 min. This treatment resulted in parasite death, as judged by staining with ethidium bromide-acridine orange (1 μg/ml) and the failure to infect mice. Antigen was stored at −20°C.

L. major LPG was purified chemically from promastigotes by sequential solvent extraction and hydrophobic chromatography on octyl-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden), as described in detail by McConville et al. (25). Each batch of LPG was carefully checked for residual protein, both colorimetrically (Protein Assay; Bio-Rad Laboratories, Richmond, Calif.) and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (25). However, contamination with protein could not be detected, and the purity of the preparations is estimated to be greater than 99.5%. The carbohydrate moiety (*L. major* CHO) was obtained by incubating the purified LPG with phosphatidylinositol-specific phospholipase C isolated from *Staphylococcus aureus* (25), a generous gift from M. Low, Columbia University, New York, N.Y., and octyl-Sepharose chromatography to separate the hydrophilic (CHO) and hydrophobic (LPG) forms.

Soluble *Schistosoma japonicum* egg antigen was prepared by homogenization and sonication of eggs, followed by centrifugation (100,000 × *g*) and collection of the supernatant. A family of *S. japonicum* glycoconjugates (termed ABCD) was obtained by affinity purification of *S. japonicum* egg antigen, using a series of monoclonal antibodies that react with carbohydrate epitopes on *S. japonicum* eggs. The procedure is described in detail elsewhere (44a), and the antigen preparations were kindly provided by W. U. Tiu.

Immunization. Multilaminated liposomes were prepared from dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidylserine (7:3, wt/wt; Sigma Chemical Co., St. Louis, Mo.). The lipids (32 mg) were dried as a film, the *L. major* LPG (10 μg) in phosphate-buffered saline (PBS) was added, and the whole mixture was vortexed. Mice were injected intraperitoneally with 10 μg of LPG in liposomes together with 100 μg of *C. parvum* (Wellcome Research Laboratories, Beckenham, England). Control groups of mice were injected with *C. parvum* alone or with *C. parvum* and liposomes. Injections were repeated after 20 days, and mice were challenged intradermally with 2×10^6 live *L. major* promastigotes 14 days after the second immunization. Experimental groups consisted of five to seven mice.

Assessment of antibody titers. Antibody titers in sera from immunized mice were assessed by using an enzyme-linked immunosorbent assay. The mice were bled 7 days after the second immunization (i.e., before infection with parasites) and 28 days after the challenge with live parasites. Sera from mice of the same experimental group were pooled. The enzyme-linked immunosorbent assays were performed in 96-well round-bottom polyvinyl chloride plates (Dynatech Industries, Inc., Chantilly, Va.) in a volume of 50 μl per well. For determination of anti-*L. major* antibodies, serial dilutions of sera were dispensed into wells precoated with 10^5 *L. major* promastigotes that had been fixed with 0.25% glutaraldehyde in PBS (15). For determination of anti-LPG antibodies, the wells were precoated with poly-L-lysine (5 μg/ml in distilled water) and *L. major* LPG (5 μg/ml). Before the addition of serum, unsaturated binding sites were blocked with 1% skim milk powder–0.05% Tween 20 in PBS. After the serial dilutions of serum were incubated for 2 h at room temperature, the plates were washed repeatedly in PBS, horseradish peroxidase-coupled sheep anti-mouse immunoglobulins (Fab fragments; Silenus Laboratories, Melbourne, Australia) were added, and the plates were incubated for 2 h at 37°C. For assessment of immunoglobulin isotypes, horseradish peroxidase-coupled goat anti-mouse immunoglobulin isotype-specific reagents (Southern Biotechnology Associates, Birmingham, Ala.) were used. After a washing, the substrate 2,2′-azino-di-(3-ethylbenz-thiazoline-sulfonic acid) (ABTS; Sigma) was added, and the absorbance in the wells was read 30 min later with a Titertek Multiskan ML (Flow Laboratories), using dual wavelengths (414 and 492 nm). Control wells lacking antibody consistently gave an absorbance value of <0.01 optical density units. The units of antibody activity were calculated by correlating the dilution of the test sample with the dilution of the standard sample at a given optical density value in the linear range. The standard serum was an internal standard that was chosen from a series of test sera (from LPG-immunized mice), as it gave representative titers for all immunoglobulin isotypes. A 10^{-4} dilution of the standard serum at the respective optical density value in the linear range was defined as the titer corresponding to an antibody activity of 1,000 U.

Limiting-dilution analysis. Spleen cell suspensions were prepared 7 days after the second immunization of mice with *L. major* LPG in liposomes and *C. parvum*. L3T4$^+$ cells were obtained by treatment with anti-Ly-2 antibodies, from hybridoma YTS 169.4 (4), followed by incubation with rabbit complement, as described elsewhere (40). In some experiments, the lymphocytes were immediately tested in limiting-dilution analysis to determine the frequency of interleukin-2 (IL-2)- and IL-3-producing precursor cells. In other experiments, the responder cells were subjected to an additional cycle of in vitro restimulation and rest (37). A total of 3×10^6 cells were cultured with 5×10^6 syngeneic irradiated (3,000 rad) spleen accessory cells and 3×10^6 UV-irradiated *L. major* promastigotes in a total volume of 1.5 ml in flat-bottom culture wells. After 4 days, viable cells were isolated from the bulk cultures by centrifugation over Ficoll-Hypaque and placed into rest cultures consisting of 4×10^5 responder cells and 5×10^6 syngeneic spleen accessory cells, in the absence of antigen, for another 7 days. Viable cells were again isolated over Ficoll-Hypaque and tested by limiting-dilution analysis for the presence of IL-2- and IL-4-secreting cells.

Limiting numbers of lymphocytes were cultured in round-bottom microdilution wells (Linbro; Flow Laboratories) with 2×10^5 irradiated (3,000 rad) syngeneic spleen accessory cells from normal mice in 0.2 ml of a modified RPMI 1640 culture medium (supplemented with 32 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 10^{-4} M 2-mercaptoethanol, and 10% fetal calf serum) containing 15% supernatant from concanavalin A-stimulated rat spleen cell cultures as a source of IL-2. In some experiments, limiting-dilution cultures were supplemented with soluble *L. major* CHO (40 μg/ml) that had been prepared by affinity purification of *L. major* culture supernatant on a column containing monoclonal antibody WIC-79.3 (17). For each T-cell concentration ranging from 10^4 to 150 cells per well, replicates of 24 wells were set up in the absence or presence of 2×10^5 UV-irradiated *L. major* promastigotes as a source of antigen. After 7 days of incubation (37°C, 10% CO_2), microcultures were washed three times to remove the supernatant from the concanavalin A-stimulated rat spleen cell cultures and were restimulated by adding 2×10^5 irradiated (3,000 rad) T-cell-depleted syngeneic spleen cells in the absence or presence of antigen (2×10^5 UV-irradiated *L. major* promastigotes), in 0.2 ml of culture medium. Twenty-

four hours thereafter, 0.15 ml of supernatant was collected from each well for the determination of lymphokine activity. Cultures containing T cells and accessory cells but no antigen were used as controls for each T-cell concentration. Antigen-stimulated microcultures were scored positive when the values of lymphokine activity exceeded the arithmetic mean of the control wells by more than 3 standard deviations. Minimal estimates of the precursor frequency for each activity were obtained by the maximum-likelihood method from the Poisson distribution relationship between the number of responding cells and the logarithm of the fraction of negative cultures (11).

Assays for lymphokine activity. Microculture supernatant was tested for IL-2 or IL-3 activity by incubation with the cloned IL-2-dependent T-cell line CTLL (10) or the IL-3-dependent hematopoietic cell line 32D (12, 27), respectively. The cell lines were kindly provided by Anne Kelso of the Walter and Eliza Hall Institute. A 50-μl volume of supernatant was cultured with 4×10^3 CTLL cells or 2×10^3 32D cells in a total volume of 0.1 ml of RPMI 1640 culture medium in flat-bottom microwells (Linbro; Flow Laboratories). After 20 h (CTLL assay) or 44 h (32D assay), 1 μCi of [^{3}H]thymidine was added for a further 4 to 5 h. Cells were harvested onto glass fiber filter strips by using an automated cell harvester and counted in a liquid scintillation counter.

For simultaneous determination of IL-2 and IL-4 activities, supernatants from individual limiting-dilution cultures were divided into triplicate aliquots. Each aliquot of 50 μl was tested for its ability to stimulate proliferation of 3×10^3 cells of the mouse HT-2 T-cell line (45) in a 0.1-ml flat-bottom culture well. After 48 h, responsiveness was measured by using a modified colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (44). One aliquot was incubated in the absence of antibodies, to test for IL-2 and IL-4 activities. A second aliquot was tested in the presence of anti-IL-2 antibodies, from the hybridoma S4B6 (32), for the determination of IL-4 activity. The third aliquot was tested in the presence of both anti-IL-2 and anti-IL-4 antibodies from the hybridoma 11B11 (34).

Assay for DTH reactivity. The test antigen was administered into the left ear of each mouse after the optimal concentration had been determined according to a dose-response curve for each individual antigen. A 10-μl volume of the appropriate antigen concentration in PBS was injected intradermally, using a 100-μl Hamilton syringe mounted with a 30-gauge Yale needle. The right ear of each mouse remained untreated. After 15, 24, and 48 h, the thicknesses of both ears were measured with an engineering micrometer, and the degree of swelling was expressed as the difference in thickness of the antigen-injected and the control ears. Experimental groups of five to eight mice were infected with *L. major* promastigotes and tested for DTH reactivity at various time points after infection.

Preparation of tissues for histology. Skin test sites were prepared for histological examination immediately after skin test reading. Mice were killed, and excised tissue was fixed in Bouin solution, embedded in paraffin wax, sectioned at 5 μm, and stained with hematoxylin-eosin.

RESULTS

Immunization of mice with *L. major* LPG induces an increase in frequency of *L. major*-reactive T cells. Previous studies in our laboratory have shown that purified *L. major* LPG, injected with killed *C. parvum* as an adjuvant, vaccinates genetically susceptible mice against cutaneous leishmaniasis (17, 25). In order to analyze whether immunization of susceptible mice with LPG and *C. parvum* induces the activation and expansion of *L. major*-specific T cells, we determined the frequency of precursor T cells that responded to *L. major* antigen by production of lymphokines.

TABLE 1. Frequency of *L. major*-reactive precursor T cells in mice immunized with *L. major* LPG[a]

Treatment	Reciprocal of frequency of lymphokine-producing cells (95% confidence limits)	
	IL-2	IL-3
LPG in liposomes + *C. parvum*	11,990 (8,795–18,832)	10,314 (7,716–15,576)
C. parvum	24,154 (16,000–49,261)	27,100 (18,348–51,813)
None	ND[b]	95,740 (55,726–339,564)

[a] Limiting numbers of T cells from BALB/c.H-2^k mice were cultured with syngeneic accessory cells in the presence or absence of *L. major* antigen and were restimulated 7 days later. At 24 h thereafter, supernatants from individual cultures were tested for IL-2 activity on CTLL cells or for IL-3 activity on 32D cells.

[b] ND, Not determined.

A limiting-dilution system was used for frequency estimation. Lymphocytes were collected 7 days after the second immunization of BALB/c.H-2^k mice with *L. major* LPG in liposomes and *C. parvum*. Lymphocytes from syngeneic control mice were used for comparison. Limiting numbers of nylon wool-purified T cells were stimulated in vitro with UV-inactivated *L. major* promastigotes and accessory cells in the presence of supernatant from concanavalin A-stimulated rat spleen cell cultures. As a control, a second series of limiting-dilution cultures was set up in the absence of antigen for each responder-cell population. For detection of *L. major*-reactive T cells, lymphokine production was found to be much more antigen specific, and gave considerably lower background activity, than lymphoproliferation. Hence, supernatants from individual microcultures were split and assayed for IL-2 and IL-3 activities as a readout, in the limiting-dilution analysis, for responsiveness. Table 1 summarizes the frequencies of *L. major*-specific precursor T cells from LPG-immunized and control mice. The data show that the frequency of lymphokine-producing T cells increased more than twofold in mice immunized with LPG (IL-2, 1/11,900; IL-3, 1/10,314), compared with those of mice injected with *C. parvum* alone (IL-2, 1/24,154; IL-3, 1/27,100). Treatment with *C. parvum* alone also resulted in some stimulation of T-cell activity over that seen in untreated mice (frequency [*f*] = 1/95,740).

In a further experiment, it was attempted to characterize the type of T cell that is being activated. For this purpose, purified L3T4$^+$ cells from BALB/c mice were used in limiting-dilution analysis. and the culture supernatants were tested on HT-2 cells, in the presence or absence of anti-IL-2 antibodies, for IL-2 and IL-4 production. As reported previously (37), it was difficult to detect IL-4-producing cells in cultures of freshly prepared lymphocytes from antigen-primed mice. However, IL-4 secretion could be demonstrated in limiting-dilution cultures set up after a cycle of in vitro restimulation with *L. major* antigen and an inteval of culture in the absence of antigen (37). The frequency of restimulated *L. major*-reactive L3T4$^+$ cells with lymphokine activity (IL-2 and IL-4) was very much higher in LPG-treated mice (1/258; 95% confidence limits, 1/198 to 1/370) than in control mice treated only with liposomes and *C. parvum* (1/6,274; 95% confidence limits, 1/3,473 to 1/32,124). When the culture supernatants were assayed in the presence

TABLE 2. Effect of soluble *L. major* CHO on frequency of *L. major*-reactive precursor T cells[a]

Expt	Cells	Culture supplement	Reciprocal of frequency of lymphokine-producing cells (95% confidence limits) in the presence of:	
			No antibodies	Anti-IL-2
1	T	None	4,328 (3,121–7,056)	ND[b]
		L. major CHO	18,053 (11,954–36,310)	ND
2	L3T4$^+$	None	935 (670–1,551)	6,742 (4,814–11,169)
		L. major CHO	3,195 (1,831–12,558)	15,020 (10,404–27,043)

[a] All BALB/c mice were treated with LPG in liposomes and *C. parvum*. After a cycle of in vitro stimulation with *L. major* antigen followed by an interval of culture in the absence of antigen, limiting numbers of selected spleen cells were cultured with syngeneic accessory cells and *L. major* antigen. Cultures were restimulated 7 days later, and 24 h thereafter, supernatants were collected and tested for lymphokine activity on HT-2 cells in the presence or absence of anti-IL-2 antibodies. Limiting-dilution cultures were set up and restimulated in the presence or absence of 40 μg of soluble *L. major* CHO per ml.
[b] ND, Not determined.

of anti-IL-2 antibodies, residual activity (f = 1/1,197; 95% confidence limits, 1/919 to 1/1,712) could be detected only in cultures containing T cells from LPG-immunized animals (for control mice, f < 1/100,000). All this remaining activity was inhibited when the supernatants were tested in the additional presence of anti-IL-4 antibodies (data not shown), indicating that it could in fact be attributed to IL-4. These data demonstrate that immunization of mice with LPG results in the induction of both IL-2- and IL-4-producing *L. major*-specific L3T4$^+$ cells.

The addition to limiting-dilution cultures of the soluble CHO moiety of *L. major* LPG, purified from promastigote culture supernatant, caused a significant reduction in frequency of *L. major*-reactive T cells (Table 2). This evidence for competitive inhibition by soluble *L. major* CHO suggests that a large proportion of *L. major*-reactive T cells from LPG-immunized mice recognizes a CHO epitope of LPG. The addition of mannan or dextran did not have any effect, demonstrating the specificity of the inhibition (data not shown). Conversely, *L. major* CHO did not block the response of lymphocytes from infected BALB/c mice to *L. major* promastigotes (data not shown).

Immunization of mice with *L. major* LPG induces a specific IgG antibody response. At 7 days after the second immunization of BALB/c.H-2^k mice with *L. major* LPG in liposomes and *C. parvum* (i.e., before infection with parasites) as well as 28 days after the subsequent challenge with viable *L. major* promastigotes, sera were collected from immunized and control mice. The titers of serum antibodies binding to either whole *L. major* promastigotes or purified LPG were determined in an enzyme-linked immunosorbent assay. The majority of antibodies detected in immunized and/or infected mice belonged to the IgM and IgG1 subclasses, but there were also significant titers of IgG2a and IgG3 antibodies (Fig. 1). No significant antibody response to *L. major* antigens could be detected in untreated mice or in mice injected with *C. parvum* alone. Antibodies binding to epitopes on whole *L. major* promastigotes (Fig. 1, top) could be demonstrated in sera from LPG-immunized mice before and after infection with parasites, as well as in sera from *L. major*-infected control mice. On the other hand, antibodies binding to purified *L. major* LPG could be found only in mice treated with LPG (Fig. 1, bottom), with the exception of a low IgG1 titer in sera from infected control mice. It is of particular interest that anti-LPG as well as anti-*L. major* antibodies of the IgG3 isotype were detected exclusively in LPG-immunized mice. No IgE antibodies could be detected in sera of any of the experimental groups (data not shown). The total immunoglobulin content of the sera was determined in an independent assay, using anti-mouse immuno-

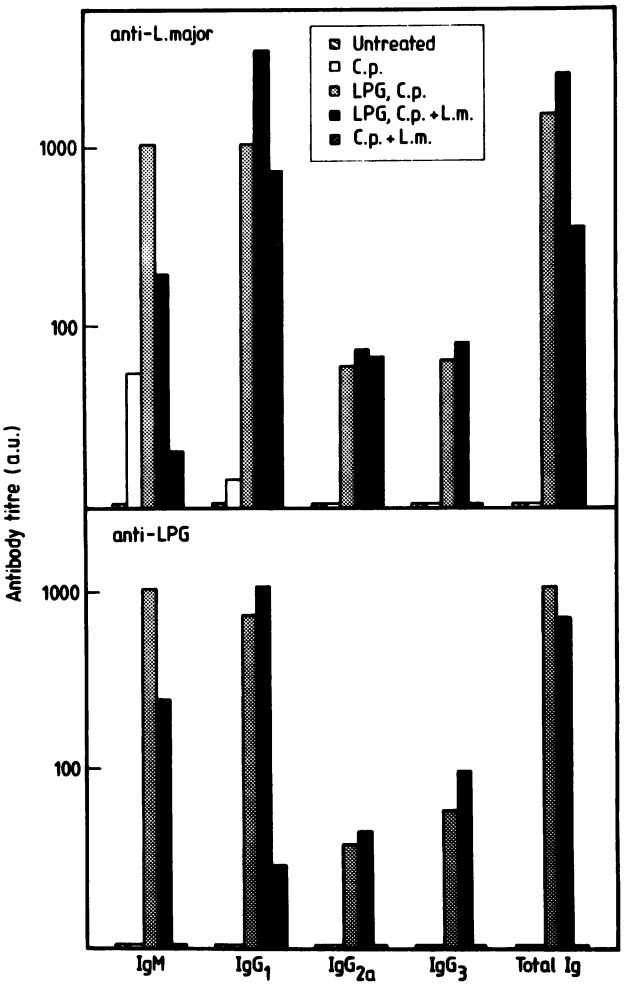

FIG. 1. Specific antibody titers in sera from mice immunized with *L. major* LPG. After administration of LPG in liposomes and *C. parvum* (LPG, C.p.), as well as after subsequent infection with *L. major* parasites (LPG, C.p. + L.m.), sera were collected from LPG-immunized and corresponding control mice (C.p.; C.p. + L.m.). The titers of serum antibodies binding to either whole *L. major* parasites (top) or purified *L. major* LPG (bottom) were determined by using an enzyme-linked immunosorbent assay. a.u., Arbitrary units, as defined in Materials and Methods.

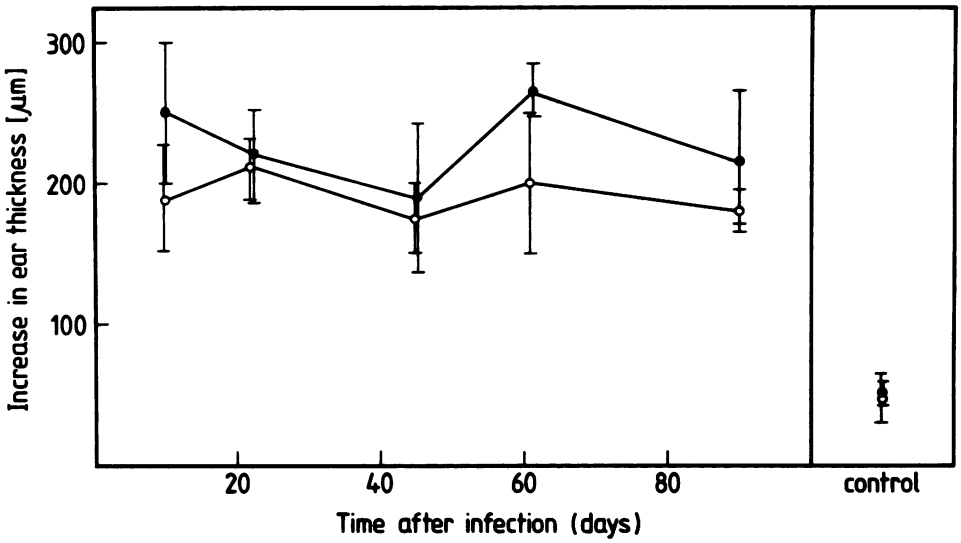

FIG. 2. DTH reactivity of *L. major*-infected C57BL/6 (●) and BALB/c (○) mice to whole *L. major* promastigotes. Ear swelling was determined 24 h after local antigen challenge and at various days after infection. Animals were tested only once, and uninfected mice were used as a control. Vertical bars indicate the standard deviation of the arithmetic mean.

globulins rather than isotype-specific antibodies as the second-step reagent. Therefore, the titers are not directly comparable to those determined in the isotype assays; in the experiments reported here, they were lower than the sum of the isotype titers. In conclusion, the generation of LPG-specific IgG antibodies in response to immunization of mice with purified *L. major* LPG in liposomes indicates that this glycolipid antigen can activate T cells in vivo to provide helper signals for the maturation of IgG-secreting B cells.

***L. major* LPG induces DTH reactivity in infected mice.** The data obtained from experiments with genetically susceptible mice immunized with *L. major* LPG in liposomes and *C. parvum* provide strong evidence for T-cell recognition of the purified LPG, as judged by an increased frequency of *L. major*-reactive T cells and the apparent ability to mediate T-cell help for antibody production. In order to extend these results to an examination of T-cell reactivity to purified LPG in vivo, we have assessed the capacity of LPG to elicit DTH reactivity in *L. major*-infected mice.

In a control experiment, genetically susceptible BALB/c and genetically resistant C57BL/6 mice were challenged intradermally with a preparation of 10^6 UV-inactivated *L. major* promastigotes at various time points after infection with *L. major* parasites. At 15, 24, and 48 h after antigen injection into the ear, the increase in thickness of the injected ear was determined. The swelling peaked at 24 h and persisted beyond 48 h after ear challenge. The values for the 24-h time point (Fig. 2) demonstrate that despite the difference in susceptibility to infection between BALB/c and C57BL/6 mice, both strains exhibited a marked ear response to this crude preparation of *Leishmania* antigens (13). A positive reaction remained stable for 90 days of infection, at which time C57BL/6 mice had healed completely, whereas BALB/c mice exhibited typical large lesions. Histological examinations of the affected ear tissue showed a marked infiltration with mononuclear cells for both strains and thus confirmed the tuberculin-type nature of the DTH reaction measured (data not shown). Sex- and age-matched uninfected controls did not show a significant DTH reactivity.

Using a similar experimental protocol, *L. major*-infected C57BL/6 and BALB/c mice were injected in the ears with 2.5 µg of purified *L. major* LPG. Resistant C57BL/6 mice were able to develop a DTH reaction to LPG throughout the course of disease and showed significant ear swelling even after their lesions had healed (Fig. 3). Challenge of *L. major*-infected mice with 0.1 µg of a glycoconjugate isolated from the helminth worm *S. japonicum* did not result in ear swelling. In mice sensitized with *S. japonicum* eggs, however, this antigen did elicit a response. The fact that *L. major*-infected C57BL/6 mice developed a specific DTH response to purified *L. major* LPG suggests that LPG can activate antigen-specific T cells.

When *L. major*-infected BALB/c mice were challenged with LPG, ear swelling was found at the onset of infection but not at later stages of chronic disease (Fig. 3). Furthermore, histological examinations showed that, in contrast to C57BL/6 mice, the LPG-induced DTH reaction in BALB/c mice was characterized by infiltration with polymorphs rather than mononuclear cells (data not shown). This finding suggests that the LPG-induced DTH response is qualitatively different in these two strains of mice.

Lack of DTH reactivity to the CHO moiety of *L. major* LPG. In contrast to the intact *L. major* LPG that can protect mice against cutaneous leishmaniasis (17, 25), the hydrophilic CHO moiety derived from the LPG, when injected in Freund complete adjuvant, was shown to exacerbate disease (29). It was therefore of interest to assess the T-cell reactivity to *L. major* CHO, using the ear assay for DTH. At 25 and 61 days of infection, BALB/c and C57BL/6 mice were injected in the ears with *L. major* CHO that had been prepared by enzymatic hydrolysis of the lipid moiety from LPG. The glycoconjugate failed to induce significant swelling (Table 3). Using a dose of CHO that corresponded to that of LPG which elicited a marked response (2.5 µg), there was no DTH reactivity to *L. major* CHO.

DISCUSSION

In the present report, we provide evidence for T-cell recognition of a purified *Leishmania* glycolipid antigen that can vaccinate mice against cutaneous leishmaniasis. This is one of a growing number of examples of T-cell recognition of

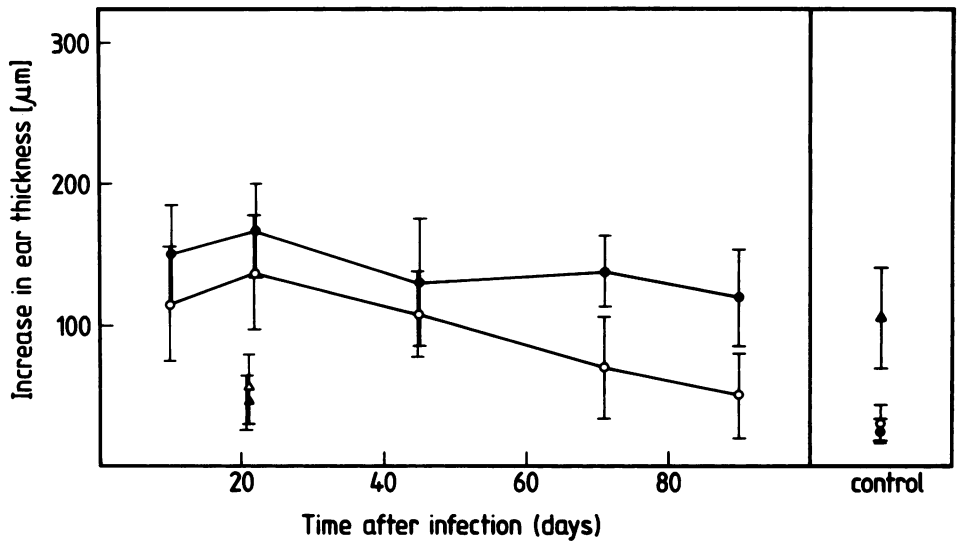

FIG. 3. DTH reactivity of *L. major*-infected C57BL/6 (closed symbols) and BALB/c (open symbols) mice to purified *L. major* LPG (●, ○) or *S. japonicum* glycoconjugate (▲, △). Ear swelling was determined 24 h after local antigen challenge and at various days after infection. Animals were tested only once. Control groups consisted of uninfected mice challenged with *L. major* LPG (●, ○), or of mice sensitized with *S. japonicum* eggs (▲).

carbohydrate antigens (1, 7, 26, 36, 38, 43; for a review, see reference 5).

For analysis of T-cell responsiveness to *L. major* LPG, we used genetically susceptible mice that had been immunized with LPG in liposomes and *C. parvum* as well as genetically resistant and susceptible mice infected with *L. major* promastigotes. The LPG preparation used for these experiments was highly purified and free of detectable protein. The data demonstrate that immunization of mice with *L. major* LPG increased the frequency of *L. major*-reactive T cells with the capacity to secrete lymphokines. Immunization also resulted in the production of T-cell-dependent IgG antibodies to LPG. Finally, a most important finding was that LPG was able to elicit a specific tuberculin-type DTH reactivity in *L. major*-infected mice. This provides evidence, though still circumstantial, for in vivo activation of T cells by the purified *L. major* LPG.

T-cell reactivity to *L. major* antigen in vitro was significantly decreased by adding the soluble CHO moiety of LPG to the limiting-dilution cultures; i.e., *L. major* CHO presumably competed for T-cell recognition of relevant antigenic structures. This finding strongly suggests that a large proportion of *L. major*-reactive T cells from LPG-immunized mice is directed against a CHO epitope of LPG. It was noted that treatment of mice with *C. parvum* alone also resulted in a moderate increase in frequency of T cells releasing IL-2 and IL-3. *C. parvum*, given alone, probably induces a polyclonal activation of these T cells, some of which would be directed to *Leishmania* antigens, whereas immunization with LPG in adjuvant presumably leads to the additional stimulation of antigen-specific T cells. IL-4 activity, on the other hand, was detected only in LPG-immunized mice. We are currently analyzing in further detail the influence of LPG on the cytokine patterns in *L. major*-infected and uninfected mice of various strains.

TABLE 3. DTH reaction to the CHO moiety of purified *L. major* LPG in *L. major*-infected mice

Time after infection (days)	Increase in ear thickness (µm ± SD)[a]			
	BALB/c		C57BL/6	
	CHO	*L. major*	CHO	*L. major*
25	27 ± 12	190 ± 74	36 ± 24	146 ± 48
61	27 ± 11	200 ± 49	54 ± 30	266 ± 18

[a] The difference in thickness of the injected ear and the control ear was determined 24 h after challenge in the ears with either whole *L. major* promastigotes or 2.5 µg of *L. major* CHO prepared by enzymatic cleavage of purified *L. major* LPG. SD, Standard deviation.

DTH reactivity upon challenge of infected mice with a preparation of whole *L. major* promastigotes did not correlate with the degree of susceptibility of the inbred mice used. Both genetically resistant C57BL/6 and genetically susceptible BALB/c mice were able to develop a DTH response over a period of 90 days after infection. At this time, lesions had completely resolved in C57BL/6 mice, whereas BALB/c mice suffered from chronic disease. In both strains of mice, the DTH reaction to this crude preparation of *L. major* antigens peaked at 24 to 48 h after challenge in the ear. Histological examination did not reveal a qualitative difference, but infiltration with mononuclear cells was less pronounced in tissues from BALB/c mice. These results indicate that a DTH response to crude antigen is not necessarily associated with a protective immune response, as has been observed by others (reviewed in reference 20). On the other hand, the DTH reaction in response to the purified *L. major* LPG correlated well with resistance to cutaneous infection. Resistant C57BL/6 mice were able to respond to LPG throughout the course of disease and after healing, whereas susceptible BALB/c mice developed LPG-specific reactivity only at the onset of infection. Moreover, histological examination of the skin test site revealed a predominant infiltration with polymorphonuclear cells in BALB/c mice, whereas only mononuclear cells were found in C57BL/6 mice. This finding suggests that the LPG-specific response in BALB/c mice did not fulfill all the criteria of a classical DTH reaction. Thus, DTH reactivity is different in genetically susceptible and genetically resistant mice, a conclusion that is supported

by a recent report on differences in the kinetics of DTH responses to *Leishmania* antigen preparations, depending on the state of resistance of the inbred mice used (6). Gamma interferon has recently been shown to mediate lymphocyte recruitment into the skin during DTH reactions (19). This raises the possibility that LPG is able to stimulate the production of this lymphokine by T cells, but further studies are required to consolidate such an interpretation.

In contrast to *L. major* LPG, which vaccinates mice against cutaneous leishmaniasis, the CHO moiety derived by enzymatic cleavage of LPG, when given with Freund complete adjuvant, can exacerbate subsequent disease (29). Since the induction of protective immunity as well as its impairment have been attributed to *L. major*-specific L3T4$^+$ T-cell subpopulations (23), these findings favor the hypothesis that the LPG molecule activates resistance-promoting L3T4$^+$ T cells, whereas the CHO split product of LPG activates L3T4$^+$ T cells that facilitate disease (30). This interpretation is supported by our finding that *L. major*-infected mice of a resistant genotype develop a consistent DTH response to LPG, whereas genetically susceptible mice with chronic disease fail to respond. On the other hand, we could not detect any DTH reactivity to purified *L. major* CHO, neither in resistant nor in susceptible mice. It is possible that *L. major* CHO is recognized by T helper cells of type 2 that are not able to mediate DTH reactions, whereas LPG stimulates T helper cells of type 1 that are capable of inducing DTH (33), possibly via secretion of gamma interferon (19). However, it should be noted that it is not clear yet whether the concept of different types of T helper cells, which has been established for cloned T cells, can similarly be applied to L3T4$^+$ cells in vivo. Using T-cell clones, evidence that protective and exacerbative T cells belong to different T helper subsets and respond to distinct antigens has also been provided by a recent report (41).

The presented evidence for T-cell recognition of the purified LPG molecule is based on the ability of LPG to elicit T-cell functions both in vivo and in vitro. It will be of particular importance to extend the reported findings by using in vitro T-cell assay systems. They will facilitate functional characterization at a clonal level and a more precise quantification of the T-cell subsets involved in an anti-LPG immune response. We have been unable to detect direct T-cell responsiveness to soluble LPG in vitro. Current studies are aimed at increasing the efficiency of antigen presentation by using an appropriate antigen carrier and different sources of antigen-presenting cells. The final aim of these efforts is the identification and characterization of L3T4$^+$ T cells promoting either resistance or susceptibility to cutaneous leishmaniasis. The analysis of their lymphokine patterns will be of particular interest because of recent evidence (22) for differences in lymphokine production by lymphocytes from healer and nonhealer mice infected with *L. major*. The findings suggested that effective cellular immunity is reflected in the expansion of type 1 T helper cells, whereas the inability to control disease is accompanied by the expansion of type 2 T helper cells (33).

It has long been controversial whether T cells are capable of recognizing polysaccharide antigens. DTH reactivity to bacterial carbohydrates has repeatedly been reported (1, 7, 38; reviewed in reference 5), but to our knowledge there has not been any report yet documenting direct and unequivocal T-cell responsiveness to pure carbohydrate or glycolipid antigens in vitro. However, there is accumulating evidence demonstrating the capacity of such antigens, obtained from bacteria, to modulate T-cell responses in vivo and in vitro (26, 36, 43). The present report extends this growing evidence to a highly purified parasite glycolipid antigen.

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