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Characterization of Cellular Immune Response to Chemically Defined Glycoconjugates from *Leishmania mexicana* subsp. *amazonensis*

MAURÍCIO M. RODRIGUES,[1] MÁRCIA T. XAVIER,[2] LÚCIA M. PREVIATO,[2] AND MARCELLO A. BARCINSKI[1]*

Institute of Biophysics[1] and Institute of Microbiology,[2] Federal University of Rio de Janeiro, 21941, Rio de Janeiro, Brazil

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Two defined glycoconjugates (GP-10/20 and FR II Phe) purified from *Leishmania mexicana* subsp. *amazonensis* were analyzed with respect to their ability to induce cellular responses in immunized and infected mice. Each glycoconjugate was recognized by specific immune cells, as assessed by the proliferative response of lymph node cells of immunized mice. The response to GP-10/20 depended on helper T cells and antigen-presenting cells and was restricted by a major histocompatibility complex class II gene product. A specific anti-GP-10/20 T-cell line was established, and it was able to transfer a delayed-type hypersensitivity (DTH) response to normal mice. Both antigens were also recognized during an ongoing disease, as assessed by DTH response of infected mice. By this response, it was possible to distinguish susceptible from resistant strains of mice. In the course of the disease in resistant mice a correlation between the size of the primary lesion and the DTH response to GP-10/20 was observed. The presence of the glycoproteins on both promastigote and amastigote forms of the parasite, the antigenic similarities between both fractions, and the distribution of the GP-10/20 antigen in other trypanosomatids were studied. The results showed that both antigens were present on promastigotes and amastigotes. GP-10/20 shared no epitopes with FR II Phe, was included as part of the crude preparation leishmanin, and had some cross-reactive determinants with *Leishmania donovani* and *Crithidia deanei*.

Cutaneous leishmaniasis is a disease characterized by a single cutaneous lesion that resolves either spontaneously or with the aid of pentavalent antimonial therapy. Healing is accompanied by the development of cellular immunity against antigens of the parasite. In a very few patients, the disease can develop into a diffuse form which is usually fatal. Patients with diffuse cutaneous leishmaniasis are anergic to parasite antigens. The full spectrum of the disease can be reproduced in different inbred strains of mice according to their genetic constitution (7, 19). When *Leishmania major* is the etiological agent, helper T cells are required for the resistance of CBA mice, and suppressor cells are associated with the susceptibility of BALB/c mice (13). A clone of suppressor T cells specific for parasite antigens and able to increase the lesion size in infected BALB/c mice has been described previously (12). All of these observations support the notion that immunological phenomena are critical in the pathogenesis of cutaneous leishmaniasis caused by *L. major*. Much less is known about the immunopathological phenomena involved in cutaneous leishmaniasis and diffuse cutaneous leishmaniasis induced by *Leishmania mexicana* subsp. *amazonensis*. It has been shown that susceptible (BALB/c) mice, unlike resistant strains, are unable to evince a delayed-type hypersensitivity (DTH) response to parasite antigens (1, 2). The DTH response is believed to be related to the ability to resist infection.

Extensive experimental work is being done lately on the characterization of biologically active molecules extracted from different microorganisms. These molecules are potentially useful for studies of the taxonomy (20, 21) of parasites, as well as for differential diagnosis (24) prophylaxis, and for a better understanding of phenomena underlying host-parasite interactions (17).

* Corresponding author.

In this study, we examined the immune responses of susceptible and resistant strains of mice to two purified glycoconjugates extracted from *L. mexicana* subsp. *amazonensis*. Immunized and infected mice exhibited a specific response to the purified antigens. Susceptible and resistant strains differed in the intensity of their DTH reactions to the purified glycoconjugates. Antigenic determinants of the glycoconjugates were present in both differentiation forms of the parasite. The importance of studying such purified antigens for a better understanding of the pathogenesis of leishmaniasis is discussed.

MATERIALS AND METHODS

Animals. Male and female mice of strains C57BL/10 (B10), C57BL/10.A (B10.A), and BALB/c (4 to 20 weeks old, raised in our own facilities) were used throughout the studies.

Parasites and growth conditions. *L. mexicana* subsp. *amazonensis* H-21 was provided by the Wellcome Parasitology Unit, Instituto Evandro Chagas, Belém, Brazil. *Leishmania donovani* 1S was kindly donated by Dennis Dwyer, National Institutes of Health. Promastigotes were grown in screw-cap tubes containing brain heart infusion broth (37 g/liter), folic acid (0.02 g/liter), and hemin (0.01 g/liter dissolved in 0.2 M NaOH) for 5 days at 28°C. Cells were harvested by centrifugation, washed three times with Hanks balanced salt solution (HBSS), and used at once or frozen at −20°C. *Crithidia deanei* (wild-type strain) was grown in liver infusion tryptose medium for 2 days at 28°C. The cells were harvested, washed in phosphate-buffered saline, and heat killed (80°C, 15 min) before use. *L. mexicana* subsp. *amazonensis* amastigotes were obtained from infected hamsters or BALB/c mice 40 to 60 days after they had been inoculated in the hind footpads with 10^5 to 10^6 amastigotes. The lesion was aseptically dissected out, washed in sterile HBSS, finely teased, and ground in a glass homogenizer

containing cold HBSS. The suspension was then centrifuged at 200 × *g* to remove large debris, and the amastigotes from the supernatant were washed at least twice in cold HBSS. The parasites were counted in a Neubauer chamber and adjusted to the desired concentration.

Extraction and purification of *L. mexicana* subsp. *amazonensis* antigens. Frozen cells were rapidly thawed and extracted as described by Mendonca-Previato et al. (18). The aqueous extract (AE) was fractionated by gel filtration chromatography on a Bio-Gel P-10 column (120 by 2 cm). The material that emerged in the void volume was designated GP-10/20. The remaining cellular pellet was extracted with a hot phenol-water solution, and the aqueous layer thus obtained was dialyzed, lyophilized, and purified by gel filtration chromatography on a Bio-Gel P-100 column (100 by 1 cm). The void volume was designated FR II Phe. Leishmanin was prepared from promastigotes of *L. mexicana* subsp. *amazonensis* that were grown and harvested as described above. The cells were washed, frozen, and thawed 10 times, solubilized in 8 M urea, dialyzed overnight against a large volume of phosphate-buffered saline, and centrifuged at 33,000 × *g* for 1 h. The supernatant was under sterile conditions filtered and stored at −20°C until use. The antigen concentrations are referred to as mass (dry weight).

Conventional antigens. Purified protein derivative (PPD; Connaught Medical Research Laboratories, Ontario, Canada) was diluted in HBSS and used at the indicated concentrations.

Immunization. Mice were immunized in the hind footpads with one of the following antigens: (i) aqueous extract of *L. mexicana* subsp. *amazonensis* (50 μg per animal); (ii) living promastigotes of *L. mexicana* subsp. *amazonensis* (10^7 per animal); (iii) promastigotes of *L. donovani* (10^7 per animal); (iv) amastigotes of *L. mexicana* subsp. *amazonensis* (10^7 per animal); (v) heat-killed promastigotes of *C. deanei* (10^7 per animal); (vi) purified GP-10/20 (50 μg per animal). Each antigenic fraction was dissolved or suspended in HBSS and mixed with an equal volume of complete Freund adjuvant (CFA; 0.4 mg of *Mycobacterium tuberculosis* $H_{37}RA$ per ml; Difco Laboratories, Detroit, Mich.), and 50 μl of the emulsion was injected into each hind footpad.

Infection. Amastigotes of *L. mexicana* subsp. *amazonensis* were obtained as described above, and the concentration was adjusted to 1 × 10^6 to 2 × 10^6 parasites per ml in HBSS. Mice of each strain were inoculated subcutaneously with 0.1 ml in the left hind footpad. Infection was quantified by measuring the increase in thickness of the footpad with an engineering caliper. The size of the lesion during the course of the infection was calculated as the difference in thickness between the pads in infected and noninfected mice.

Measurement of DTH responses. Mice that had been infected in the left footpad were injected in the right footpad with 50 μl of purified antigen or with leishmanin. The degree of swelling was expressed as the difference in millimeters between the thickness of the footpad before injection and the thickness measured after 6, 24, and 48 h, or as the percentage of swelling according to the following relationship: percent swelling = (thickness after injection − thickness before injection)/thickness before injection.

Preparation of responder and stimulator cells. At 1 to 5 weeks after immunization the draining lymph nodes were collected, and single-cell suspensions of whole lymph node cells (LNC) were prepared. Lymph node lymphocytes (LNL) were prepared by eluting LNC through nylon wool columns (10). Normal spleen cell suspensions were prepared in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) and treated with 25 μg of mitomycin C (Sigma Chemical Co., St. Louis, Mo.) per ml for 1 to 2 h at 37°C. After extensive washing, spleen cells were adjusted to the proper concentration in culture medium and used as a source of antigen-presenting cells (APC).

DTH transfer via a short-term T-cell line specific for GP-10/20. Responding T cells were LNL at 5 × 10^6 cells per well. Stimulator cells (10^6 cells per well) were normal spleen cells that had been treated with mitomycin C. Responding and stimulator cells were mixed in 2 ml of culture medium and cultured in 24-well plates (Linbro Inc., Hamden, Conn.) in the presence of 25 μg of GP-10/20 or 20 μg of PPD per ml. After 1 week of culture, the nonadherent viable cells were recovered, washed, and adjusted to the proper density in HBSS with antigen (GP-10/20) at the indicated concentration. Responder cell suspensions were injected in the hind footpad of normal syngenic mice, and the DTH response was measured as described above.

T-cell proliferation assay. Culture medium consisted of RPMI 1640 medium supplemented with gentamicin (10 μg/ml; Schering, Rio de Janeiro), L-glutamine (2 mM; Sigma), 2-mercaptoethanol (5 × 10^{-5} M; Eastman Kodak Co., Rochester, N.Y.), and 5% fetal calf serum (Microbiológica, Rio de Janeiro) or 5% heat-inactivated normal human serum. Whole LNC were cultivated at 37°C for 3 days in a humid environment containing 5% CO_2. Cells were cultivated at a density of 4 × 10^5 per well in 96-well round-bottom microtiter plates (Linbro) containing the appropriate antigen concentration. In some experiments a monoclonal anti-I region-associated antigen (Ia) antibody (I-E^k/C^k, Litton Bionetics, Charleston, S.C.) was used to block T-cell proliferation. A monoclonal antibody against the helper T-cell antigen L_3T_4 (a kind gift of Ethan M. Shevach, National Institute of Allergy and Infectious Diseases) was also employed. This antibody (monoclonal antibody [MAb] α L_3T_4) was obtained as described previously (28) and was added to the cultures at the indicated concentrations to yield final volumes of 200 or 220 μl per well.

At 18 to 20 h before harvesting, each well received 1 μCi of tritiated thymidine ([*methyl*-^{3}H]TdR; specific activity, 6.7 Ci/mM; New England Nuclear Corp., Boston, Mass.). Cultures were harvested into fiber glass filters with the aid of a semiautomatic harvesting device. The amount of [*methyl*-^{3}H]TdR incorporated was measured in a liquid scintillation counter. Results are expressed as means ± standard deviations (SD) of triplicate cultures. The changes in counts per minute (cpm) were calculated by the following (exp, experimental; ctr, control):

$$\text{cpm(exp)} - \text{cpm(ctr)} \pm \sqrt{\text{SD}^2\text{(exp)} + \text{SD}^2\text{(ctr)}}$$

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gel electrophoresis was carried out under reducing conditions with a discontinuous buffer system on 10-cm slab gels containing 15% polyacrylamide (11). The protein-containing bands were stained with Coomassie brilliant blue, and the carbohydrate-containing bands were stained with the periodic acid-Schiff reagent.

RESULTS

LNC respond to parasite extract. As described previously for other species of *Leishmania* (14, 26), proliferation of immune LNC in culture is stimualted by AE of *L. mexicana*

subsp. *amazonensis* promastigotes and by leishmanin (Table 1). The observed response is dose dependent and specific. LNC from mice immunized with CFA do not respond to leishmanial antigens (data not shown).

LNC respond to purified antigens from *L. mexicana* subsp. *amazonensis*. To investigate the antigen specificity of proliferating LNC, the purified glycoconjugates GP-10/20 and FR II Phe were used. The carbohydrate and protein moieties of these glycoconjugates have been described previously (M. T. Xavier, J. O. Previato, and L. Mendonca-Previato, Arq. Biol. Tecnol. **26**:204, 1983). GP-10/20 has two sharp bands of M_r ~10,000 and 20,000 on sodium dodecyl sulfate-polyacrylamide gels (15.0%) when stained for protein and carbohydrate (Fig. 1A and B). This fraction contained 50% protein and 15% carbohydrate on a dry weight basis. FR II Phe showed a large band of M_r ~14,000, strongly stained with the periodic acid-Schiff reagent (Fig. 1C), and displayed a very weak reaction for protein. This fraction contained 3% protein and 92% carbohydrate on a dry weight basis. Mice immunized with 10^7 living promastigotes of *L. mexicana* subsp. *amazonensis* were tested for the presence of specifically primed cells by measuring the proliferation in response to GP-10/20 with LNC from mice of three different haplotypes ($H\text{-}2^{a.b.d}$). FR II Phe was tested with BALB/c ($H\text{-}2^{d}$) immune LNC at different times after immunization. A tendency for an optimal response at lower doses (10 μg/ml) of antigen in vitro was observed with this last fraction (Fig. 2). The lack of proliferative response to the purified glycoconjugates in BALB/c mice immunized with PPD or with the parasite growth medium (data not shown) shows that the response is not due to a nonspecific mitogenic effect of the purified glycoconjugates.

Proliferative response to GP-10/20 is helper T-cell dependent. To define the role of helper T cells, MAb α L_3T_4 (GK1.5) was used. It has been reported that the addition of α L_3T_4 in culture can inhibit all functions of helper T-cell clones stimulated with soluble antigens (28). A 10% concentration of α L_3T_4 completely suppressed the proliferative response of LNC to GP-10/20 (Fig. 3B). The suppression was reduced by diluting the monoclonal antibody. The α L_3T_4 had no effect on the B-cell proliferative response to lipopolysaccharide B of *Escherichia coli* (LPS) (Fig. 3A), showing that the suppressive effect in the antigen-specific T-cell proliferation is not due to a nonspecific toxicity of the reagent.

TABLE 1. Proliferative response of LNC to leishmania antigens

Mouse strain	Antigen	Concn (μg/ml)	[*methyl*-^{3}H]TdR incorporation (change in cpm ± SD, ×10^3)
BALB/c[a]	AE of *L. mexicana* subsp. *amazonensis*	0.1	1.93 ± 0.40
		1	3.54 ± 0.30
		20	19.56 ± 2.60
B10	AE of *L. mexicana* subsp. *amazonensis*	0.5	8.26 ± 1.00
		5	15.23 ± 0.90
		25	19.97 ± 1.60
BALB/c[b]	Leishmanin	20	33.51 ± 2.60

[a] Mice were immunized with 50 μg of aqueous extract of *L. mexicana* subsp. *amazonensis* in CFA 2 weeks before the experiment. Background values were 6,080 ± 347 cpm for B10 and 6,450 ± 532 cpm for BALB/c mice.

[b] Mice were immunized with 10^7 amastigotes of *L. mexicana* subsp. *amazonensis* in CFA 2 weeks before the experiment. Background values were 12,034 ± 1,584.

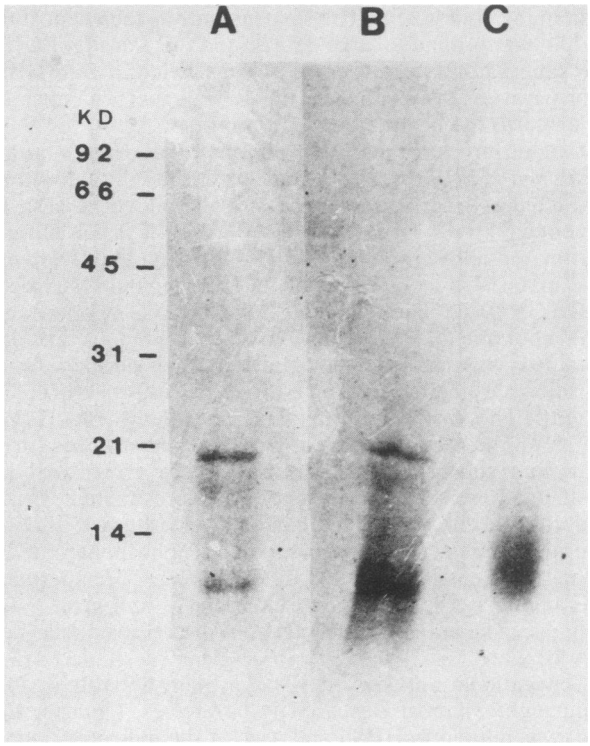

FIG. 1. Polyacrylamide gel electrophoresis of *L. mexicana* subsp. *amazonensis* preparations. Lanes A and B, GP-10/20 stained with Coomassie brilliant blue and periodic acid-Schiff reagent, respectively; lane C, FR II Phe stained with periodic acid-Schiff reagent. KD, Molecular mass in kilodaltons.

Helper T-cell recognition of GP-10/20 is dependent on APC and involves gene products of the major histocompatibility complex I region. LNL were purified as described above. Spleen cells from a nonimmune mice were treated with mitomycin C to use as APC. Immune LNL were cultured for 3 days with either the antigen, APC, or both. Recognition of the antigen required the presence of APC (Table 2). Since antigen recognition by helper T cells is generally considered to be restricted by gene products of the major histocompatibility complex (MHC) I region (Ia antigens), we used an anti-Ia^k MAb in an attempt to block the specific proliferative response to GP-10/20. Table 3 shows that the MAb induces a partial (42%) blockade of LNC proliferation but has no effect on the B-lymphocyte blastogenic response to LPS. These results are consistent with the hypothesis that some of the helper T cells recognize GP-10/20 in association with the epitope that is blocked specifically by the MAb anti-Ia^k.

Purified antigens GP-10/20 and FR II Phe are present in amastigotes of *L. mexicana* subsp. *amazonensis*. As a means of assessing whether the purified glycoconjugates were present in the intracellular form of the parasite, BALB/c mice were immunized with amastigotes that had been obtained from a lesion of a BALB/c mouse and emulsified in CFA. Proliferative response of LNC to both antigens is shown in Table 4. These data imply that these glycoconjugates bear nondifferentiation epitopes.

T-cell anti-GP-10/20 are able to transfer a local DTH response to a normal syngenic mouse. To investigate whether the proliferative lymph node T cells could induce DTH responses stimulated by the antigen, we attempted to estab-

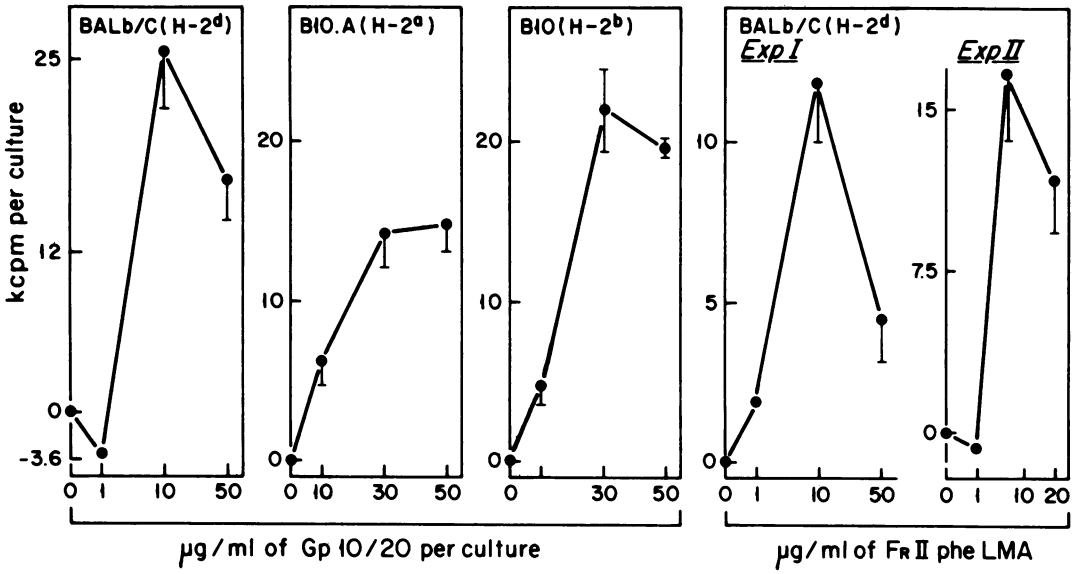

FIG. 2. Proliferative response of immune LNC to purified antigens from *L. mexicana* subsp. *amazonensis*. Mice from the indicated strains were immunized with 10^7 living promastigotes in CFA. LNC were isolated and stimulated in vitro with the indicated doses of GP-10/20 and FR II Phe. Background values of LNC proliferative response in the absence of antigens were 5,012 ± 79 cpm for B10.A cells, 5,916 ± 528 cpm for B10 cells, and 14,741 ± 1,103 cpm for BALB/c cells. In experiments with FR II Phe the background values were 7,778 ± 119 cpm (Exp I) and 14,741 ± 1,103 cpm (Exp II). [*methyl*-^{3}H]TdR was measured after 72 h in culture.

lish short-term T-cell lines with specificity for GP-10/20 molecules. LNC from B10.A mice immunized with GP-10/20 were cultivated for 1 week with GP-10/20 or PPD in the presence of syngenic APC. The resulting responder cells were washed and suspended in HBSS containing 25 μg of GP-10/20 per ml and injected (100 μl) in the hind footpad of a syngenic mouse. The DTH responses were measured 24, 40, and 48 h after cell transfer. A strong specific response was observed when mice were injected with the anti-GP-10/20 T-cell line in addition to the antigen, but not when injected with the antigen alone. A less marked DTH response was observed when the T-cell line was anti-PPD (Table 5). This evidence supports the notion that an anamnestic in vivo response is important in the generation of a DTH reaction.

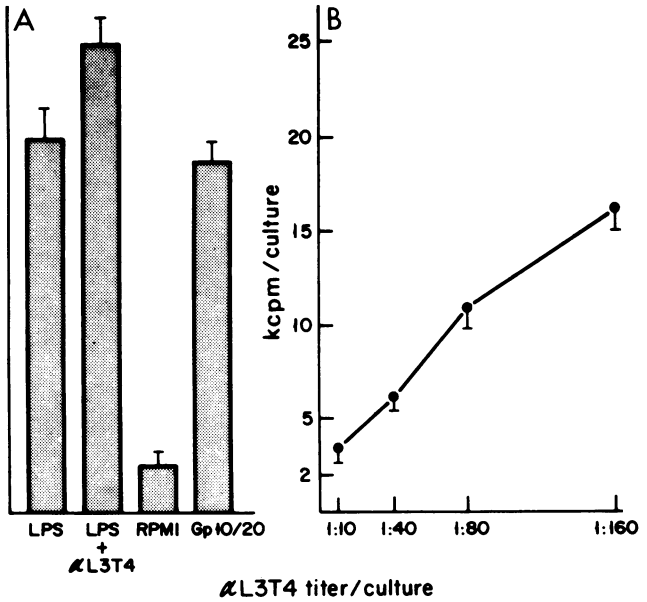

FIG. 3. Helper T-cell dependence of LNC proliferative response to GP-10/20. Responder LNC isolated from B10.A mice that had been immunized with 10^7 living promastigotes in CFA were cultured with GP-10/20 (30 μg/ml), LPS (20 μg/ml), or RPMI 1640 medium alone. Anti-L_3T_4 supernatant was used at a final dilution of 1:10 in an LPS-stimulated culture (A) or titrated in GP-10/20-stimulated cultures (B). [*methyl*-^{3}H]TdR was measured after 72 h in culture.

Leishmanin bears T-cell activating epitopes not present in GP-10/20. To compare the antigenic differences between leishmanin and GP-10/20, B10.A mice were immunized with purified GP-10/20. LNC were collected and stimulated with either antigens or FR II Phe. The plateau of the dose-response curve for [*methyl*-^{3}H]TdR incorporation was similar for cultures stimulated by GP-10/20 and leishmanin (Fig. 4). This suggests that most of the clones that proliferate in response to GP-10/20 also respond to leishmanin. However, in mice immunized with whole parasites (*L. mexicana* subsp. *amazonensis* amastigotes) the pattern was completely different. The response to leishmanin exceeded by far the response to the purified glycoconjugates (Table 4). It was then concluded that only part of the T-cell clones against leishmanin recognize GP-10/20. On the other hand, T cells against GP-10/20 were not stimulated by FR II Phe, showing that they have little or no similarity in their molecular structures.

GP-10/20 is present in other trypanosomatids. The LNC

TABLE 2. APC dependence of LNL proliferative response to GP-10/20[a]

Addition	[*methyl*-^{3}H]TdR incorporation (cpm per culture)
APC	1,582 ± 48
GP-10/20	952 ± 113
APC–GP-10/20	20.692 ± 435

[a] LNL were recovered from B10.A mice immunized with 10^7 living promastigotes of *L. mexicana* subsp. *amazonensis* emulsified in CFA 2 weeks prior to the assay. APC were mitomycin C-treated spleen cells from nonimmune syngenic mice.

TABLE 3. Inhibition of T-cell response to GP-10/20 by MAb anti-Ia^k

Stimulus (concn [μg/ml])	[*methyl*-^{3}H]TdR incorporation (change in cpm ± SD)		Inhibition (%)
	MAb absent	MAb present	
GP-10/20 (30)[a]	11,787 ± 613	6,837 ± 1,066	42.0
LPS (50)[b]	26,532 ± 2,100	25,960 ± 1,500	2.5

[a] B10.A mice were immunized with 10^7 living promastigotes emulsified in CFA 2 weeks before the assay. Background values were 534 ± 109 cpm.
[b] B10.A mice were immunized with CFA alone. Background values were 4,250 ± 322 cpm.

response of mice immunized with *L. donovani* Sudan and *C. deanei* (wild-type strain) was stimulated in a dose-dependent manner by the presence of GP-10/20 in the cultures (Table 6). Apparently, this glycoconjugate has antigenic epitopes that are neither species nor genus specific.

Infected resistant and susceptible mice respond to GP-10/20 and to FR II Phe. As a means of assessing the recognition of the purified glycoconjugates by immune cells of infected animals, B10.A (resistant) and BALB/c (susceptible) mice were infected with 10^5 *L. mexicana* subsp. *amazonensis* amastigotes in one of the hind footpads. Twelve weeks later the DTH responses to the purified glycoconjugates and to leishmanin, as a positive control, were measured in the infected mice of both strains and in uninfected controls. The purified antigens evoked a DTH response in both strains of mice (Fig. 5). However, the intensity of the response in the resistant strain was higher than that in the susceptible strain. Figure 6 shows the time course of the size of the primary lesion, which correlates with the number of parasites, and of the DTH response to GP-10/20 in the resistant strain of mice. The high correlation coefficient (0.96) suggests that the intensity of the DTH response is dependent on the antigenic load. Both the difference in DTH intensity in susceptible and resistant mice and the correlation with the size of the lesion observed with the purified antigens already have been described with leishmanin (2).

DISCUSSION

The role of cell-mediated immunity in the fate of the cutaneous lesion in leishmaniasis is at present a debatable issue. DTH reactions have been associated with the acquisition by the host of immune mechanisms effective in the elimination of the parasite and in protection from reinfections (8, 13, 23). Recently, a cloned T-cell line able to specifically suppress the DTH response to leishmanial antigens was shown to enhance the size of the lesion on susceptible mice (12). However T-cell lines positive for the L_3T_4 marker capable of transferring parasite-specific DTH responses and of activating macrophages to destroy leishmania were shown to exacerbate the lesions when transferred to infected resistant and susceptible mice (27). Also, protective immunity has been achieved with an immunization schedule that fails to raise a DTH response (9). Those studies were made with whole organisms or the soluble fractions of crude promastigote extracts (leishmanin) of *L. major*. Less is known about the murine model of leishmaniasis induced by *L. mexicana* subsp. *amazonensis*, although evidence exists of similarities in some of the genetic, immunological, and pathological findings among the different models (1, 19, 20).

TABLE 4. Proliferative response of LNC from mice immunized with anastigotes of *L. mexicana* subsp. *amazonensis*[a]

Antigen	Concn (μg/ml)	[*methyl*-^{3}H]TdR incorporation (change in cpm ± SD, ×10^3)
	1.0	3.07 ± 2.0
GP-10/20	10.0	15.80 ± 1.59
	50.0	19.29 ± 3.8
	1.0	3.22 ± 2.0
FR II Phe	10.0	9.34 ± 2.6
	20.0	7.96 ± 1.8
Leishmanin	20.0	33.51 ± 2.6

[a] Mice were immunized with 10^7 amastigotes of *L. mexicana* subsp. *amazonensis* emulsified in CFA 2 weeks before the assay. Background value was 12,034 ± 1,584 cpm.

TABLE 5. Transfer of DTH response by anti-GP-10/20 T-cell lines[a]

Addition	Footpad swelling (change in thickness [mm] ± SE, ×10^2) at:		
	24 h	40 h	48 h
GP-10/20	6 ± 6	0 ± 0	0 ± 0
GP-10/20–anti-PPD T cells	16 ± 6	10 ± 10	6 ± 11
GP-10/20–anti-GP-10/20 T cells	53 ± 6	43 ± 6	37 ± 6

[a] Responder T cells isolated from B10.A mice that had been immunized with 50 μg of GP-10/20 per animal were cultured for 1 week in the presence of syngenic APC and either 25 μg of GP-10/20 per ml or 20 μg of PPD per ml. Recovered cells were adjusted to 10^7 viable cells per ml in HBSS containing 25 μg of GP-10/20 per ml, and 0.1 ml was injected in the hind footpads of B10.A mice.

The use of whole organisms or crude antigenic extracts raises some considerations. *Leishmania* spp. undergo changes in expressed proteins during their differentiation process (3, 5, 6), and amastigote and promastigote forms of leishmania do not share the same antigenic pattern (amount and quality). Even within promastigotes differences in infectivity (22) and in lectin binding (21a) have been described in parasites at different points of their growth curve. Furthermore, no evidence excludes the possibility that different antigens are related to different immune effector mechanisms. A specific epitope has been shown to be related to the suppressor mechanism in lepromatous leprosy (17).

To circumvent some of the limitations described above, we used purified and well-defined glycoconjugates extracted from *L. mexicana* subsp. *amazonensis* as antigens.

The results of this study show that the two glycoconjugates are immunogenic and antigenic not only for infected and

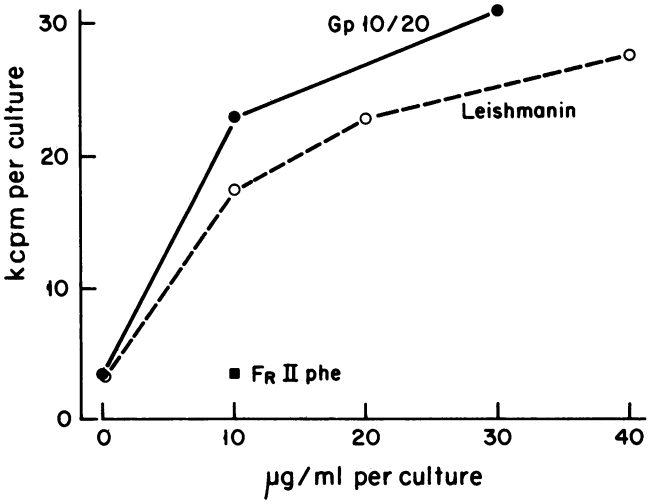

FIG. 4. LNC proliferative response to leishmanial fractions of mice immunized with purified GP-10/20 in CFA. LNC of immunized B10.A mice were isolated and stimulated with the indicated doses. [*methyl*-^{3}H]TdR uptake was measured after 72 h in culture.

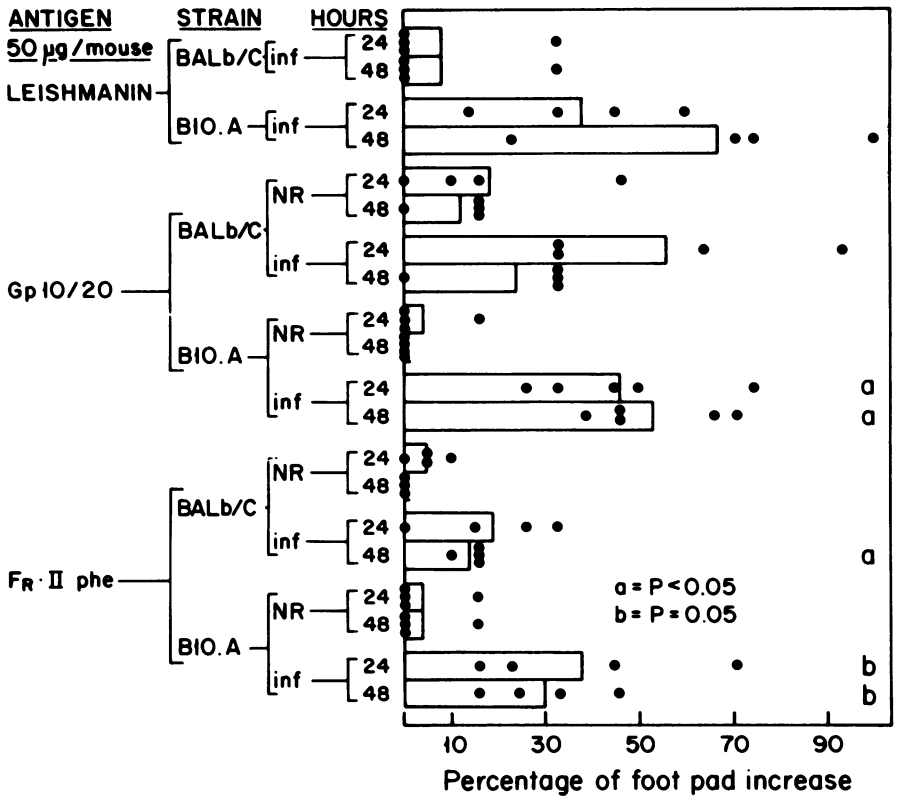

FIG. 5. DTH responses of infected susceptible and resistant mice to the purified glycoconjugates. Indicated strains of mice had been infected 12 weeks previously with 10^5 amastigotes in the left hind footpad. The percent increase in footpad thickness before and after infection of the antigen was calculated as described in the text. Statistical significance of the difference at the same time point between infected (inf) mice and age- and sex-matched uninfected (NR) mice was calculated by the Mann-Whitney nonparametric test.

immunized mice of different *H-2* haplotypes, but for mice with different degrees of susceptibility to the disease (Fig. 2). BALB/c, B10, and B10.A mice respond in a lymph node proliferative assay to GP-10/20, and BALB/c mice respond to FR II Phe; BALB/c and B10.A exhibit a DTH response to both antigens during an ongoing infection (Fig. 5). Under the conditions used in this study, the intensity of the DTH response is greater in the resistant (B10.A) than in the susceptible (BALB/c) strain of mice. Interestingly, the DTH response of BALB/c mice to GP-10/20 ($P = 0.10$ at 24 h; $P = 0.20$ at 48 h), which is an antigen contained in leishmanin, suggests that the unresponsiveness to the crude extract may be due to quantitative rather than qualitative differences in the immunogenic properties of some of its molecular constituents.

The purified glycoconjugate GP-10/20 is recognized in immunized animals by nylon wool nonadherent cells via APC (Table 2) and class II MHC gene products (Table 3). The LNC proliferative response was shown to be dependent on L_3T_4-positive helper T cells (Fig. 3). The functional characteristic of GP-10/20-specific T cells maintained in vitro was seen by the ability of those cells to transfer DTH response (Table 5). Our data confirm, for the purified glycoconjugate, the prevailing view on antigen presentation to helper T cells by Ia-bearing accessory cells (15, 25).

The antigens GP-10/20 and FR II Phe are present in both promastigote and amastigote forms of the parasite (Fig. 5

TABLE 6. Presence of GP-10/20 in other trypanosomatids

Immunogen	GP-10/20 concn (µg/ml)	[*methyl*-^{3}H]TdR incorporation (change in cpm ± SD, ×10^3)
L. donovani[a]	1	2.5 ± 0.5
	30	6.4 ± 1.2
	50	8.6 ± 0.3
C. deanei[b]	30	11.7 ± 2.0

[a] B10 mice were immunized with 10^7 promastigotes of *L. donovani* emulsified in CFA. Background values were 3,051 ± 701 cpm.

[b] B10 mice were immunized with 10^7 heat-killed *C. deanei* emulsified in CFA. Background values were 2,010 ± 266 cpm.

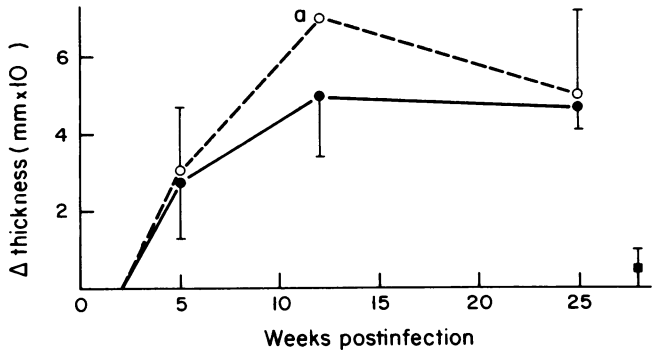

FIG. 6. Time course of the increase in size of the primary lesion (○) and the DTH response (●) to GP-10/20 in infected, resistant B10 mice. The mice were infected with 2×10^5 amastigotes in the left hind footpad. The size of the lesion is the average size from 10 mice. DTH response is the average from four mice injected with 50 µg of GP-10/20 in the right footpad. The DTH response was assessed by the difference (Δ) between footpad thickness before and 24 h after injection of the antigen. Controls (■) were four uninfected normal mice injected with the same amount of antigen. a, Standard deviation was 0.3 mm.

and Table 4). It was also observed that *L. donovani* promastigotes and *C. deanei* culture forms bear a cross-reactive epitope with GP-10/20 (Table 6). Many cross-reactive as well as species- and subspecies-specific epitopes recently have been described with the use of antileishmanial MAbs (4, 16) or murine T-cell clones (27). In a cross-panel analysis employing MAb specific for *Leishmania* spp., cross-reaction was observed with GP-10/20 and several MAbs specific for *Leishmania mexicana* (D. McMahon-Pratt, personal communication). The possibility that cross-reactive (public) and species-specific (private) epitopes are part of the same molecule is now being investigated.

The antigens described here could turn out to be useful tools for studying the mechanism involved in the generation of the specific suppressor and effector mechanisms underlying susceptibility or resistance to the disease.

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