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Immunology

Immunization with *Leishmania* receptor for macrophages protects mice against cutaneous leishmaniasis

(glycoconjugate/glycolipid/vaccine)

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ABSTRACT **The *Leishmania major* receptor for macrophages is a lipid-containing glycoconjugate that is recognized by the monoclonal antibody WIC-79.3. When *L. major* promastigotes were incubated with Fab fragments of WIC-79.3 prior to injection into genetically susceptible mice, their infectivity was decreased. Fab fragments from an irrelevant control antibody of the same class had no effect. The *L. major* glycolipid was purified from detergent-solubilized promastigotes by affinity chromatography on immobilized WIC-79.3 and used to vaccinate mice that are genetically resistant or susceptible to disease. Genetically resistant mice could be protected totally from cutaneous disease with as little as 5 μg of glycolipid. A high but not absolute level of resistance was also induced in the susceptible mice, in which the disease is otherwise fatal. No protection was obtained with the carbohydrate fragment of the glycolipid alone or by injection of the glycolipid in the absence of adjuvant. Genetically susceptible mice, immunized and protected from disease as a result of multiple injections of live avirulent cloned promastigotes of *L. major*, produced antibodies to the glycolipid of *L. major*. No antibodies were detected in serum from chronically diseased mice. The data suggest that this functionally important antigen of *L. major* is a candidate vaccine against cutaneous leishmaniasis.**

Old World cutaneous leishmaniasis, caused by the protozoan *Leishmania major*, is usually a self-limiting disease that is followed by long-lasting immunity and resistance to reinfection. To date, no vaccine exists against any parasitic disease of man, including leishmaniasis, but controlled exposure to living promastigotes has been used to induce protection [reviewed by Greenblatt (1)]. Cutaneous leishmaniasis should therefore be amenable to vaccine-based control. Ideally, the vaccine would consist of a defined antigen preparation.

To facilitate the development of leishmania vaccines, much use has been made of the mouse model for cutaneous leishmaniasis, which shares many features with the disease in man (2–4). Mice express a spectrum of disease patterns similar to that observed in man. Most laboratory mouse strains are "resistant," in that cutaneous lesions resolve spontaneously. In contrast, BALB/c mice and H-2 congeneic mice on a BALB/c background are susceptible and develop fatal disease. Genetically based variation in susceptibility therefore enables vaccination protocols to be tested under conditions of high stringency by using BALB/c mice or conditions of lower stringency by using C3H/He or C57BL/6 mice (5, 6).

We have recently identified and characterized a glycolipid antigen present on the promastigote membrane (7). This antigen was also found on the surface of the infected macrophage (7) and was subsequently shown to be the parasite receptor for macrophages (8). The *Leishmania* glycoconjugate is thus the molecule directly involved in the initiation of infection. It therefore seemed possible that immunization with this molecule might prevent infection. In this paper, we provide experimental evidence suggesting that this is the case.

MATERIALS AND METHODS

Mice. C3H/He and BALB/c and its congeneic partner lines BALB/c.H-2^b (BALB/B) and BALB/c.H-2^k (BALB/K) mice were produced in a pathogen-free facility and maintained conventionally as described (4, 9).

Parasites. The cloned, virulent *L. major* parasite line V121 was produced from the human isolate LRC-L137 (10) and maintained by passage in BALB/c mice. Promastigotes were grown *in vitro* in blood/agar cultures (4) or in RPMI 1640 medium with 10% fetal calf serum.

Preparation of Glycolipid from *L. major* Promastigotes. V121 promastigotes were grown *in vitro* for 4–6 days and washed in phosphate-buffered saline (P_i/NaCl) (pH 7.3), and 10^9 parasites were solubilized in 10 ml of 1% Triton X-100 in P_i/NaCl (TX-100/P_i/NaCl) containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride and 10 mM iodoacetamide). Insoluble material was removed by centrifugation at 28,000 × *g* for 30 min.

The detergent-soluble material was loaded onto a column containing monoclonal antibody WIC-79.3 bound to CNBr-activated Sepharose (7 mg/ml of gel) (Pharmacia). The column was washed extensively in detergent-free P_i/NaCl, and the bound glycolipid antigen was eluted with 6 M guanidine·HCl. The antigen was dialyzed in P_i/NaCl before use. In initial experiments, the Triton X-100 on the column was exchanged with octyl glucoside (30 mM in P_i/NaCl) before elution of antigen with 6 M guanidine·HCl. The octyl glucoside was then removed by dialysis in P_i/NaCl. Subsequently, it was found that the presence of detergent was no longer necessary after the glycolipid had bound to the antibody column, and the yield of purified antigen was similar in the two protocols. Since the monoclonal antibody WIC-79.3 is directed to a carbohydrate epitope in the glycolipid, the same column and a similar protocol were used for the purification of the water-soluble carbohydrate fragment of the antigen (excreted factor, EF) released by the parasites into culture medium (8). In this case, no detergent was used.

To quantitate the amount of glycolipid or glycoconjugate obtained, the Dubois–Gillis method was used (11) with glucose as standard.

Antibodies. The IgG1 monoclonal antibody WIC-79.3 is described in detail elsewhere (12–14). It binds specifically to the *L. major* promastigote membrane glycolipid and to the carbohydrate fragment of the glycolipid that is released from the parasite surface into culture medium in which the para-

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Abbreviations: EF, excreted factor; IRMA, immunoradiometric assay.

sites grow. The antibody was purified by ion-exchange chromatography on DEAE-Sephacel (Pharmacia) with a linear salt gradient and then was immobilized onto CNBr-activated Sepharose (Pharmacia) according to the manufacturer's recommendations.

Fab fragments of the purified WIC-79.3 antibody were generated by papain treatment as described (8). As a control, the IgG1 myeloma MOPC-21 was used.

Competitive RIA and Two-Site Immunoradiometric Assay (IRMA). To determine the titer of antibodies to the WIC-79.3 target epitope in mouse serum, the WIC-79.3 monoclonal antibody-based competitive RIA described by Greenblatt *et al.* (14) was used. The EF used in this assay was a gift of C. L. Greenblatt and was prepared by phenol extraction and gel filtration on Sephadex G-50 (15). This glycoconjugate represents the A1 serological prototype of *L. major* "EFs" (16, 17). In this assay, the monoclonal antibody WIC-79.3 can be replaced by another antibody, L-5-16, described by Handman and Hocking (18), that appears to have identical specificity but lower affinity.

For the IRMA, monoclonal antibody was incubated with polyvinyl chloride plates at 10 μg/ml overnight and, after 1 hr of incubation with 0.5% bovine serum albumin in P_i/NaCl, dilutions of serum or EF in 3% selected normal rabbit serum in P_i/NaCl containing 0.05% Tween 20 were added together with 30,000 cpm of ^{125}I-labeled monoclonal antibody (specific activity, 10 μCi/μg; 1 Ci = 37 GBq). After overnight incubation at room temperature, plates were washed and cut, and wells were assayed for radioactivity in a Packard autogamma counter. Using known amounts of EF, this IRMA was shown to be capable of detecting about 10 ng of EF per ml.

Vaccination and Assessment of Lesions After Challenge. Antigens were mixed with 100–200 μg of *Corynebacterium parvum* (Wellcome) and injected intraperitoneally. Alternatively, they were emulsified with an equal volume of Freund's complete adjuvant (Difco) and injected intraperitoneally. Details of subsequent injections and time of challenge are indicated in *Results*.

Immunized mice were challenged with V121 promastigotes cutaneously near the base of the tail, and lesions were scored as described (9). The scoring system was as follows: 1 = small swelling or resolving scar; 2 = larger swelling or small lesion <5 mm in diameter; 3 = lesion 5–10 mm; 4 = lesion >10 mm or evidence of systemic disease.

RESULTS

Fab Fragments of WIC-79.3 Passively Protect Mice from Infection with *L. major*. Our previous studies showed that Fab fragments of monoclonal antibody WIC-79.3 could block attachment of promastigotes to a macrophage cell line *in vitro* (8). The next question was whether they would reduce the infectivity of promastigotes *in vivo*. Promastigotes of the cloned *L. major* line V121 were incubated for 30 min on ice with Fab fragments of WIC-79.3 or MOPC-21 or with P_i/NaCl and then injected cutaneously into BALB/c mice. Fig. 1 summarizes results of four such experiments. When highly susceptible BALB/c mice were injected with small numbers of promastigotes that had been incubated with high concentrations of Fab fragments of the monoclonal antibody WIC-79.3, there was a substantial reduction in infectivity compared to controls (Fig. 1*B*). When mice were challenged with larger numbers of promastigotes treated with lower concentrations of antibody, the development of lesions was delayed but not abolished (Fig. 1 *A* and *C*).

In a separate experiment, BALB/c mice were injected with 10^3 WIC-79.3 antibody-secreting hybridoma cells intraperitoneally. Four days later, they were injected cutaneously with 5×10^3 promastigotes. Only 3 of 16 mice injected with the hybridoma developed tumors. These were the only mice that did not develop lesions for up to 40 days after infection with *L. major* promastigotes. All control mice, as well as mice that did not develop tumors, had lesions by 40 days.

Vaccination with the Water-Soluble Glycoconjugate Does Not Protect Mice. Initial experiments involved the intraperitoneal injection of BALB/c mice with the water-soluble glycoconjugate purified from parasite culture supernatant by affinity chromatography on the monoclonal antibody WIC-79.3. The priming injection consisted of the antigen in Freund's complete adjuvant and was followed by two aque-

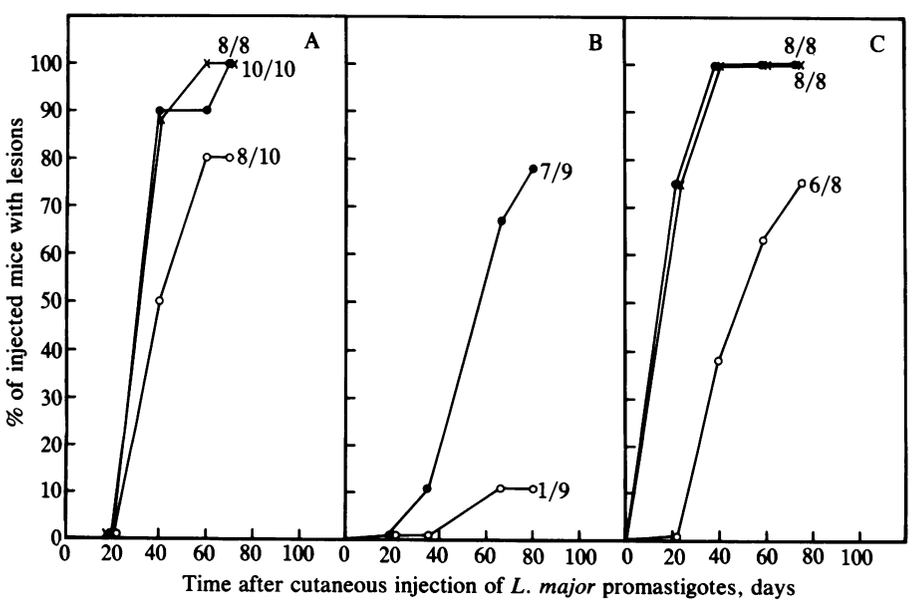

FIG. 1. Determination of the infectivity of promastigotes of *L. major* incubated with antibodies prior to cutaneous injection in BALB/c mice. Parasites were incubated with Fab fragments of the monoclonal antibody WIC.79-3 (○) or Fab fragments of the myeloma protein MOPC-21 (●) or with P_i/NaCl alone (×). Numbers of mice bearing lesions in each group are indicated at the termination of experiments. (*A*) Promastigotes (5×10^4) incubated with 400 μg of the various antibodies per ml injected into each mouse. (*B*) A pool of an experiment in which groups of mice received graded numbers of parasites (10^2, 10^3, or 10^4) incubated with 1 mg of antibody per ml. (*C*) Promastigotes (5×10^3) incubated with 500 μg of antibody per ml injected into each mouse.

ous booster injections. Mice were immunized with a total of 15 μg, 0.15 μg, or 0.015 μg of antigen prior to cutaneous challenge with 10^4 live promastigotes. No protection was observed with this immunization protocol. Low levels of antibodies to the vaccinating glycoconjugate could be detected by competitive RIA using radioiodinated WIC-79.3 in mice immunized with 15 μg of glycoconjugate but not in mice immunized with the smaller doses (data not shown).

In two other experiments, genetically susceptible BALB/c.H-2^b mice were injected intraperitoneally with 500 or 660 μg of *L. major* LRC-L137 glycoconjugate (EF) (15) together with 100 or 200 μg, respectively, of *C. parvum*, 3 weeks prior to challenge with live promastigotes. No alteration in the time course or pattern of lesion development was observed between experimental and control groups. The control groups consisted of mice injected with the adjuvant alone and mice that were not injected prior to challenge.

In all of the experiments described above the mean lesion score was ≥2 in all groups at day 40 and the lesions persisted through day 100.

Vaccination with the Lipid-Containing Glycoconjugate Protects Mice from Infection. In this series of experiments, mice that are genetically resistant or susceptible to disease caused by *L. major* were injected intraperitoneally with 2–5 μg of the glycolipid prior to challenge with live promastigotes.

Injection of the genetically resistant C3H/He mice with the glycolipid together with 100 μg of *C. parvum* induced complete protection from disease and none of the mice developed lesions (Fig. 2*A*). Six of the 7 control mice had lesions on day 40 after injection of 2×10^6 promastigotes. Healing was evident in control mice by day 60, when 4 mice still displayed lesions.

Injection of the glycolipid into the genetically susceptible BALB/c or BALB/c.H-2^k mice induced substantial reduction of disease but not absolute protection. BALB/c mice vaccinated with 2 μg of glycolipid in Freund's complete adjuvant and challenged with 1×10^4 promastigotes showed a delay of 50 days in the development of lesions (Fig. 2*B*). After 75 days only 10 of the 16 mice vaccinated showed lesions and their lesion score was significantly less than the controls. All control mice displayed lesions on day 50 after challenge, whereas only 25% of the vaccinated mice started to show signs of lesions by day 60.

BALB/c.H-2^k mice, which are slightly more resistant than BALB/c mice, were vaccinated with 5 μg of glycolipid together with *C. parvum* and were challenged with 2×10^6 promastigotes (a very high dose). Partial protection was achieved (Fig. 2*A*). Vaccination of BALB/c mice with the glycolipid alone injected intraperitoneally or together with the egg lecithin adjuvant Lipovant (Accurate Chemicals, Westbury, NY) did not confer protection.

We considered the possibility that protection was due to a contaminating protein that copurified with the glycolipid. To examine this possibility, the glycolipid was purified from promastigotes that had been biosynthetically labeled with [^{35}S]methionine (19). Analysis of this glycolipid preparation by NaDodSO$_4$/polyacrylamide gel electrophoresis and fluorography failed to detect any radiolabeled proteins, even after 1 week's exposure of the gel. In addition, serum from vaccinated mice did not specifically immunoprecipitate any radiolabeled parasite proteins (data not shown). These results are consistent with the notion that the immunizing antigen was indeed the glycolipid and not contaminating proteins from the parasite detergent lysate.

Evidence for a Difference in Responsiveness to the Lipid-Containing Glycoconjugate in Vaccinated Resistant Mice Compared with Chronically Diseased Mice. Genetically susceptible BALB/c and BALB/c.H-2 congeneic mice can be protectively immunized against chronic cutaneous disease by injection of living avirulent cloned promastigotes of isolate LRC-L137 (6). Serum from vaccinated and repeatedly challenged resistant mice was tested for the presence of antibodies to the glycoconjugate by competitive RIA. Serum from chronically diseased mice as well as from control mice infected with other parasites was also examined. Inhibitory activity was present in serum from resistant mice (50% inhibition at a serum dilution of >1:80) but not in serum from diseased or control mice.

The negative result with serum from diseased mice could have been due to the presence of circulating antigen that has complexed all antibody. However, no antigen could be detected in the serum of chronically diseased mice by using a two-site IRMA with a sensitivity of detection of <10 ng of

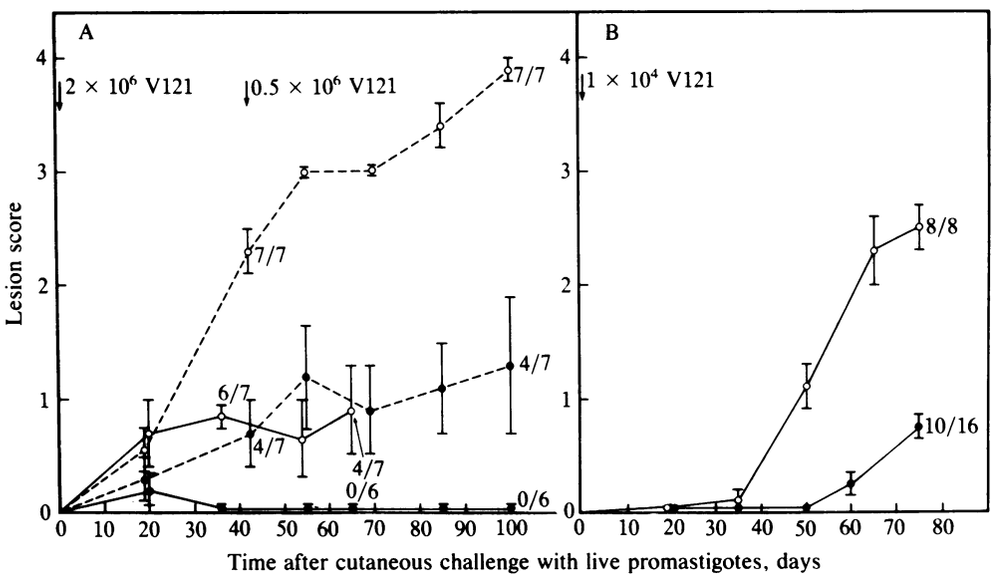

FIG. 2. Development of lesions in mice immunized with *L. major* glycolipid in adjuvant (●—●, ●----●) or adjuvant alone (○—○, ○----○) and challenged with live *L. major* promastigotes. (*A*) Solid line, C3H/He mice. Dashed line, BALB/c.H-2^k. The adjuvant used was *C. parvum*. (*B*) BALB/c mice. The adjuvant used was Freund's complete adjuvant. Data are expressed as lesion score ± SEM. Arrows at days 0 and 42 indicate time and dose of challenge.

glycoconjugate per ml. This supports the conclusion that diseased mice, in contrast to immune mice, do not produce detectable levels of antibodies directed to the *L. major* glycoconjugate.

DISCUSSION

The *L. major* glycolipid is an attractive candidate for a vaccine molecule because of its biological function as the parasite receptor for the macrophage (8) and because of its subsequent expression on the surface of the infected macrophage (12, 18). Immunization with this molecule could induce a first level of immune attack by antibodies, which may prevent parasite attachment to macrophages. Parasites that are not internalized rapidly by macrophages die in the extracellular space in a few minutes (20). If, however, one parasite succeeds in gaining entrance into a macrophage, infection may occur. In this case, the glycoconjugate displayed on the infected macrophage may induce a second level of immune attack by specific T cells, leading to macrophage activation and parasite killing (21–24).

Our previous studies (8) showed that incubation of *L. major* promastigotes with Fab fragments of the monoclonal antibody WIC-79.3 inhibited attachment of the parasites to macrophages *in vitro*. In the present study, we have extended this observation to the mouse model for cutaneous leishmaniasis. Incubation of 10^2–10^3 promastigotes with Fab fragments of WIC-79.3 monoclonal antibody before intradermal injection into the highly susceptible BALB/c mice prevented development of lesions. Control incubation in Fab fragments of MOPC-21 had no effect. As expected from passive immunization of this type, protection was achieved only at a high concentration of antibody and a small number of challenge organisms. However, the number of organisms used far exceeds the number believed to be introduced by the sandfly in natural infections (25). This result is consistent with evidence that a single virulent *L. major* promastigote is capable of inducing infection in BALB/c mice (14). One may expect that a few parasites in the higher inoculum doses may break through and infect macrophages.

Active immunization with the purified glycolipid was also effective. A total of about 2 μg of antigen in Freund's complete adjuvant was sufficient to significantly delay the development of lesions in the highly susceptible BALB/c mice. Although 87% of control mice had large lesions by day 50 after challenge, none of the vaccinated mice did. By day 75, however, about 60% of the vaccinated mice showed small lesions. In BALB/c.H-2^k mice immunized with 5 μg of glycolipid and *C. parvum* as adjuvant, three of seven mice were totally resistant, whereas the others developed lesions that generally remained small. In the genetically resistant C3H/He mice, which are similar to the majority of the human population exposed to *L. major*, vaccination with 5 μg of glycolipid conferred complete protection from disease. In these mice, no lesions developed up to 100 days after infection with large numbers of virulent parasites.

Protection was dependent on the intact glycolipid and on the use of particular adjuvants. Injection of the carbohydrate-containing fragment of the molecule alone did not confer protection in any experiment. In one experiment the purified glycolipid was treated with phospholipase CIII from *Bacillus cereus* (8) and the carbohydrate-containing fragment was separated and injected with *C. parvum* into BALB/c.H-2^k mice. No protection was observed in these vaccinated mice. However, genetically resistant mice could be partially protected with the water-soluble, lipid-free antigen chemically cross-linked to the synthetic adjuvant muramyl dipeptide (26). It is possible that this cross-linked conjugate is similar in its properties to the glycolipid and is processed by the immune system in a similar way.

Mice that were protectively immunized with live avirulent promastigotes of *L. major* and were resistant to infection with the virulent cloned line V121 were shown by competitive RIA to produce antibodies to the epitope recognized by the monoclonal antibody WIC-79.3. The presence of antibody directed to the target epitope(s) of WIC-79.3 in serum from resistant mice may reflect the operation of helper T cells in addition to T cells with the capacity to activate macrophages. The apparent lack of antibodies to the target epitopes of WIC-79.3 in chronically diseased mice may reflect the operation of T cells with suppressive, disease-promoting activities (27, 28). To date, model vaccines against cutaneous leishmaniasis have consisted of intact, irradiated, or disrupted promastigotes [reviewed by Howard and Liew (29)], killed infected macrophages, and living avirulent cloned promastigotes (5, 6). In all of these cases the subcutaneous route of injection was totally ineffective. Results presented in this paper suggest the possibility of a molecularly defined *Leishmania* vaccine. It remains to be determined whether the route of injection is also a limitation in the case of the glycolipid vaccine and whether injection of larger amounts of glycolipid will obviate the need for adjuvants of the type used in this study.

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