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Immunogenicity of Soluble and Particulate Antigens from *Leishmania donovani*: Effect of Glucan as an Adjuvant

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The protective efficacy of glucan as an adjuvant with killed promastigotes of *Leishmania donovani* was compared with that of soluble or particulate fractions of the parasite. When these vaccine preparations were injected either intravenously or subcutaneously in CF-1 mice, glucan potentiated resistance against *L. donovani* infections as reflected by significant reductions in hepatic amastigote counts relative to infected control mice. The leishmanial antigens alone afforded no protection. Serum direct agglutination titers to leishmanial antigens were highest in all groups given the vaccine intravenously, whereas the delayed-type hypersensitivity response to the antigen was positive only in groups immunized subcutaneously with glucan as an adjuvant. Some index of protection and immune response against visceral infection with the parasite was seen in groups vaccinated with glucan and soluble antigens. However, the protection afforded by glucan and particulate antigens of *L. donovani* more closely paralleled the resistance of mice treated with glucan and unfractionated killed promastigotes. Further antigenic analysis of particulate fractions of *L. donovani* may optimize effective immunization when used with appropriate adjuvants, e.g., glucan.

Parasites of the genus *Leishmania* are kinetoplastid protozoa which infect humans and other mammalian species. In these hosts, the leishmaniae have a predilection for macrophages of the reticuloendothelial system, where they multiply as obligatory intracellular organisms. The species *L. donovani* is the causative agent of human visceral leishmaniasis (Kala-azar), a disease associated with significant morbidity and mortality in endemic geographic areas (18, 29). The induction of protective immunity utilizing homologous killed vaccines would be a major step toward the control of such severe forms of leishmaniasis. However, the protection of experimental animals against leishmanial infections by the use of nonviable or attenuated parasites as vaccines has not been consistently achieved and has frequently produced disappointing results (14, 15; E. Marva and C. L. Greenblatt, J. Protozool. **26**:49, 1979). Immunogenic enhancement with appropriate adjuvants may thus be required to elicit protective immunity.

We have recently demonstrated the feasibility of such immunoprophylaxis with glucan, a β-1,3-glucopyranose derivative of yeast cell walls (10). Intravenous injections of glucan provided nonspecific protection against *L. donovani* infections in both mice (3, 11) and hamsters (2) as measured by lowered organ parasite burdens relative to control groups. Importantly, glucan was found to have a potent adjuvant effect when injected either intravenously or subcutaneously into mice with Formalin-killed promastigotes (10a, 11). This specific immunity was evident when the mice were challenged with viable parasites up to 80 days after immunization. In the latter investigations, glucan treatment alone afforded minimal nonspecific protection, whereas animals immunized with injections of the killed parasite alone exhibited no resistance to infection (10a, 11).

In these previous immunization studies, intact Formalin-killed promastigotes were used as an antigenic stimulus in the presence of glucan. The further characterization of protective antigens essential for the induction of specific immunity is a necessary step toward optimizing effective vaccine preparations. The identification of antigenic subcellular fractions of *Leishmania* sp. and the differentiation of common and strain-specific antigens (9) may allow the eventual determination of suitable antigens that stimulate protective immunity. It has also been proposed that leishmaniae may contain two types of antigens: those which stimulate specific resistance to infection, i.e., particulate antigens; and those which depress specific resistance to infection, i.e., soluble antigens (21). In the present study, we compared the efficacy of glucan as an adju-

vant with particulate and with soluble fractions of *L. donovani* relative to intact Formalin-killed parasites or frozen-thawed preparations. Specifically, these vaccine preparations were tested both intravenously and subcutaneously in CF-1 mice. The criteria for the induction of protective immunity were based on lowered organ parasite counts, changes in serum direct agglutination titers, and delayed-type hypersensitivity (DTH) responses to leishmanial antigens.

MATERIALS AND METHODS

Experimental animals and parasite. Female CF-1 mice were obtained commercially and maintained in air-conditioned quarters with food and water provided ad libitum. The mice were 8 to 10 weeks old at the beginning of the experiments. *L. donovani* 2S (23) was maintained by passage in outbred male hamsters. The methods used for the initiation and maintenance of promastigote stages in vitro have been described previously (2, 11). The promastigotes used to initiate infection were harvested during the log phase of growth by centrifugation (900 × *g*, 30 min) and suspended in Earle balanced salt solution (EBSS) at the desired concentration as determined by counting in a hemacytometer. The promastigotes used for infection were passaged in vitro no more than 10 times.

Preparation of glucan and parasite antigens. Glucan was prepared from bakers' yeast, *Saccharomyces cerevisiae*, as described by the method of DiLuzio et al. (6). The promastigotes were killed by freezing and thawing or by Formalin treatment. For the latter treatment, the promastigote forms used for immunization were harvested by the above method, washed three times in EBSS, and killed by suspension in 0.1% Formalin overnight. The dead promastigotes were washed three times and suspended in EBSS at the desired concentration. For the frozen-thawed preparation, the promastigotes, suspended in EBSS, were frozen at −70°C and then thawed at 37°C. This procedure was repeated three times. The criteria for the determination of promastigote death were the absence of parasite motility, failure of parasites to grow in vitro when inoculated into culture medium, and failure to initiate promastigote growth by in vitro cultivation from the ground spleens of immunized but unchallenged mice.

The particulate and soluble promastigote antigens were prepared from the frozen-thawed parasites. The preparation, on ice, was further fractionated by sonication at 30 MHz for a 1-min burst five times (Branson cell disruptor). The complete disruption of cellular integrity was verified by light microscopic examination. The preparation was centrifuged in a Sorvall (model RC-5B) for 11,100 × *g* for 20 min. The supernatant was decanted and passed through 0.2-μm membrane filters (Millipore Corp.). The particulate pellet was washed three times with EBSS and suspended to the original volume.

For the intravenous immunization, the mice received 0.2 ml of 1×10^7 killed promastigotes or the equivalent in soluble or particulate antigens with or without glucan (0.6 mg). The immunizing injections were given at 4-day intervals for four injections before challenge with 2×10^7 viable promastigotes on day 80 after the last immunization. The latter time interval, i.e., 80 days, was selected to preclude nonspecific protection by intravenous glucan (3) which may have masked its adjuvanticity with the killed parasite vaccine. For the subcutaneous study, the immunizing injections were given on days 48, 44, 40, and 21 before challenge with 1.0×10^7 promastigotes.

Monitoring of infection course. At designated times after infection, the mice in each treatment group were killed in chloroform vapor, and body spleen and liver weights were recorded. Impression slides from the spleen and liver were fixed in methanol and stained with Giemsa, and the parasite burdens in the organs were determined by the method of Stauber (23). DTH responsiveness was monitored by the footpad injection of washed promastigotes suspended in 0.5% phenol-saline (0.025 ml; 2.0×10^8/ml). The thickness of the inoculated foot was measured with dial calipers 24 h after injection, and the results were expressed as the percentage of increased thickness as compared with the uninjected contralateral foot. We have previously observed that the injection of the phenol-saline vehicle causes no significant increase in footpad swelling. Antibody detection was by the direct agglutination test, with trypsin-Formalin-treated promastigotes (10^8/ml) as the antigen (25).

Statistics. The results were analyzed by the Student unpaired *t* test with a probability of 0.05 considered significant. The data are expressed as the mean plus or minus the standard error of the mean.

RESULTS

Organ parasite counts. The intravenous administration of frozen-thawed promastigotes (FTP) or particulate and soluble antigens alone afforded no protection against hepatic amastigote proliferation when the mice were challenged with a viable inoculum 80 days after the last immunizing injection (Table 1). On the contrary, hepatic amastigote burdens were significantly higher than those of the untreated group of mice receiving FTP or soluble antigen alone on days 15 and 28, respectively, after infection. The glucan-treated group, however, did exhibit a significant reduction in hepatic parasites on day 15 postinfection relative to untreated controls (Table 1).

The groups receiving glucan with killed intact promastigotes exhibited significant reductions on all days postinfection relative to the untreated group (Table 1). The groups receiving glucan in combination with particulate antigen likewise exhibited significant decreases in hepatic amastigotes on days 15 and 28 postinfection. On the other hand, the conjoint administration of glucan and the soluble antigen had no effect on the early infection course but did suppress hepatic parasites on day 42.

As with the intravenous immunization study, the mice injected subcutaneously with promastigote antigens alone exhibited no evidence of

TABLE 1. Course of *L. donovani* infection in mice after intravenous immunization

Group[a]	Mean hepatic parasite count (± SEM; × 10^7) at (day postinfection):		
	15	28	42
Control groups			
Untreated	15 ± 1.0	24 ± 10	14 ± 3.6
FTP	15 ± 9.2	54 ± 12[b]	19 ± 3.8
Particulate	13 ± 9.4	15 ± 2.4	11 ± 3.3
Soluble	21 ± 2.0[b]	32 ± 9.8	17 ± 6.9
Glucan	11 ± 1.1[b]	10 ± 4.1	32 ± 18
Adjuvant group			
FKP + glucan	4.9 ± 0.9[b]	2.7 ± 0.7[b]	0.7 ± 0.1[b]
FTP + glucan	8.1 ± 3.6[b]	6.8 ± 0.8[b]	3.1 ± 1.46[b]
Particulate + glucan	8.0 ± 1.9[b]	5.6 ± 1.9[b]	15 ± 8.3
Soluble + glucan	15 ± 1.1	18 ± 2.6	3.3 ± 0.8[b]

[a] The mice received (intravenously) killed promastigotes (10^7) or the equivalent in particulate or soluble antigens with or without glucan (0.6 mg). The immunizing injections were given at 4-day intervals for four injections before challenge with 2.0 × 10^7 viable promastigotes on day 80 after the last immunization. The number of mice in each group was seven. FKP, Formalin-killed promastigotes.

[b] $P < 0.05$ compared with the untreated group.

increased resistance to *L. donovani* infection (Table 2). However, a significant decrease in hepatic parasites was observed in the FTP-particulate group receiving glucan as an adjuvant on all days postinfection. No protection was afforded by the subcutaneous injection of glucan with soluble antigens on day 14, but protection was seen on days 28 and 42 postinfection.

Immunological parameters. Serum direct agglutination titers to leishmanial antigens were used as an indication of humoral responsiveness. In the control groups, the highest $\log_2$ dilution titers occurred in the mice receiving intravenous FTP or particulate antigens (Table 3). The combination of glucan and the particulate antigen or intact parasite preparations appeared to boost the humoral response in the early (day 15) and late (day 42) stages of the infection.

Subcutaneous immunization had less effect on humoral responsiveness than did the intravenous route. Some increase in agglutination titers, i.e., range 7 to 10 $\log_2$ dilution titers, was observed on day 42 postinfection in the sera from all groups. However, no difference between the adjuvant and control groups with regard to the antibody titers was noted.

Footpad swelling in response to phenol suspensions of promastigotes was used as an index of cellular immune responsiveness. This DTH response was negative in all groups receiving intravenous treatments when tested 21 and 80 days after the last immunization injection. The

TABLE 2. Course of *L. donovani* infection in mice after subcutaneous immunization

Group[a]	Mean hepatic parasite count (±SEM; ×10^6) at (day postinfection):		
	14	28	42
Control groups			
Untreated	14 ± 2.6	67 ± 31	25 ± 12
FTP	12 ± 3.1	42 ± 22	23 ± 20
Particulate	8.4 ± 2.1	61 ± 5.7	35 ± 20
Soluble	51 ± 25	88 ± 33	20 ± 15
Glucan	23 ± 11	79 ± 45	48 ± 20
Adjuvant group			
FTP + glucan	5.4 ± 0.8[b]	3.8 ± 1.8[c]	2.6 ± 1.1[b]
Particulate + glucan	2.6 ± 0.7[b]	9.1 ± 0.7[b]	0.5 ± 0.2[b]
Soluble + glucan	23 ± 11	8.3 ± 3.6[b]	6.7 ± 2.9[b]

[a] The mice received (subcutaneously) 1 × 10^7 killed promastigotes or the equivalent in soluble or particulate antigens with or without glucan (0.6 mg). The immunizing injections were given on days 48, 44, and 21 before challenge with 1.0 × 10^7 promastigotes on day 0. The number of mice in each group was 7.

[b] $P < 0.05$ compared with the untreated group.

TABLE 3. Direct agglutination titers in mice after intravenous immunization

Group[a]	Direct agglutination titer[b] at (day postinfection):			
	0	15	28	42
Control group				
Untreated	0	7	9	7
FTP	8	14	>24	13
Particulate	6	14	21	14
Soluble	4	7	8	5
Glucan	0	6	10	6
Adjuvant group				
FKP + glucan	9	18	>24	>24
FTP + glucan	9	17	23	20
Particulate + glucan	8	15	>24	19
Soluble + glucan	7	12	11	8

[a] The immunization schedule was the same as that described in Table 1, footnote *a*. FKP, Formalin-killed promastigotes.

[b] The data are expressed as the highest $\log_2$ dilution titers in sera pooled from seven mice in each group.

intravenously treated groups also remained negative on days 15, 28, and 42 after infection. In the groups of mice receiving subcutaneous injections, all control groups were likewise negative at the time of infection (day 21 after the last injection) and on all days postinfection. The mice receiving subcutaneous glucan with parasite antigens were the only groups exhibiting positive DTH responses (Table 4). At the time of challenge and on day 14 after infection, all mice receiving glucan and FTP were positive; a proportion of mice in the particulate-glucan group were positive, whereas all mice in the soluble-glucan group were negative. The DTH reactivity of the mice receiving glucan and particulate antigen was also significantly greater than that of the group receiving the soluble antigen on day 28 postinfection.

DISCUSSION

Several approaches to vaccination against virulent leishmanial infections have been attempted in humans. These include the use of homologous virulent organisms, heterologous nonpathogenic organisms, or killed organisms. The inoculation of fully virulent *L. tropica major* promastigotes in human subjects induces a localized cutaneous lesion which resolves and confers protective immunity (8, 12). However, the use of self-limiting infections against more severe forms of the disease in humans, i.e., visceral and mucocutaneous leishmaniasis, is impractical in view of the risk of parasite dissemination. Effective immunization with nonpathogenic heterologous isolates or killed vaccine preparations has either not been substantiated (18, 19), or the data have been inconclusive (20).

Immunization trials with killed or attenuated *Leishmania* parasites have also been attempted in laboratory animals with variable results. A loss of infectivity was induced in *L. enriettii* by gamma radiation, but multiple vaccinations conferred no protection against the homologous parasite in guinea pigs (15). On the other hand, Coutinho (4) found that killed promastigotes of *L. enriettii* provided a limited degree of protection in guinea pigs. Marva and Greenblatt (E. Marva and C. L. Greenblatt, J. Protozool. 26:49, 1979) were unable to demonstrate protective immunity in mice vaccinated with *L. tropica* promastigotes which had been inactivated by exposure to acriflavine in vitro. Likewise, Lainson and Bray (14) found that intradermal injections of Formalin-killed *L. mexicana* with Freund adjuvant conferred no immunity on either mice or hamsters. More recently, Beacham et al. (1) found that sonicated preparations of *L. braziliensis* did afford a degree of protection in the African white-tail rat against cutaneous infection with that parasite.

The present study extends our previous observation that vaccination with killed promastigotes of *L. donovani* provides no protection in CF-1 mice when given either subcutaneously or intravenously (10a, 11). The subcutaneous administration of glucan alone also failed to elicit nonspecific resistance against *L. donovani* infection

TABLE 4. DTH response to leishmanial antigens in mice after subcutaneous immunization

Group[a]	Mean DTH response[b] (± SEM) at (day postinfection):			
	0	14	28	42
FTP + glucan	33.2 ± 5.9 (7/7)	42.9 ± 4.4 (7/7)	44.7 ± 7.9 (2/7)	17.7 ± 5.3 (5/7)
Particulate + glucan	36.0 ± 6.0 (5/5)	12.3 ± 8.9 (2/7)	43.2 ± 6.8[c] (6/7)	29.1 ± 4.3 (7/7)
Soluble + glucan	Negative (0/5)	Negative (0/7)	21.5 ± 3.9 (2/7)	23.9 ± 2.7 (5/7)

[a] The immunization schedule was the same as that given in Table 2, footnote *a*. The DTH response was measured as the percent increase in footpad swelling. All other control groups were negative for footpad swelling.

[b] The numbers in parentheses represent the number of mice with positive DTH reactions divided by the total number in the group.

[c] $P < 0.05$, particulate antigen plus glucan versus soluble antigen plus glucan.

and conferred minimal protection when given intravenously. Nonspecific protection by systemic glucan administration is a function of the time after treatment. Glucan induces a transient hyperplasia and hypertrophy of the major reticuloendothelial organs (5), and this hyperfunctional reticuloendothelial system coincides with maximal resistance against *L. donovani* infections in mice (3). Since the challenge with the viable inoculum in the present study was 80 days after the last intravenous vaccination, it is likely that an effective degree of nonspecific reticuloendothelial system stimulation by glucan was no longer present. As an adjuvant, glucan was effective in enhancing host resistance when given by both injection routes with the FTP preparation, and the degree of adjuvanticity observed was comparable to that previously demonstrated with intact Formalin-killed promastigotes (10a, 11). The fact that subcutaneous vaccination was effective is particularly important since the effects of intravascular glucan (see above) are potential contraindications for its clinical use. It also should be noted that glucan has been used as an intralesional treatment against malignant melanoma in patients and elicits few adverse side effects (17). An additional feature of the subcutaneously vaccinated group was the demonstrable DTH response to leishmanial antigens that was not seen in other control groups. The induction of such measurable immunological parameters may have important predictive value with regard to resistance to infection. This measure of cellular immunity did correlate well with enhanced resistance to infection in those groups immunized subcutaneously. The DTH response, however, was of no predictive value in mice immunized intravenously since all groups exhibited a negative reaction. Other indices of cellular immunity may thus be necessary to demonstrate enhanced immunocompetence after intravascular immunization.

Antibody titers were slightly higher in the mice immunized intravenously with glucan and leishmanial antigens, thus further indicating the adjuvant activity of the compound. Whether these enhanced antibody titers were of any consequence to the course of infection or are contingent upon the route of injection cannot be ascertained from present data. Cellular rather than humoral mechanisms, however, have been proposed as being primarily responsible for acquired immunity against visceral leishmaniasis (18, 24). Additionally, antibody titers were comparatively low in the subcutaneously vaccinated mice despite the observation of strong resistance to infection in the adjuvant-treated mice.

Protection against *L. donovani* with vaccine preparations from parasite antigenic extracts has not been adequately investigated. Preston and Dumonde (21), in a preliminary report, observed strong immunity in guinea pigs vaccinated with ribosomal fractions of *L. enriettii* and Freund adjuvant as evidenced by resistance to cutaneous infections and the development of DTH responses. In contrast, soluble antigens were not protective and appeared to impair resistance to infection. The latter response was attributed to immunological paralysis induced by the soluble antigens. In the present study, we observed that the particulate fraction of the parasite more closely paralleled the immunogenicity of the intact Formalin-treated or FTP preparation. This was shown by the comparable serum agglutination titers in the intravenously vaccinated group and positive DTH response in the adjuvant group receiving subcutaneous immunization. Organ parasites were likewise suppressed by both routes of vaccination in groups receiving the particulate antigen and glucan. On the other hand, the soluble antigen was not devoid of immunogenicity, and a significant proportion of the mice did exhibit some evidence of humoral and cellular immune responsiveness when glucan was given as an adjuvant. With the exception of a higher organ parasite number on day 15 postinfection in the mice injected intravenously with the soluble antigens, no other evidence of impaired resistance to infection compared with the untreated controls was seen. This differs from the augmented *L. enriettii* infections induced with soluble leishmanial antigens in guinea pigs (21). Such effects may well reflect intrinsic differences in the pathogenicity of these two leishmanial species, as well as the difference in animal models.

Ribosome-derived and ribonucleic-acid-rich extracts of some bacteria, fungi (7, 12, 17, 18, 28) and, more recently, of the kinetoplastid protozoon *Trypanosoma cruzi* (16) have been shown to confer a degree of protective immunity against infection in experimental animals. Studies aimed at further antigenic analysis of ribosomal and other subcellular fractions of *L. donovani* are merited to differentiate common and strain-specific antigens. The employment of effective adjuvants, e.g., glucan, with immunogenic fractions of the parasite may provide a practical and rational approach to potential immunoprophylaxis against visceral leishmaniasis.

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