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Leishmania mexicana Cysteine Proteinase-Deficient Mutants Have Attenuated Virulence for Mice and Potentiate a Th1 Response

James Alexander,* Graham H. Coombs,2† and Jeremy C. Mottram‡

Leishmania mexicana mutants lacking cysteine proteinase genes cpa (Δcpa), cpb (Δcpb), or both cpa and cpb (Δcpa/Δcpb) have been generated by targeted gene disruption. Δcpa mutants produce a disease phenotype in BALB/c mice close to that of wild-type L. mexicana, but Δcpb mutants are much less infective, producing very slowly growing small lesions, and Δcpa/Δcpb double mutants do not induce lesion growth. Immunologic analysis of Ab isotype during infection and splenocyte IFN-γ, IL-2, and IL-4 production following stimulation with Leishmania Ag or Con A indicates that there was a significant shift from a predominantly Th2-associated immune response in mice infected with wild-type L. mexicana to a Th1-associated response in mice inoculated with Δcpb or Δcpa/Δcpb. Significantly, Δcpa altered the balance of the immunologic response to a lesser extent than did the other mutants. Similar disease outcomes and switches in the Th1/Th2 balance were also observed when other L. mexicana-susceptible mouse strains were infected with the mutants. BALB/c and C57BL/6 mice vaccinated with Δcpa/Δcpb and CBA/Ca mice vaccinated with Δcpb or Δcpa/Δcpb were subsequently more resistant, to varying degrees, than were untreated mice to infection with wild-type parasites, as measured by development of lesions and parasite burden. These data implicate leishmanial cysteine proteinases not only as parasite virulence factors but also in modulation of the immune response and provide strong encouragement that cysteine proteinase-deficient L. mexicana mutants are candidate attenuated live vaccines. The Journal of Immunology, 1998, 161: 6794–6801.

The kinetics of the developing immune response and the ensuing disease phenotypes following infection with the obligate intracellular parasite Leishmania have been the subject of intense study during recent years. Thus, it is widely recognized that acquired protective immunity against murine cutaneous leishmaniasis is dependent on the ability to mount an IL-12-driven CD4+ Th1-type response (1–4). This lymphocyte subset produces IFN-γ, which mediates protection by up-regulating macrophage inducible nitric oxide synthase expression and nitric oxide production, which is microbicidal for the parasites (4). Consequently, neutralization of IL-12 or IFN-γ or inhibition of nitric oxide production results in disease exacerbation (2, 5–9). The immunologic pathways leading to the development of nonhealing progressive disease are less well characterized and more contentious. Thus, although a large number of studies have indicated that susceptibility to L. major (4, 10, 11), L. mexicana (12) and L. amazonensis (13) is related to a developing Th2 response and IL-4 production with down-regulation of Th1-associated activities, further studies on several Leishmania species suggest that the inability to mount a Th1 response rather than the presence of a Th2 response may determine susceptibility (13–15). Nevertheless, studies using IL-4-deficient mice from a number of genetic backgrounds have demonstrated an absolute requirement for this cytokine in determining susceptibility to L. mexicana, as in the absence of IL-4 lesions develop at the site of cutaneous infection (12). As most mouse strains develop nonhealing lesions when infected with L. mexicana (16), this parasite must be particularly adept at subverting a protective immune response.

Of particular interest with regard to the factors that determine the outcome of L. mexicana infection is the idea that cysteine proteinases (CPs) might be potentiating Th2 responses by enhancing IL-4 production (17). L. mexicana contains multiple, highly active CPs, many of which are stage regulated (18). Three L. mexicana CP genes are known. These are cpa, a single-copy gene encoding a nonabundant cathepsin L-like CP (19); cpb, a multicopy gene that encodes the major cathepsin L-like CPs of the intracellular mammalian amastigote form of the parasite (20); and cpc, a single-copy gene encoding a cathepsin B-like CP (21). Mutants lacking each of these genes have been generated by targeted gene disruption (21–23). cpa-deficient mutants were found to resemble wild-type L. mexicana in both in vitro and in vivo phenotypic tests (22). The cpb-deficient mutants proved to be more interesting. It was discovered that although cpb is not essential for growth or differentiation of the parasite in vitro, the null mutant was 5–10-fold less infective to explanted mouse macrophages, indicating that the enzyme is a virulence factor (23). The data suggest that cpb-deficient mutants can only survive in a subpopulation of macrophages, although the parasites that successfully infect these macrophages grow normally. The cpb-deficient mutants were found to infect BALB/c mice but produced only very small, slowly growing lesions.

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3 Abbreviations used in this paper: CP, cysteine proteinase; cpa, gene encoding a L. mexicana CP; Δcpa, L. mexicana cell line with the cpa gene deleted; cpb, gene encoding a multicopy L. mexicana CP; Δcpb, L. mexicana cell line with all the cpa genes deleted; Δcpa/Δcpb, L. mexicana cell line with both cpa and cpb genes deleted.
lesions (23). Mutants lacking both the cpa and cpb genes were also created, and these were found to have a phenotype similar to that of the cpb-deficient mutant in terms of their ability to infect macrophages in vitro (23), although the infectivity for mice of the cpa/cpb-deficient double mutant had yet to be studied.

These results demonstrate that L. mexicana CPs play an important part in sustaining an infection in the mammalian host. One clear possibility is that they modulate the host immune response to the parasite. The current study was therefore undertaken to determine whether the absence of CPs in the mutant parasites results in a shift in the Th2/Th1 balance and whether such a shift results in protective immunity. If so, such CP-deficient parasites would clearly be vaccine candidates of high potential.

Materials and Methods

Parasites

Promastigotes of L. mexicana (NYMC/BZ62/M379) were grown in HOMEM medium, pH 7.5, containing 10% (v/v) heat-inactivated FCS at 25°C as described elsewhere (19). The following antibiotics were added in combination, as appropriate, for maintenance of drug-selectable markers in the respective mutants: phleomycin (Cayla, Toulouse, France) at 10 μg/ml, nourseothricin hydrosulfate (Hans-Knoll Institute, Tubingen, Germany) at 25 μg/ml, puromycin (Sigma, Poole, U.K.) at 10 μg/ml, and hygromycin (Boehringer Mannheim, Mannheim, Germany) at 50 μg/ml.

Mice

BALB/c, C57BL/6, CBA/Ca, 129Sv/Ev, and 129Sv/Ev IFN-γ receptor-deficient and C57BL/6 recombinant activating gene-deficient (RAG2−/−) mice were bred and maintained at the Universities of Glasgow and Strathclyde. In the preliminary study, groups comprised 4 mice each. In later comparative and vaccine studies, however, groups of 20 female, 8–10-wk-old mice were infected s.c. in the shaven rump with 5 × 10^8 stationary-phase promastigotes of wild-type or CP-deficient L. mexicana. In each experiment, groups of normally 5 but no fewer than 4 mice were used from each group per sample point for immunologic analysis; the data presented are means ± SEM from the groups of animals. Each experiment was repeated twice (C57BL/6) or more (BALB/c and CBA/Ca). Lesion size was measured using a slide gauge micrometer. In the preliminary experiments undertaken at the University of Glasgow, lesion volume was measured (23). Thereafter, at the University of Strathclyde, lesion diameters were measured, and whole parasite burdens from excised disrupted lesions were assessed using a Neubauer hemocytometer (24).

Detection of Leishmania-specific Abs by ELISA

Peripheral blood was obtained from infected animals by tail bleeding into heparinized capillary tubes. All plasma samples were stored at −20°C before analysis for specific Ab content. Leishmania-specific IgG1 and IgG2a end-point titers were measured by ELISA as previously described (12). Briefly, each well of an Immunolon-1 microtiter plate (Dynatech Laboratories, Billinghamurst, U.K.) was coated with 1 μg of leishmanial lycase Ag (freeze/thawed wild-type promastigotes in PBS, pH 9.0) by overnight incubation at 4°C. Following incubation of serial dilutions of plasma samples for 1 h at 37°C, bound Abs were detected by incubation with either rat anti-mouse IgG1 horseradish peroxidase conjugate or rat anti-mouse IgG2a horseradish peroxidase conjugate (Southern Biotechnology Associates, Birmingham, AL) followed by conjugate washing with tetramethylbenzidine (0.06 mg/ml) in 0.1 M sodium acetate buffer, pH 5.5, containing 0.03% H₂O₂. The color reaction was stopped by adding 10% (v/v) sulfuric acid, and the absorbance was measured at 450 nm. Results are expressed as end-point dilutions, for which the end point is defined as the final plasma concentration that yielded an absorbance higher than a negative control plasma sample included in the assay. Comparisons between groups of mice were made with a Mann-Whitney U test.

Splenocyte responses

Spleens were aseptically removed at appropriate times postinfection, as detailed for individual experiments, and cell suspensions were prepared by gently teasing apart the tissue in RPMI 1640 supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.05 mM β-mercaptoethanol, and 10% (v/v) heat-inactivated FCS (Life Technologies, Paisley, U.K.). Following centrifugation at 200 × g for 10 min at 4°C, cells were resuspended in 3 ml of Boyle’s solution (0.17 M Tris-HCl, pH 7.2, and 0.16 M ammonium chloride) at 37°C for 3 min to deplete red blood cells. Spleen cell suspensions were then centrifuged at 200 × g for 10 min at 4°C, resuspended, and resuspended in 2 ml of complete RPMI 1640 (as above). Viable cells were enumerated by trypan blue exclusion, and the suspensions were adjusted to 5 × 10⁶ cells/ml. Aliquots of the cell suspension (100 μl) were added to 96-well, flat-bottom tissue culture plates (Costar, Cambridge, MA), and 100-μl aliquots of Con A (5 μg/ml) or L. mexicana lycase Ag (5, 10 or 25 μg protein/ml) were added as appropriate. Cultures were then incubated in 5% CO₂/95% air for 60 h at 37°C, whereupon cultures were pulsed with 0.25 Ci of [3H]thymidine (sp. act. 2 Ci/mmol) and incubated for another 12 h. Supernatants were collected from parallel cultures at this time for quantification of cytokine production (see below). Pulsed cells were then harvested onto filter paper using a cell harvester (Skatron, Lier, Norway), and thymidine uptake was determined by liquid scintillation on a beta counter (Pharmacia LKB Bio- tech, Milton Keynes, U.K.).

IFN-γ, IL-2, and IL-4 assays

IFN-γ, IL-2, and IL-4 production by stimulated cells (stimulated by Leishmania Ag or Con A) and nonstimulated cells from mice infected with wild-type or CP-deficient parasites were measured by capture ELISA. Briefly, the wells of Immunolon-I microtiter plates (Dynatech Laboratories) were coated with capture Ab at 2.0 μg/ml (IFN-γ, R4-6A2; IL-2, JES6-1A.2; PharMingen, San Diego, CA); IL-4, 11B11 (Genzyme, Cambridge, U.K.) in PBS (pH 9.0) or carbonate buffer (0.05 M, pH 9.5) by overnight incubation at 4°C. Wells were then washed three times with PBS, pH 7.4/0.05% Tween-20 and blocked by incubation with 10% (v/v) FCS for 1 h at 37°C. The culture supernatants and appropriate recombinant standard of IFN-γ and IL-4 (PharMingen; IL-2, Genzyme) were then added to individual wells. For standard curves, IFN-γ (0–10 ng/ml), IL-2 (0–1350 pg/ml), and IL-4 (0–1000 pg/ml) were used. Following incubations at 37°C for 2 h, the wells were washed three times with PBS, pH 7.4/0.05% Tween-20, and then biotinylated rat anti-mouse IFN-γ (XMG1.2, Pharmingen; 1 μg/ml), biotinylated rat anti-mouse IL-2 (JES6-5H4, Pharmingen; 1 μg/ml), or biotinylated goat polyclonal anti-IL-4 (Genzyme; 1 μg/ml) was added and incubated for 1 h at 37°C. For the detection of bound biotinylated rat Ab, 100 μl of streptavidin-alkaline phosphatase conjugate (diluted 1/1000, PharMingen) was added to each well for 45 min at 37°C and following further washing, binding was visualized with substrate consisting of p-nitrophenyl phosphate (1 mg/ml, Sigma) in glycine buffer (0.1 M, pH 10.4). The absorbance was subsequently measured at 405 nm on a Titertek Multiscan Plate Reader. For detection of bound biotinylated goat Ab, 100 μl of streptavidin-horseradish peroxidase conjugate (diluted 1/500, Genzyme) was added to each well for 30 min at 37°C and following further washing was incubated with tetramethylbenzidine as described above. Cytokine concentrations in the cell cultures were determined by the standard curve regression coefficient, r = 0.990 or better. All assays were conducted in triplicate. Comparisons between groups were made using the Student’s t test. P values of <0.05 were considered significant.

Vaccination studies

Two mouse strains (BALB/c and CBA/Ca) that develop nonhealing lesions when infected with L. mexicana and have been used previously for vaccine studies were examined (25), as well as C57BL/6 mice, which normally develop nonhealing lesions when infected with this parasite. The protocols for each mouse strain were modified to reflect their response to CP mutants. At 2 and 4 mo after vaccination by inoculation s.c. in the flank with 5 × 10⁶ stationary-phase promastigotes of Δcpb/Δcpb, BALB/c mice were infected s.c. in the shaven rump with 5 × 10⁶ wild-type parasites, and disease progression was compared with that of nonvaccinated mice. CBA/Ca and C57BL/6 mice were vaccinated with Δcpb or ΔΔcpb (s.c. inoculation of 5 × 10⁶ stationary-phase promastigotes) and challenged 8 wk later with 10⁵ stationary-phase promastigotes of wild-type L. mexicana. Lesion growth was monitored up to 20 wk and compared with that of nonvaccinated control animals.

Results

Lesion growth in mice inoculated with wild-type L. mexicana or CP-deficient mutants

BALB/c mice inoculated s.c. with 5 × 10⁶ stationary-phase promastigotes of wild-type L. mexicana developed rapidly growing, nonhealing lesions (Fig. 1). While lesion growth following s.c. infection with the cpb-deficient mutants (Δcpa) was slower than that recorded with wild-type infections, lesion growth resulting from infection with cpb-deficient mutants (Δcpb) was considerably slower.
6 mo after infection with wild-type (WT), Δcpa (○), or Δcpb (△). The lesions resulting from infection with Δcpb were slow to appear (first appearance at wk 31) and very small (mean lesion volume at wk 37 was 3.5 mm³), whereas inoculation of Δcpa/cpb did not result in lesions. The data are means from each group of mice. Error bars have been omitted for clarity; however, the data for each group were significantly different from those for each of the other groups subsequent to lesions becoming apparent.

FIGURE 1. Cutaneous lesion growth in BALB/c mice infected with 5 × 10⁶ stationary-phase *L. mexicana* promastigotes of wild-type (●), Δcpa (○), or Δcpb (△). The lesions resulting from infection with Δcpb were slow to appear (first appearance at wk 31) and very small (mean lesion volume at wk 37 was 3.5 mm³), whereas inoculation of Δcpa/cpb did not result in lesions. The data are means from each group of mice. Error bars have been omitted for clarity; however, the data for each group were significantly different from those for each of the other groups subsequent to lesions becoming apparent.

Ab response generated following infection with wild-type or CP-deficient *L. mexicana*

Plasma levels of *Leishmania*-specific IgG1 and IgG2a were determined 6 mo postinfection (Fig. 2). Animals infected with wild-type *L. mexicana* had pronounced *Leishmania*-specific Ab levels, primarily of the IgG1 subclass. Mice infected with Δcpa also had large Ab titers, primarily of the IgG1 subclass but with significantly higher IgG2a titers than those following infection with wild-type *L. mexicana* (p < 0.01). Mice infected with Δcpb had significantly less IgG1 Ab than animals infected with wild-type *L. mexicana* (p < 0.01), and there was a distinct and significant increase in the IgG2a/IgG1 ratio. Mice infected with Δcpa/cpb had very little detectable *Leishmania*-specific Ab.

**FIGURE 2.** Analysis of plasma IgG1 and IgG2a levels in BALB/c mice 6 mo after infection with wild-type (WT), Δcpa, Δcpb, or Δcpa/cpb stationary-phase *L. mexicana* promastigotes. Values represent mean end-point dilutions ± SEM (n = 4).

In vitro splenocyte proliferative responses following infection with wild-type or CP-deficient *L. mexicana*

Both wild-type and CP-deficient mice were able to mount Ag-specific and Con A-induced proliferative responses at all time points examined (2, 3, 4, 6, and 9 mo) in all experiments. From 2 mo onward the Ag-specific and from 6 mo onward the Con A-induced proliferative responses were significantly greater in mice inoculated with Δcpa/cpb (p < 0.05 for Con A and Ag) and mice inoculated with Δcpb (p < 0.05 for Con A and Ag) than in those mice inoculated with wild-type parasites (Fig. 3).

**FIGURE 3.** Splenocyte proliferation responses induced by Con A (5 μg/ml) or *L. mexicana* lysate (5 μg/ml) in BALB/c mice infected with *L. mexicana* wild-type parasite (WT), Δcpb, or Δcpa/cpb, 6 mo postinfection. Data are represented as mean stimulation index ± SEM (n = 5).

IFN-γ production by stimulated splenocytes from infected mice

IFN-γ production from splenocytes isolated from BALB/c mice 9 mo postinfection and stimulated with parasite Ag (5 μg protein/ml) or Con A are shown in Fig. 4, a and b. Similar results were found for different amounts of Ag used and at earlier time points postinfection (not shown). Ag stimulation resulted in significantly increased IFN-γ production in comparison with background levels (p < 0.05) by all splenocyte cultures (Fig. 4a). However, production was significantly greater by Ag-stimulated splenocytes from animals infected with CP-deficient mutants than with wild-type *L. mexicana* (Δcpa and Δcpb, p < 0.05; Δcpa/cpb, p < 0.005). Moreover, Ag-induced IFN-γ production was significantly greater from splenocytes isolated from mice infected with Δcpb (p < 0.05) and Δcpa/cpb (p < 0.01) than from splenocytes isolated from Δcpa-infected mice. Con A stimulation increased IFN-γ production to significantly greater levels than background in all splenocyte cultures. Under these conditions, splenocytes derived from mice infected with Δcpa (p < 0.05) and Δcpb (p < 0.02) but not Δcpa/cpb produced significantly more IFN-γ than splenocytes derived from mice with wild-type infections (Fig. 4b).

**FIGURE 4.** IFN-γ production from splenocytes of mice infected with wild-type or Δcp-deficient *L. mexicana*.

**FIGURE 3.** Splenocyte proliferation responses induced by Con A (5 μg/ml) or *L. mexicana* lysate (5 μg/ml) in BALB/c mice infected with *L. mexicana* wild-type parasite (WT), Δcpb, or Δcpa/cpb, 6 mo postinfection. Data are represented as mean stimulation index ± SEM (n = 5).

IL-2 and IL-4 production by stimulated splenocytes from infected mice

In follow-up studies, we concentrated on comparing primarily the developing immune response in mice inoculated with Δcpa/cpb and wild-type parasites. In addition to confirming differences in splenocyte IFN-γ production, we measured IL-2, IL-4, IL-5, IL-10, and IL-12 production. While no differences in splenocyte IL-5, IL-10, and IL-12 production were observed between mice infected with Δcpa/cpb and those infected with wild-type parasite, profound differences in IL-2 and IL-4 production were observed, depending on the infectious agent, at 2 and particularly 4 and 6 mo...
postinfection (Fig. 5, a and b). While splenocytes from animals infected with wild-type parasites failed to produce a significant Ag-induced increase in IL-2 production, those infected with Δcpa/Δcpb produced, following stimulation with Ag, IL-2 significantly over background (p < 0.01). Ag-stimulated splenocyte IL-4 production was, however, significant over background for both animals infected with Δcpa/Δcpb (p < 0.001) and those infected with wild-type parasites (p < 0.001). However, the increase in splenocyte IL-4 production was significantly greater in wild-type parasite-infected animals than in animals inoculated with Δcpa/Δcpb (p < 0.001).

Infectivity of CP-deficient mutants for other mouse strains

Δcpa/Δcpb parasites failed to induce lesion growth in C57BL/6, CBA/Ca, 129Sv/Ev, IFNγR−/− 129SvEv, and RAG2−/− C57BL/6 mice up to 6 mo postinfection (results not shown). Small lesions were induced in C57BL/6 and RAG2−/− mice, but not in any of the other strains used, by inoculation with Δcpb (results not shown). All animals infected with wild-type parasites developed large, nonhealing lesions. The immunologic responses generated by wild-type parasites or CP-deficient mutants in the wild-type mouse strains were examined and were similar to those observed in BALB/c mice. In C57BL/6 mice, Ag-induced splenocyte cytokine production in animals inoculated 6 mo previously with Δcpa/Δcpb consisted entirely of IFN-γ (p < 0.01) and IL-2 (p < 0.05), with virtually no IL-4 produced above background levels (Fig. 6, a through c). However, Ag-stimulated splenocytes from animals inoculated with wild-type parasites produced not only significant levels of IFN-γ (p < 0.05) but also IL-4 (p < 0.01), with little or no IL-2 above background levels. Although IFN-γ levels following Ag stimulation were similar in wild-type and Δcpa/Δcpb infected mice groups, the increase in splenocyte IFN-γ production in Δcpa/Δcpb-inoculated animals over background (5.8 ± 1.5 and 0.11 ± 0.05 pg/ml, respectively) was 10-fold greater than that produced over background by splenocytes from wild-type parasite-infected mice (6.0 ± 3.2 and 0.99 ± 0.43 pg/ml, respectively). Con A-induced splenocyte IFN-γ production was also significantly greater (p < 0.05) in mice inoculated with Δcpa/Δcpb than in mice inoculated with wild-type parasites.

Vaccine potential of CP-deficient mutants

BALB/c mice vaccinated 2 or 4 mo before infection with wild-type parasites produced more slowly growing lesions (Fig. 7, a and b), which contained significantly fewer parasites, than similarly infected nonvaccinated mice. At wk 8 postinfection with wild-type L. mexicana, the wild-type parasite burdens in mice vaccinated 2 and 4 mo previously with Δcpa/Δcpb were significantly less than those of nonvaccinated mice (for vaccinated mice: 2 mo, 2.7 × 10^6 ± 5.4 × 10^6; 4 mo, 1.6 × 10^6 ± 3.2 × 10^6; for nonvaccinated mice: 2 mo, 6.2 × 10^7 ± 2.3 × 10^7; 4 mo, 8.4 × 10^7 ± 2.4 × 10^7;
p < 0.002 and p < 0.001, respectively). Whereas all nonvaccinated and the vast majority of vaccinated mice went on to develop nonhealing lesions, 2 of the 10 mice infected 4 mo after vaccination failed to develop lesions up to 12 wk postchallenge. C57BL/6 mice vaccinated with Δcpa/cpb also developed significantly smaller lesions following challenge infection with wild-type parasites than nonvaccinated mice (Fig. 8, p < 0.005 wk 14 postchallenge). The lesions in the vaccinated C57BL/6 mice but not the control mice began to decrease in size after this period.

CBA/Ca mice were also used in vaccine studies, as they have been shown to be more amenable to vaccination against L. mexicana than BALB/c mice (25) and also fail to develop lesions following challenge with Δcpb. Nine of 10 nonvaccinated mice developed nonhealing cutaneous lesions by wk 14 following infection with wild-type L. mexicana promastigotes. However, only 1 of 10 mice vaccinated with Δcpb and 1 of 10 mice vaccinated with Δcpa/cpb had developed lesions over the experimental period following the same challenge with wild-type parasites (Table I). Analysis of splenocyte Ag-specific IFN-γ production at 8 and 14 wk post-challenge infection demonstrated the significantly enhanced ability of Δcpa/cpb-vaccinated mice to produce this cytokine over control nonvaccinated animals infected with wild-type parasites (Fig. 9).

Discussion

One significant phenotype of the L. mexicana CP-deficient mutants is the difference in their ability to form lesions in the highly susceptible BALB/c murine infection model. The virulence of Δcpa to BALB/c mice is similar to that of wild-type L. mexicana (Ref. 22 and Fig. 1), whereas deletion of the multicopy cpb genes results in mutants that form only very slowly growing small lesions (Ref. 23 and Fig. 1). The finding that cpb-deficient double mutants do not form lesions in BALB/c mice suggests that deletion of both
genes has a synergistic effect in reducing infectivity. Furthermore, the finding that Δcpa/cpb mutants infect macrophages at a similar rate as do Δcpb mutants confirms that the in vivo situation is more complex than that in vitro and implicates host factors, in addition to the susceptibility of macrophages, as playing parts in determining whether the parasites cause lesions. The difference between the ability of the CP-deficient mutants to infect mice was also observed in C57BL/6 mice. Two other strains (CBA/Ca and 129Sv/Ev) did not form lesions when inoculated with either Δcpb or Δcpa/cpb, whereas nonhealing lesions resulted from inoculation with wild-type L. mexicana. An examination of the humoral and cellular immune responses of BALB/c mice following infection with the CP-deficient or wild-type L. mexicana suggests that the CPs of the parasite may not only be important virulence factors but may also play a crucial role in modulating the host immune response.

The resistance of BALB/c mice to cutaneous lesion growth following infection with CP-deficient mutants was closely related to the ability of their splenocytes to produce IFN-γ following specific Ag stimulation. However, as the CP-deficient mutants failed to induce lesions in IFN-γR−/− mice, raised IFN-γ is perhaps a beneficial consequence of infection with these parasites but not the reason for their low virulence. Nevertheless, as they cause a significant elevation of parasite-specific IFN-γ production, these CP mutants may be excellent vaccine candidates. Previous reports for the L. mexicana complex (12, 13, 15) have shown that there is a strong correlation between IFN-γ production and resistance to infection, sometimes irrespective of the presence of IL-4 (13, 15). In addition, CPs have previously been reported to induce IL-4 production (17), and throughout the course of infection in this study, splenocytes from mice infected with wild-type parasites produced significantly more IL-4, following stimulation, than those infected with Δcpa/cpb mutants. Indeed, Ag-induced splenocyte IL-4 production was barely detectable in the majority of C57BL/6 mice infected with Δcpa/cpb. These results are consistent with the finding that lesions failed to develop following L. mexicana infection in susceptible mice totally deficient in IL-4 (12). Furthermore, although lesions developed in IL-4−/− splenocyte-reconstituted SCID mice following infection with L. mexicana, these healed, unlike IL-4−/− splenocyte-reconstituted SCID mice, which continued on to develop fatal infection (26). Thus, while healing of L. mexicana lesions and resistance to lesion growth may take place in the presence of IL-4 and is associated with IFN-γ production, a nonhealing response is probably dependent on the presence of lymphocyte-derived IL-4. Taken together, our results further emphasize the finding originally described by Heinz et al. (10), and now well documented, of the counterregulatory activities of IFN-γ and IL-4 in determining the outcome of cutaneous leishmaniasis.

Table I. Incidence of cutaneous lesion development in nonvaccinated or CP-deficient mutant-vaccinated CBA/Ca mice subsequently challenged with wild type L. mexicana stationary phase promastigotes

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>No. of Mice With/ Without Lesions</th>
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<tr>
<td>Group 1</td>
<td>—</td>
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<tr>
<td>Group 2</td>
<td>Δcpa/cpb</td>
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<tr>
<td>Group 3</td>
<td>Δcpb</td>
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* Mice were vaccinated subcutaneously with 5 × 10⁶ Δcpb or Δcpa/cpb promastigotes 8 wk prior to challenge infection with 10⁶ stationary phase promastigotes of wild-type L. mexicana.

* Lesion development was monitored over a 14-wk period postchallenge with wild-type parasites.
While Ag-induced splenocyte IL-4 production was reduced or absent in Δcpa/cpb-infected mice, IL-2 production was significantly enhanced. No increased IL-2 production was generally observed in similarly treated wild-type parasite-infected mice. While several reports have suggested a potential disease-exacerbating role for IL-2 in cutaneous leishmaniasis (24, 27), this has been related to the ability of IL-2 to promote IL-4 production (27). In the present study this is clearly not the situation, as the cytokines appear to be counterregulatory. What may be more important in this study on L. mexicana, which is known to disseminate and visceralize under controls similar to those described for L. donovani (16), is that IFN-γ and IL-2 have been shown to synergize to protect against L. donovani (28). Indeed, we have found that although local treatment of L. mexicana lesions with rIL-2 did not reduce local parasite burdens (24), it did prevent disease dissemination (unpublished observation).

Concurrent with enhanced Ag-induced IFN-γ production by splenocytes and lymphocyte proliferation responses from mice infected with Δpb and Δcpa/cpb, there was a significant reduction in parasite-specific Ab levels. This is reminiscent of the immunologic status of humans at the resistant end of the disease spectrum (reviewed in Ref. 29). Similarly, high Ab levels and low IFN-γ production displayed by animals infected with wild-type L. mexicana are generally reminiscent of individuals with nonhealing disease (29). Although producing high specific Ab levels, splenocytes derived from Δcpa-infected mice did produce more IFN-γ than their wild-type counterparts upon Ag stimulation and consequently significantly more IFN-γ-dependent IgG2a Ab (30). Clearly, however, the increase in the ability to produce IFN-γ was not sufficient to shift the balance toward a protective Th1 response as the cpa-deficient mutants formed nonhealing lesions, albeit somewhat more slowly growing than those caused by wild-type L. mexicana. While it has been suggested from studies on BALB/c mice infected with L. major (31) that simply reducing the infectious parasite dose can have immunomodulatory effects similar to those observed here, we have found no healing responses following infection with low doses of wild-type L. mexicana in the mouse strains used in this study.

Our observations therefore support the theory that CPs have a Th2-potentiating role (17). This previous study provided preliminary evidence that the CP papain was a potent allergen that, following inoculation into the footpad, induced a 10–30-fold increase in IL-4 mRNA expression in mice within 1 h. Other CPs are also low doses of wild-type L. mexicana homologue of receptor for activated kinase) to promote IL-4 production in susceptible mice has also been observed for these parasites (36, 37).

Thus, L. major-susceptible mice made tolerant to LACK respond with a Th1 rather than a Th2 response when challenged with wild-type parasites (37). The discovery of how L. mexicana CPs mediate their effect awaits further study. Interestingly, however, leishmanial CPs have been implicated in the inhibition of macrophage Ag presentation (38, 39) by degrading MHC class II molecules in the parasitophorous vacuole (39). Limiting the ability of macrophages to present Ag has previously been shown to potentiate Th2 responses by favoring Ag presentation by accessory cells of other lineages (40).

Targeted gene deletion of an essential metabolic gene, dihydrofolate reductase-thymidylate synthase, has been used to create L. major auxotrophic mutants (41). These parasites failed to replicate in macrophages but persisted in BALB/c and nu/nu BALB/c mice for 2 mo without causing overt disease (42). By contrast, Δcpa/cpb multiplied in a small number of macrophages in vitro (23) and persisted in mice for up to 6 mo postinfection although without lesion development. While persistence of parasites without clinical infection is well documented (43) and may be necessary to maintain an anamnestic response (44), there is the risk of subsequent disease reactivation in immunodepressed individuals. It was extremely encouraging, therefore, that these CP-deficient mutants not only failed to induce overt disease in a number of L. mexicana-susceptible mouse strains such as 129Sv/Ev, but also failed to induce lesion growth in IFN-γ−/−/− 129Sv/Ev mice and in T and B cell-deficient RAG2−/−/− mice on a C57BL/6 background.

The majority of mouse strains are susceptible to L. mexicana (16, 45) and develop nonhealing lesions that may metastasize to the extremities or the viscera. Thus, this parasite in mice offers an excellent model system for putative vaccine studies against disseminating disease in a variety of susceptible genotypes. It is therefore extremely encouraging that Δcpa/cpb successfully limited lesion and parasite growth in BALB/c mice and C57BL/6 mice and that both Δpb and Δcpa/cpb virtually ablated the incidence of lesion development in CBA/Ca mice challenged with wild-type parasites following s.c. vaccination. This is particularly promising, as vaccination by this route in the absence of a cytokine or suitable adjuvant generally results in exacerbated disease (3, 4). The positive findings clearly suggest that the CP-deficient L. mexicana are vaccine candidates of high potential.

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References


