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Expression of Multiple *CPB* Genes Encoding Cysteine Proteases Is Required for *Leishmania mexicana* Virulence In Vivo

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Leishmania mexicana **mutants deficient in the multicopy *CPB* gene array have reduced virulence, demonstrated by poor lesion growth in BALB/c mice and induction of a protective Th1 response. Reinsertion of the amastigote-specific *CPB2.8* or metacyclic stage-specific *CPB2* gene into a *CPB*-deficient mutant *L. mexicana* failed to restore either a Th2 response or sustained virulence. However, reexpression of multiple *CPB* genes from a cosmid significantly restored virulence. This was characterized by increased lesion and parasite growth and the acquisition of a Th2 response, as determined by measuring interleukin-4 production and immunoglobulin G1 (IgG1) and IgE levels. These studies confirm that *L. mexicana* cysteine proteases are important virulence factors and provide an explanation for the presence in *L. mexicana* of a multicopy tandem array of *CPB* genes.**

Parasite cysteine proteases have been implicated in several processes including differentiation, nutrition, host cell infection, and evasion of the host immune response (10, 17, 19). *Leishmania mexicana* possesses three cysteine proteases of the papain family (designated clan CA), namely, the cathepsin L-like CPA (12) and CPB (25) and the cathepsin B-like CPC (4). The *CPB* genes are multicopy and are located in a single locus of 19 copies arranged in a tandem repeat (13, 14) (Fig. 1a). The first two copies of *CPB*, *CPB1* and *CPB2*, are expressed in the infective metacyclic stage of the parasite, while the others are expressed predominantly in the intracellular amastigote stage (6, 13, 14). Information about the roles and importance of the enzymes in host-parasite interactions was obtained by the generation of a *CPB*-deficient (Δ*cpb*) mutant. It was shown that Δ*cpb* promastigotes are less infective to macrophages than wild-type parasites in vitro and that they are able to form only small, slow-growing lesions in BALB/c mice (8, 13). In contrast, Δ*cpb* amastigotes were able to infect macrophages in vitro with the same kinetics as wild-type parasites but, in a similar manner to Δ*cpb* promastigotes, formed only small, slow-growing lesions in mice. Subsequent studies indicated that the absence of the *CPB* genes resulted in a protective Th1 immune response, contrary to the Th2 response normally observed when the CPB isoenzymes are present (1, 2). The pivotal role of interleukin-4 (IL-4) and the Th2 response in establishing nonhealing *L. mexicana* infections is well documented (3, 21), and significantly we have recently found that enzymatically active CPB is a potent stimulator of IL-4 and a Th2 response (15).

Reexpression of individual *CPB* genes from an extrachromosomal episome in the Δ*cpb* mutant has allowed further analysis of the importance of individual *CPB* gene products. In particular, differences in substrate specificity among the individual isoenzymes were demonstrated (8, 14) and insights into the processing and the trafficking of these enzymes were provided (5). However, only marginal recovery of lesion growth, at best, could be restored to Δ*cpb* mutants by reinserting individual *CPB* genes on episomes (8, 13). This inability to restore virulence could have been due to the loss of the *CPB* gene-containing episome by amastigotes in vivo, in the absence of antibiotic pressure. An alternative possibility is that higher CPB activity is required to enhance infectivity and that this is achieved only when several isoenzymes are present. To test these possibilities, we reintegrated individual *CPB* genes into the endogenous *CPB* locus of the Δ*cpb* mutant and also reintroduced multiple *CPB*s of the array into the Δ*cpb* mutant on an extrachromosomal cosmid. The results of our studies indicate that the expression of multiple *CPB* genes is key to the development of a Th2 response and consequently the virulence of *L. mexicana* to mice.

MATERIALS AND METHODS

Leishmania **cultivation and transfections.** The lines studied in this work were derived from *L. mexicana* (MNYC/BZ/62/M379) as described elsewhere (13). *L. mexicana* promastigotes were routinely grown in modified Eagle's medium (designated complete HOMEM medium), pH 7.5, containing 10% (vol/vol) heat-inactivated fetal bovine serum at 25°C as described elsewhere (14). *L. mexicana* parasites were transformed in vitro into axenic amastigotes as described previously (8).

All transfections were carried out as previously described (13) with 10 μg of linear DNA fragments or 5 μg of cosmid DNA. After transfection, the cells were selected in complete HOMEM medium containing the appropriate antibiotic for selection (hygromycin [50 μg/ml], nourseothricin [10 μg/ml], bleomycin [10 μg/ml], or puromycin [10 μg/ml]). Individual clones were isolated on 1% (wt/vol) agar–complete HOMEM plates and then directly transferred to complete HOMEM medium. The individual *CPB* genes were reintegrated into the *CPB* locus of *L. mexicana* as described previously (6).

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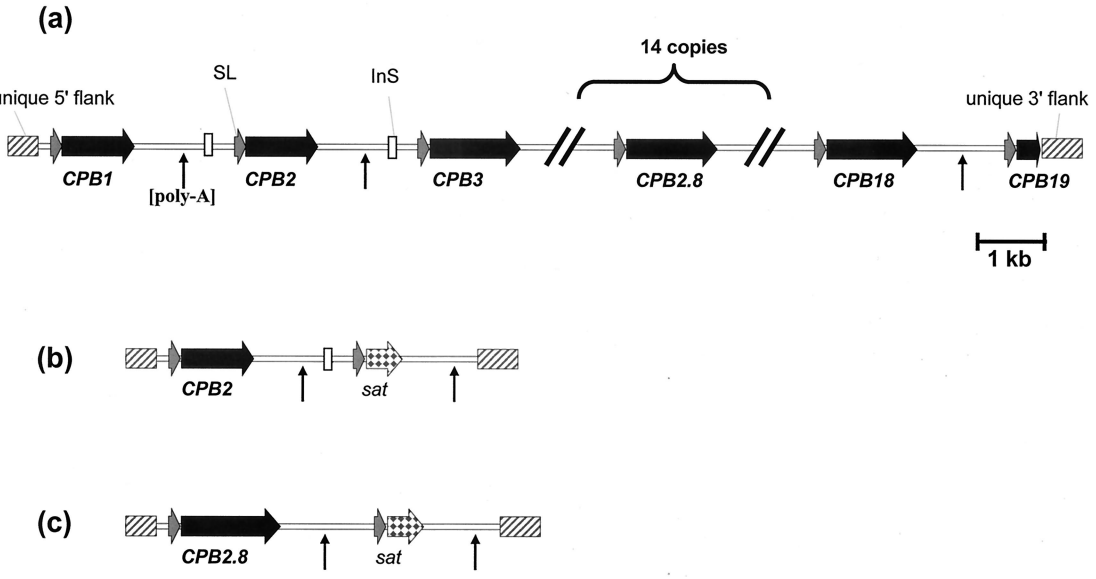

FIG. 1. Map of the *CPB* array and reintegration constructs. (a) Map of the *L. mexicana CPB* locus. InS, insertion sequence present in the intercistronic sequence downstream of *CPB1* and *CPB2*; SL, *CPB* splice leader acceptor site present upstream of each gene; vertical arrows, polyadenylation addition sites. (b and c) Maps of the *CPB2* and *CPB2.8* reintegration cassettes. The *CPB* genes, their 3′ intergenic sequences, and the *sat* gene were cloned between the unique 5′ and 3′ *CPB* flanking regions. Symbols are as in panel a.

Infectivity studies. Groups of five mice were inoculated in the footpad with 5×10^5 stationary-phase *L. mexicana* parasites resuspended in 0.025 ml of phosphate-buffered saline, pH 7.4. The resultant lesions were monitored over an 8-week period. All experiments were carried out on at least two occasions.

Screening of the *L. mexicana* cosmid library. An *L. mexicana* M379 cosmid library (D. C. Barker, Cambridge, United Kingdom) was screened with three [^{32}P]dCTP-labeled probes prepared with a Prime-it random-priming kit (Stratagene). One probe recognized the unique 5′ flanking region (5′ flank), another recognized the *CPB* gene (*CPB*), and the third recognized the unique 3′ flanking region (3′ flank), as previously described (13). Hybridization took place overnight at 65°C in 1 M NaCl–1% sodium dodecyl sulfate (SDS) with 100 μg of salmon sperm DNA/ml. The filters were washed stringently with 0.2× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% SDS at 60°C and exposed to photographic film (Agfa) at −70°C.

A cosmid (designated pGL263) containing 5′ flank, 3′ flank, and *CPB* sequences was isolated from a selected bacterial clone with a Wizard midiprep kit by following the manufacturer's instructions (Promega, Southampton, United Kingdom). After mouse infection, the cosmid was recovered from the induced lesions as described elsewhere (7).

Gelatin SDS-PAGE analysis. Cysteine protease activity was assayed by gelatin SDS-polyacrylamide gel electrophoresis (PAGE) as previously described (16). Coomassie blue stain was used to visualize the hydrolysis of 0.2% gelatin copolymerized in the 12% separating gel.

Protease activity assays. Lysates of stationary-phase promastigotes were prepared as for gelatin SDS-PAGE analyses and assayed for their ability to hydrolyze Z-Phe-Arg-7-amido-4-methylcoumarin (ZFR-AMC) at 30°C in 0.1 M sodium acetate, pH 5.2, with 2 mM EDTA and 1 mM dithiothreitol. Lysate samples were preincubated for 10 min at 30°C in assay buffer only or in the presence of cysteine protease inhibitor E-64 (10 μM; Sigma). The reaction rate was measured on a Perkin-Elmer LS 55 spectrofluorometer, with 380 nm as the excitation wavelength and 440 nm as the emission wavelength.

Detection of *Leishmania*-specific IgG1, IgG2a, and total IgE. Peripheral blood was collected from infected animals by tail bleeding into heparinized capillary tubes at 6 to 8 weeks postinfection. All plasma samples were stored at −20°C before analysis. *Leishmania*-specific immunoglobulin G1 (IgG1), IgG2a, and total IgE were measured by enzyme-linked immunosorbent assay and end point dilution as previously described (2, 20). The end point dilution represents the final plasma concentration that yielded an absorbance higher than that yielded by a negative-control plasma sample included in the assay.

Proliferation assays. Spleens and popliteal lymph nodes from *L. mexicana*-infected mice were removed aseptically, and splenocytes and lymph node suspensions (5×10^5/well) were incubated in 96-well plates at 37°C in complete tissue culture medium (RPMI 1640 [Life Technologies, Paisley, United Kingdom] with 10% [vol/vol] heat-inactivated fetal bovine serum) with or without soluble *Leishmania* antigen for 3 days. Soluble *Leishmania* antigen was prepared as described previously (24). Supernatants were assayed for IL-4 as previously described (2). All assays were conducted in triplicate.

RESULTS

Expression of *CPB* genes in the Δ*cpb* mutant. We have shown previously that the *L. mexicana CPB* locus contains 19 genes in a tandem array (Fig. 1a). To isolate cosmids containing the *CPB* array, a *CPB* gene-specific probe (13) was used to screen an *L. mexicana* cosmid genomic library and several independent clones were isolated. Analysis using specific probes revealed that cosmid pGL263 hybridized to the 5′ and 3′ flank probes, as well as the *CPB* gene itself, supporting evidence that it contained the *CPB* array. Restriction mapping, coupled to pulsed-field gel electrophoresis and sequence analysis, revealed that the cosmid contained no complete open reading frames apart from the *CPB* array.

The Δ*cpb* mutant was generated with *hyg* and *ble* drug resistance genes as selectable markers (13). As the cosmid pGL263 contains the *hyg* resistance gene (18), the Δ*cpb* mutant was reengineered to contain the *pac* gene, which confers puromycin resistance, in place of the *hyg* gene. Confirmation that *pac* had replaced *hyg* in the Δ*cpb*^{pac} cell line was obtained by assessing the sensitivity of clones to hygromycin and by PCR analysis of genomic DNA with combinations of oligonucleotide primers designed to anneal to *pac* and *CPB* flanking regions outside of the integration cassette (data not shown).

The Δ*cpb*^{pac} mutant was transfected with pGL263, and a clone resistant to hygromycin, designated the Δ*cpb*^{pac}[pGL263] mutant, was characterized. Extracts of promastigote and amastigote stages were assessed for cysteine protease activity by gelatin SDS-PAGE (Fig. 2). The Δ*cpb*^{pac}[pGL263] mutant was

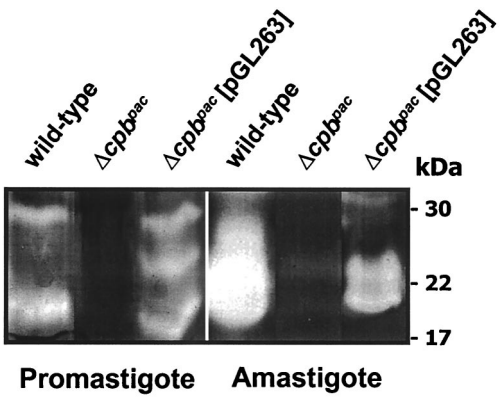

FIG. 2. Gelatin SDS-PAGE analysis of the Δ*cpb*^{pac}[pGL263] mutant. Lysates of 10^7 wild-type, Δ*cpb*^{pac}, and Δ*cpb*^{pac}[pGL263] promastigotes and 4×10^6 axenic amastigotes were analyzed by gelatin SDS-PAGE. Molecular mass standards are indicated.

found to exhibit cysteine protease activity in both life cycle stages, with higher levels detected in the amastigote. In contrast, the Δ*cpb* mutant had no cysteine protease activity that was detectable by this method.

In addition to the Δ*cpb*^{pac}[pGL263] cell line, which expresses multiple copies of *CPB*, two cell lines containing single copies of *CPB* reintroduced into the *CPB* locus of the Δ*cpb* mutant were generated (by using the constructs detailed in Fig. 1b and c) and analyzed. The Δ*cpb*::*CPB*2.8 mutant expresses the amastigote-specific *CPB2.8* gene, while the Δ*cpb*::*CPB*2 mutant expresses the metacyclic stage-specific *CPB2* gene (6). The stage-specific expression of *CPB2* and *CPB2.8* has been described previously (6, 14). In vitro, none of these cell lines have an impaired growth rate compared with wild-type parasites. Analysis of the cysteine protease activity in stationary-phase promastigotes of these cell lines with the peptidyl substrate ZFR-AMC showed that the Δ*cpb*^{pac}[pGL263] parasites had 32% of the cysteine protease activity found in wild-type promastigotes, whereas the corresponding activities in the Δ*cpb*::*CPB*2.8 and Δ*cpb* mutants were only 13 and 10%, respectively. These protease activity data are consistent with a relatively high level of *CPB* gene expression from the *CPB* cosmid, albeit less than that which occurs in wild-type parasites, but only a low level from the reintegrated *CPB* gene.

Infectivity of promastigotes of the mutant parasites. As previously observed (2), the Δ*cpb* mutants produced small and slow-growing lesions in mice compared with the rapidly growing lesions produced by wild-type *L. mexicana* (Fig. 3). The Δ*cpb*::*CPB2.8* mutant induced early lesion growth (2 weeks), similar to wild-type parasites. Lesions were significantly larger at 2 weeks than those produced by Δ*cpb* mutants ($P < 0.01$), but this was not sustained, and lesion size thereafter was similar to that for Δ*cpb* mutant-infected animals (Fig. 3a) and significantly less than that of wild-type *L. mexicana* lesions. In contrast, the increase in footpad thickness induced by the Δ*cpb*::*CPB2* mutant line was at no stage greater than that produced by Δ*cpb* mutants (data not shown). However, Δ*cpb*^{pac}[pGL263] promastigotes induced lesions which were significantly larger than those produced by Δ*cpb*^{pac} parasites at 4, 6, and 8 weeks ($P < 0.05$) postinfection and approximately 50% of the size of wild-type parasite-induced lesions (Fig. 3b). The total parasite counts at 8 weeks reflected differences in lesion size between mutants and wild-type parasites (wild-type, $[8.0 \pm 1.2] \times 10^8$; Δ*cpb*^{pac}[pGL263] mutant, $[1.1 \pm 0.6] \times 10^8$; Δ*cpb*^{pac} mutant, $[5.0 \pm 1.3] \times 10^6$). These results show that the presence of multiple *CPB* genes facilitates the establishment and sustained development of promastigote-initiated infection by *L. mexicana*.

As no selection pressure for the cosmid was maintained during in vivo infection, we verified that the pGL263 cosmid could be isolated from Δ*cpb*^{pac}[pGL263] amastigotes isolated

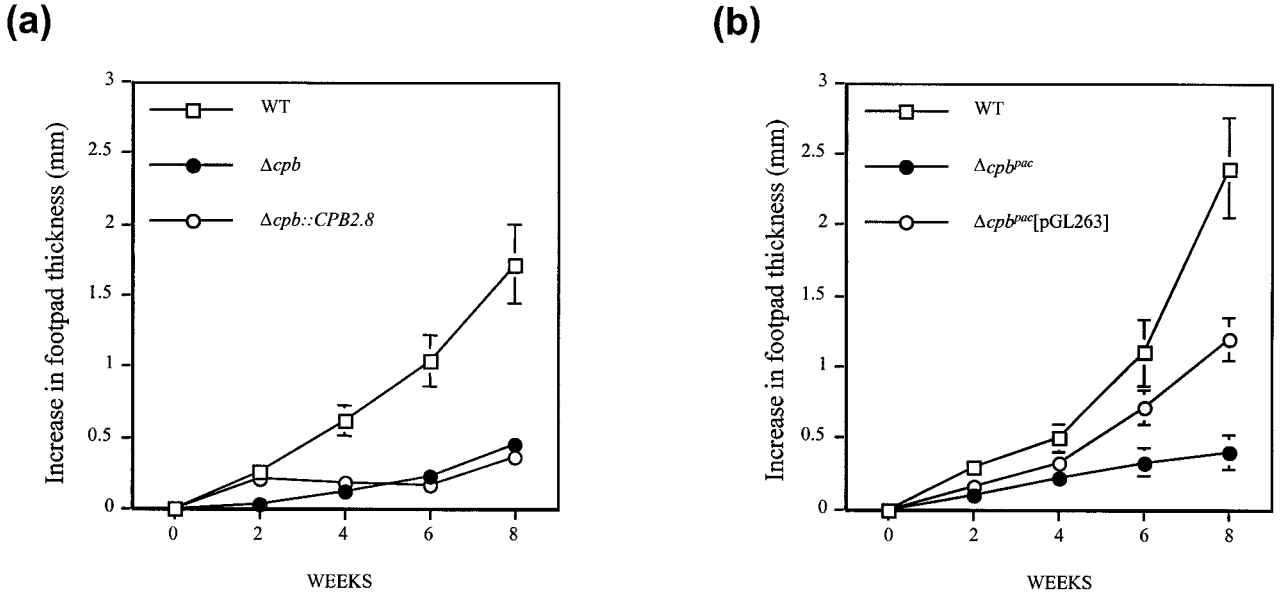

FIG. 3. Lesion development in BALB/c mice infected with *L. mexicana* mutants. (a) BALB/c mice were inoculated with 5×10^5 wild-type, Δ*cpb*, and Δ*cpb*::*CPB2.8* parasites. Footpad thickness was monitored for 8 weeks. Values represent means ± standard errors of the means ($n = 5$). (b) Same as panel a except that mice were infected with Δ*cpb*^{pac} and Δ*cpb*^{pac}[pGL263] mutants.

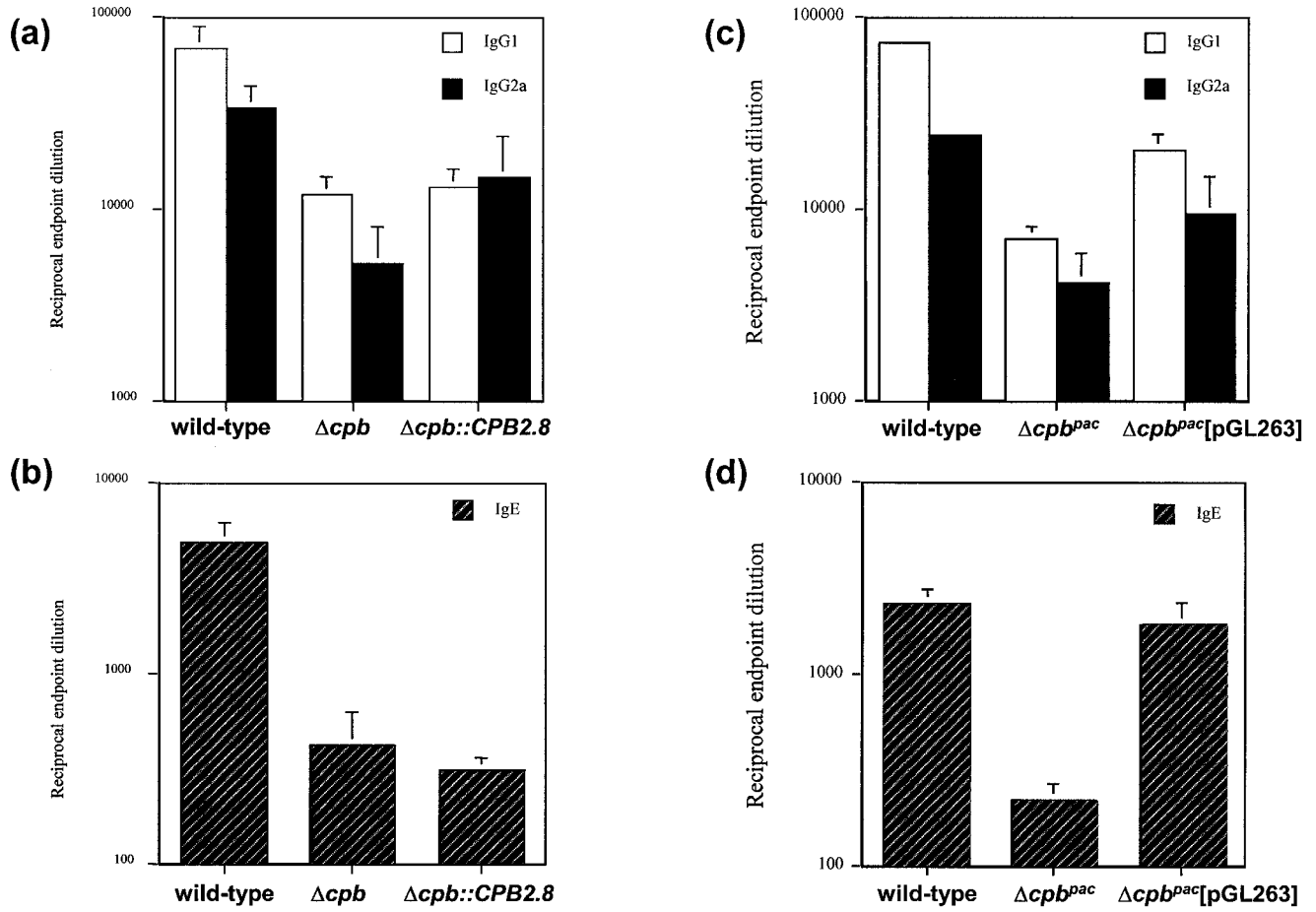

FIG. 4. Analysis of parasite-specific IgG1 and IgG2a levels (a and c) and total IgE levels (b and d) in BALB/c mice infected with *L. mexicana* mutants. The antibody titers were determined in the plasma of mice infected with wild-type, Δ*cpb*, Δ*cpb*::*CPB2.8*, Δ*cpb*^{pac}, and Δ*cpb*^{pac}[pGL263] strains at 8 weeks postinfection. Values represent the mean end point dilutions ± standard errors of the means ($n = 5$).

from a mouse lesion at 8 weeks. Isolated cosmid pGL263 DNA was analyzed by agarose gel electrophoresis and by PCR and was confirmed to contain *CPB* genes (data not shown). We also verified that the promastigote parasites, obtained after transformation in vitro of the amastigotes, were resistant to hygromycin and thus still contained the pGL263 cosmid.

Expression of multicopy *CPB* restores the Th2 response, as determined by measuring IL-4 production and IgG1 and IgE levels. We have previously shown that when BALB/c mice are infected with the Δ*cpb* mutant, they are able to mount a protective Th1 response, rather than the nonprotective Th2 response normally observed in wild-type infections (2). The latter response is characterized by elevated IL-4 production and comparatively high titers of IgG1 and IgE. To determine the type of immune response induced by the different mutant parasite lines, we measured the Ig titers in the plasma of infected mice.

As previously demonstrated, mice infected with wild-type parasites exhibited a high level of IgG1 at 8 weeks postinfection, characteristic of a Th2 response, while Δ*cpb* mutant infections led to a relatively low titer, indicative of a Th1 response (Fig. 4a). Infection with the Δ*cpb*::*CPB2.8* mutant was similar to infection with the Δ*cpb* mutant in terms of IgG1 and IgG2a antibody production. While BALB/c mice infected with wild-type parasites had significant IgE production, again indicative of IL-4 production and a Th2 response, IgE levels were significantly lower ($P < 0.001$) in mice infected with Δ*cpb* and Δ*cpb*::*CPB2.8* parasites (Fig. 4b). Conversely, following infection with the Δ*cpb*^{pac}[pGL263] mutant, BALB/c mice produced significantly more IgG1 ($P < 0.01$) than mice infected with Δ*cpb*^{pac} parasites but significantly less ($P < 0.001$) than mice infected with the wild type (Fig. 4c). Importantly, infection with the Δ*cpb*^{pac}[pGL263] mutant increased IgE levels so that they were equivalent to that produced by wild-type infection and significantly greater ($P < 0.001$) than that produced by infection with the Δ*cpb*^{pac} mutant (Fig. 4d).

The production of IgE is IL-4 dependent, so popliteal lymph node cells were harvested from mice and the production of IL-4 following stimulation with *Leishmania* antigen was determined. Popliteal lymph node cells from mice infected with Δ*cpb* and Δ*cpb*::*CPB2.8* parasites produced significantly less ($P < 0.01$) IL-4 than popliteal lymph node cells from wild-type parasite-infected mice. Reinsertion of CPB2.8 into Δ*cpb* parasites did not result in increased IL-4 production (Fig. 5a). Conversely popliteal lymph nodes from Δ*cpb*^{pac}[pGL263] mutant-infected mice produced significant quantities of IL-4 fol-

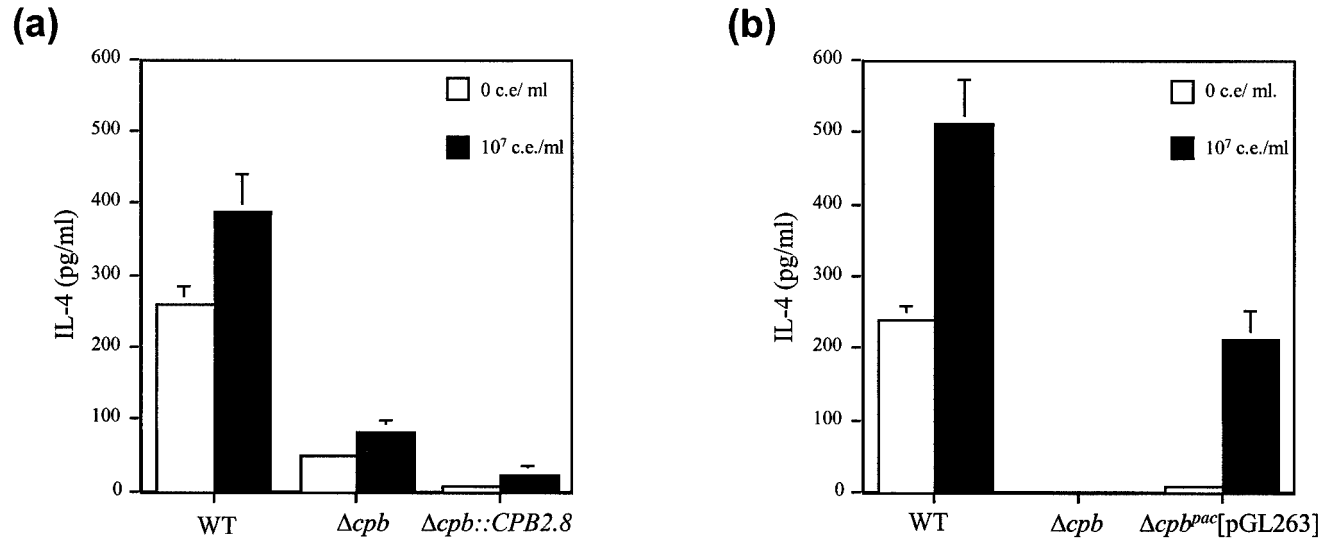

FIG. 5. Lymphocyte IL-4 responses of mice infected with *L. mexicana* mutants. IL-4 production was measured after stimulation of isolated splenocytes with 10^7 cell equivalents (c.e.) of soluble *Leishmania* antigen/ml. Error bars, standard errors of the means ($n = 5$). (a) Δ*cpb*::*CPB2.8* mutant. (b) Δ*cpb*^{pac}[pGL263] mutant.

lowing stimulation with *Leishmania* antigen (Fig. 5b). This was significantly greater than the quantity produced by mice infected with Δ*cpb* parasites ($P < 0.002$) but less than that produced by mice infected with wild-type organisms ($P < 0.005$). Similar results were obtained with splenocyte cultures (results not shown).

DISCUSSION

The aim of this study was to investigate the significance of the multiplicity of the CPB isoenzymes of *L. mexicana* in the parasite's interaction with its mammalian host. To do this we compared lesion development and the immune response in mice infected with clonal populations of Δ*cpb* null mutant parasites reexpressing different *CPB* genes. We first developed parasite lines where single *CPB* genes *CPB2* and *CPB2.8* were reintegrated in the *CPB* locus of the Δ*cpb* mutant (6). *CPB2* and *CPB2.8* were expressed in the two lines in the metacyclic and amastigote stages, respectively, as occurs in wild-type *L. mexicana*. However, wild-type *L. mexicana* expresses multiple CPB isoenzymes in the amastigote stage, where it is probable that the enzymes interact and complement each other's activities (5, 14), and so we also reexpressed multiple *CPB* genes in the Δ*cpb* mutant so that we could investigate whether the multiplicity of the CPB isoenzymes was important for the parasite's virulence. As reintegration of the complete array into the genome was not possible, we chose to reexpress the *CPB* genes from an episomal cosmid vector. The only intact genes on this cosmid were the *CPB* genes, so the effects of gene expression from the cosmid could be attributed directly to CPB and not other proteins. Gelatin SDS-PAGE and fluorogenic peptidyl substrate assays confirmed that these multiple *CPB* genes carried on the cosmid were expressed in the Δ*cpb*^{pac}[pGL263] mutant, although the expression levels were lower than those for wild-type parasites (Fig. 2).

The finding that Δ*cpb* mutants reexpressing CPB2, an isoenzyme expressed primarily in the metacyclic form, failed to promote lesion development was not unexpected. While it is conceivable that expression of metacyclic stage-specific CPB2 may support initial infection, the low levels of CPB activity expressed following transformation into the amastigote stage (6) would afford these Δ*cpb*::*CPB2* mutants no advantage over Δ*cpb* parasites. It is interesting, however, that the parasites with amastigote-specific *CPB2.8* induced increased initial lesion growth compared with Δ*cpb* parasites, even though this was not sustained. Presumably, for prolonged virulence in mice the parasites require higher levels of expression of CPB than was mediated by the single gene or, alternatively, expression of other CPB isoenzymes with different substrate specificities (6, 14). Both of these possibilities are consistent with the results obtained with the mutant expressing multiple *CPB* genes, the Δ*cpb*^{pac}[pGL263] mutant, which produced lesions that were significantly larger throughout the course of the experiment than those induced by the Δ*cpb* mutant. These data suggest that the expression of multiple CPB isoenzymes greatly facilitates virulence. However, they do not indicate whether the effect is mediated simply by enhanced CPB activity or requires several isoenzymes with subtly different substrate specificities (14). Previous studies where single *CPB* genes were reexpressed from episomes which were present in multiple copies in the parasite but did not restore virulence (8) could indicate that higher expression alone is insufficient to induce higher infectivity in vivo. Nevertheless this question requires further investigation.

The mean lesion size produced by the Δ*cpb*^{pac}[pGL263] mutant was only one-half that produced by using equivalent numbers of wild-type parasites. This could have been due to differing expression of isoenzymes from the cosmid compared with that for the natural CPB array or different overall CPB activity. The regulation of gene expression from the cosmid is likely to differ from the regulation of chromosomally located-gene expression, and indeed the gelatin SDS-PAGE data (Fig. 2) suggest that the two parasite lines are not equivalent and express different numbers or different arrays of isoenzymes.

Moreover, some parasites in the population in the lesions may have lost the cosmid, which clearly would make them less virulent. Further studies, involving reexpression of all different combinations of *CPB* genes, will be necessary to understand whether all of the isoenzymes are required for full effect and whether any in particular are playing the major role in the polarization of the immune response.

Infection with *L. mexicana* in the BALB/c mouse is associated with a polarized Th2 response, and endogenous IL-4 has been demonstrated to be necessary for early lesion development (3, 22). Moreover, susceptibility of BALB/c mice to *Leishmania major* has been shown to be the result of IL-4 production by Vβ4 Vα8CD4$^+$ T cells in response to a single T-cell epitope derived from the parasite LACK antigen (the *Leishmania* homologue of receptors for activated C kinase) (11). However, recent studies have demonstrated that LACK-tolerant BALB/c mice remain susceptible to *L. mexicana* despite resistance to *L. major* (26). As *L. mexicana* expresses LACK and as LACK is recognized by BALB/c CD4$^+$ T cells that produce IL-4, these observations clearly demonstrate that there are alternative or additional mechanisms for inducing the susceptibility of mice to *L. mexicana.* Our data suggest that the CPB enzymes may be crucially involved. Significantly, *L. mexicana* Δ*cpb* mutants have reduced infectivity for BALB/c mice and induce a polarized type 1 response (2). Of relevance to this is the report that during wild-type infections CPB occurs in large quantities in the extracellular milieu (9). Subsequently we have demonstrated that recombinant, enzymatically active CPB2.8 is a potent stimulator of IL-4 and IgE production in BALB/c mice (15), like the DerP1 antigen, which is also a cysteine protease (23). Our present findings, that reexpression of multiple *CPB* genes, but not single genes, in Δ*cpb* mutants restored a significantly increased IgE production as well as significantly elevated lymph node and splenocyte IL-4 production, are consistent with this hypothesis of a key role for CPB in the host-*L. mexicana* interactions and particularly in the modulation of the immune response.

The findings of this study confirm that the level of CPB expression or the diversity in CPB isoenzymes or both are important for parasite virulence. Moreover, this study has demonstrated a role for the multiple CPB isoenzymes in the modulation of the immune response to *L. mexicana* infection, although the mechanisms underlying this process await further characterization.

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