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Spath, Gerald F.; Lye, Lon-Fye; Segawa, Hiroaki; Turco, Salvatore J.; and Beverley, Stephen M., "Identification of a compensatory mutant (lpg2-REV) of *Leishmania major* able to survive as amastigotes within macrophages without LPG2-dependent glycoconjugates and its significance to virulence and immunization strategies." *Infection and Immunology*. 72, 6. 3622-3627. (2004).
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Infect. Immun. 2004, 72(6):3622. DOI:
10.1128/IAI.72.6.3622-3627.2004.

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Identification of a Compensatory Mutant (*lpg2*⁻*REV*) of *Leishmania major* Able To Survive as Amastigotes within Macrophages without *LPG2*-Dependent Glycoconjugates and Its Significance to Virulence and Immunization Strategies

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Received 24 December 2003/Returned for modification 7 February 2004/Accepted 2 March 2004

Different *Leishmania* species rely to different extents on abundant glycoconjugates, such as lipophosphoglycan (LPG) and related molecules, in mammalian infections. Previously, we showed that *Leishmania major* deletion mutants lacking the Golgi GDP-mannose transporter *LPG2*, which is required for assembly of the dominant phosphoglycan (PG) repeats of LPG, were unable to survive in macrophages. These *lpg2*⁻ mutants, however, retained the ability to generate asymptomatic, persistent infections in mice. In contrast, Ilg and colleagues showed that *Leishmania mexicana* *LPG2* mutants retained virulence for mice. Here we identified a partial revertant population of the *L. major* *lpg2*⁻ mutants (designated *lpg2*⁻*REV*) that had regained the ability to replicate in macrophages and induce disease pathology through a compensatory change. Like the *lpg2* parent, the *lpg2*⁻*REV* revertant was unable to synthesize *LPG2*-dependent PGs in the promastigote stage and thus remained highly attenuated in the ability to induce infection. However, after considerable delay *lpg2*⁻*REV* revertant-infected mice exhibited lesions, and amastigotes isolated from these lesions were able to replicate within macrophages despite the fact that they were unable to synthesize PGs. Thus, in some respects, the *lpg2*⁻*REV* amastigotes resemble *L. mexicana* amastigotes. Future studies of the gene(s) responsible may shed light on the mechanisms employed by *L. major* to survive in the absence of *LPG2*-dependent glycoconjugates and may also improve the potential of the *lpg2*⁻ *L. major* line to serve as a live parasite vaccine by overcoming its tendency to revert toward virulence.

Infections by the protozoan parasite *Leishmania* can cause severe diseases in humans and animals, whose manifestations range from mild cutaneous to fatal visceral pathology. Leishmaniae are transmitted in the form of highly virulent metacyclic parasites, which develop inside the midgut of phlebotomine sand flies and are inoculated into the mammalian host by biting. Inside the acidified phagolysosome of host macrophages, leishmaniae differentiate into the aflagellate amastigote stage, which resists leishmanicidal activities and inhibits macrophage activation, processes that ultimately contribute to amastigote survival and replication and disease pathology (8). In most immunocompetent hosts, parasite growth and pathology are eventually controlled by adaptive immune mechanisms (2, 20), and the infection enters a chronic phase of parasite persistence in the absence of significant pathology. There are a number of host factors which are known to modulate parasite persistence and establish a delicate balance between gamma interferon-induced leishmanicidal NO and immunosuppressive interleukin-10 produced by CD25⁺ regulatory T cells (1, 2, 9, 25). In contrast, our knowledge concerning the identities and roles of parasite factors which function differentially in medi-

ating persistence and in the acute pathogenic phase of infection is more limited.

Glycosylphosphatidyl inositol-anchored surface glycoconjugates, including lipophosphoglycan (LPG), glycoinositol phospholipids (GIPLs), and proteins, such as proteophosphoglycan (PPG) or gp63, form a dense glycocalyx in *Leishmania*. Many of these compounds are important determinants of parasite virulence, as shown by studies of purified molecules, as well as mutants defective in the synthesis of one or more of the glycoconjugates (11, 12, 14, 22–24, 28). In *Leishmania major*, LPG plays an important role in the establishment of infections in macrophages following metacyclic invasion, while both GIPLs and other alkylacyl phosphoglycerolipid moieties appear to have small roles in macrophage survival despite their abundance (12, 33). In contrast, parasite molecules that are dependent on the activity of the *LPG2*-encoded Golgi GDP-mannose transporter, such as LPG and other phosphoglycans (PGs) bearing the signature Gal-Man-P-based repeating unit, are required for both initial establishment and subsequent survival and replication of amastigotes within macrophages (24). It should be emphasized that the findings described above pertain to *L. major* and probably *Leishmania donovani* as well (M. Wilson and S. M. Beverley, unpublished data). Remarkably, studies of *Leishmania mexicana* have suggested that this species requires neither LPG, PGs, nor GIPLs for establishment or replication within macrophages (7, 10). This difference in reliance on highly abundant, structurally conserved mole-

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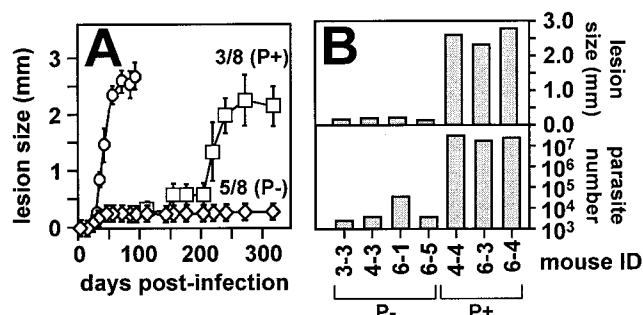


FIG. 1. Emergence of the *lpg2*⁻REV compensatory partial revertant during mouse infection. (A) Lesion formation. A total of 10⁶ WT or *lpg2*⁻ metacyclic parasites were inoculated into groups of susceptible BALB/c mice, and the infections were monitored by comparing the thickness of the injected footpad to the thickness of the uninjected footpad with a vernier caliper. Inoculation of WT parasites resulted in infections that appeared within 25 days (○), and, as shown previously, five of eight mice infected with *lpg2*⁻ parasites showed no lesion formation (P⁻) (◇). In contrast, three of eight mice infected with *lpg2*⁻ parasites in this experiment showed lesion formation beginning around day 125 (P⁺) (□); the parasites in these mice were designated *lpg2*⁻REV parasites. The bars indicate the standard deviations for WT parasites (*n* = 4), P⁻ *lpg2*⁻ parasites (*n* = 5), and P⁺ *lpg2*⁻ parasites (*n* = 3). (B) Limiting dilution assay. Representative P⁻ or P⁺ mice from the experiment shown in panel A were sacrificed on day 346 postinfection, and the numbers of parasites in the infected footpads were determined by limiting dilution. The lesion sizes (upper panel) and the parasite burdens (lower panel) of individual animals are indicated.

cules among *Leishmania* species was unanticipated and is not well understood (4, 29).

Significantly, the *L. major lpg2*⁻ parasite maintained the ability to persist at the infection site in the absence of overt pathology for more than 2 years (24). This ruled out the possibility that LPG2-dependent factors (such as PGs) are required in the persistent, asymptomatic phase of the disease. To explain the fact that the *lpg2*⁻ parasite is unable to survive in macrophages yet is able to survive indefinitely in animals, we suggested a model in which persistent parasites resided in another, presumably more hospitable compartment within the host, a compartment from which the parasite would be unable to induce disease through perturbation of macrophage function. A corollary of this safe haven model is that parasites that had recovered the ability to survive within macrophages would similarly recover the ability to induce disease pathology. Here we present evidence which supports this prediction and discuss its relevance to *Leishmania* virulence and the use of live parasite-based immunization strategies.

MATERIALS AND METHODS

Parasite strains and culture. All parasites were derivatives of *L. major* strain LV39 clone 5 (Rho/SU/59/P) and were cultivated in M199 medium (13). The *lpg2* line obtained by homologous gene replacement (Δ LPG2::HYG/ Δ LPG2::HYG) has been described previously (24). P⁺ and P⁻ derivatives of this line (Fig. 1A) were transfected with the construct pSNBR-LPG2 (B4831) and plated on semi-solid M199 medium containing 20 μ g of G418 per ml.

Monoclonal antibodies, lectins, immunofluorescence, and flow cytometry. Monoclonal antibody WIC79.3 recognizes *L. major* Gal-substituted Gal-Man-P repeating units (6), and anti-trypansomal tubulin antibody was provided by D. Russell (Cornell University, Ithaca, N.Y.). Flow cytometry with fluorescein-conjugated ricin agglutinin (Sigma, St. Louis, Mo.) was performed with live *Leishmania*. Parasites in the logarithmic growth phase (10⁵ parasites/ml) were

washed twice in cold phosphate-buffered saline and incubated with 5 μ g fluorescein-conjugated ricin agglutinin per ml in phosphate-buffered saline for 10 min at 4°C. The analysis was performed with a Becton Dickinson FACS-Calibur system (excitation wavelength, 488 nm; detection wavelength, 520 nm). Cells were fixed with 4% paraformaldehyde for 5 min at room temperature, permeabilized by incubation with ice-cold 100% ethanol for 15 min at 4°C, and sequentially incubated with monoclonal anti-PG antibody WIC79.3 (27) and fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G. Parasites were counterstained with polyclonal anti-tubulin and Texas Red-conjugated anti-rabbit immunoglobulin G.

Mouse infection and limiting dilution assay. Virulence was assessed by using 6- to 8-week-old female BALB/c mice (Charles River Laboratories Inc., Wilmington, Mass.) following subcutaneous inoculation of 10⁶ metacyclic parasites into the hind footpads (26). The metacyclic parasites were isolated by density centrifugation as described previously (21). Infections were monitored by comparing the thickness of the injected footpad to the thickness of the uninjected footpad with a vernier caliper. Parasites in the infected tissue were enumerated by a limiting dilution assay (26).

Macrophage infection. Starch-elicited peritoneal exudate macrophages (PEM) from BALB/c mice were infected as described previously (22). Metacyclic parasites were opsonized by 30 min of incubation in Dulbecco modified Eagle medium containing 4% C5-deficient mouse serum (16) and were allowed to invade macrophages in Dulbecco modified Eagle medium containing 0.7% bovine serum albumin for 2 h at 33°C at multiplicities of infection of 10 and 3 parasites per PEM for wild-type (WT) and *lpg2* parasites, respectively. Amastigotes were isolated from progressive lesions by tissue homogenization and differential centrifugation, and PEM were infected for 2 h at 33°C at a ratio of three parasites per PEM. Intracellular growth was determined by nuclear staining and fluorescence microscopy as described previously (22).

RESULTS

Emergence of an *lpg2*⁻ parasite population showing late pathology. In previous studies performed in three different laboratories, it was found that in most infections of susceptible BALB/c mice with the *lpg2*⁻ null mutant, parasites were able to persist indefinitely without pathology (24, 30). In these experiments, footpad inoculation was used to infect groups of female BALB/c mice with 10⁶ WT or *lpg2*⁻ metacyclic parasites that were isolated by Ficoll density centrifugation (21). WT metacyclic parasites elicited lesions that appeared within 25 days (Fig. 1A), while in most experiments *lpg2*⁻ metacyclic parasites failed to induce pathology, in agreement with previous findings (24). However, in two experiments involving inoculation with higher numbers of parasites, we observed that a few mice developed lesions, which developed late (after 150 to 200 days) but progressed rapidly thereafter. For example, in the experiment shown in Fig. 1A, three of eight of the *lpg2*⁻ parasite-infected mice showed this same late-lesion profile (P⁺ mice), while the remaining mice showed no pathology, as observed previously (24). The numbers of parasites were determined by a limiting dilution assay (26) on day 346 postinfection, and the results showed that, as expected, infected mice that did not exhibit overt pathology (mice 3-3, 4-3, 6-1, and 6-5) contained a low level of persistent parasites (\sim 10³ parasites) (Fig. 1B). In contrast, in mice with the late-lesion pathology (mice 4-4, 6-3, and 6-4), the numbers of parasites were nearly 10,000-fold higher (Fig. 1B).

The timing of lesion appearance differed from the timing observed previously for LPG-deficient *lpg1*⁻ *L. major*, which requires LPG for establishment of a macrophage infection but not for replication as amastigotes (22). Prompted by the supposition that this finding might reflect a new genetic difference between the late-lesion *lpg2*⁻ parasites and the canonical persistent *lpg2*⁻ parasites, we recovered parasites separately from

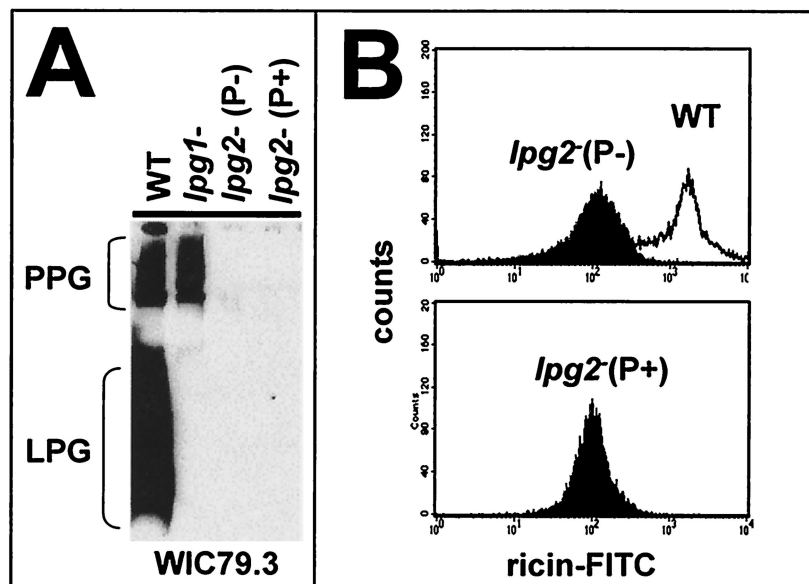


FIG. 2. Characterization of P+ and P- *lpg2*⁻ promastigotes. (A) Western blot analysis. Promastigotes from WT, *lpg1*⁻, and *lpg2*⁻ P+ and P- populations were subjected to Western blot analysis with the anti-PG antibody WIC79.3 (6). The positions of WT PPG and LPG are shown. (B) PG flow cytometry. A total of 10⁵ promastigotes from a logarithmic culture were incubated with ricin-fluorescein isothiocyanate (FITC) at 4°C prior to analysis. (Upper panel) WT and *lpg2*⁻ P- parasites; (lower panel) *lpg2*⁻ P+ parasites. The parental *lpg2*⁻ population was indistinguishable from the P- and P+ populations (data not shown).

mice which never exhibited lesion formation (designated P-) and mice which had lesions (designated P+) (Fig. 1A).

Late-pathology *lpg2*⁻ P+ parasites do not make PGs in the promastigote form. Following recovery, the two populations of parasites were allowed to differentiate into promastigotes in vitro, and PG expression was analyzed by examining reactivity with anti-PG antibody WIC79.3 in a Western blot analysis (Fig. 2A) and by flow cytometry with the β-Gal-binding lectin ricin agglutinin (Fig. 2B). WT promastigotes expressed an abundance of LPG and PPG, as shown by Western blotting, while the *lpg1*⁻ mutant (defective in LPG core synthesis) showed the expected synthesis of PPG but did not express LPG (Fig. 2A). Importantly, the *lpg2*⁻ P- and P+ populations maintained the total PG-deficient phenotype expected for the *lpg2*⁻ promastigotes, showing that they were neither WT contaminants nor second-site revertants that exhibited restored promastigote PG synthesis. It should be emphasized that the *LPG2* coding region had been removed completely from the *lpg2*⁻ line by homologous gene replacement (24), and thus reconstitution of *LPG2* itself was impossible. Interestingly, inspection of the available *L. major* genome sequence data revealed the presence of several members of the nucleotide sugar transporter family whose functions are unknown, in addition to the anticipated UDP-Gal transporters (A. Capul and S. M. Beverley, unpublished data). However, activation of an alternative GDP-Man transport activity in P+ *lpg2*⁻ promastigotes was ruled out by these data.

P- and P+ *lpg2*⁻ parasites breed true. When 10⁶ metacyclic parasites were inoculated into mice, the P- *lpg2*⁻ parasites yielded persistent infections similar to those seen previously; as in previous studies, no infected mouse went on to develop lesions or pathology over a 1-year period (Fig. 3A and data not shown). Notably, restoration of *LPG2* expression restored PG

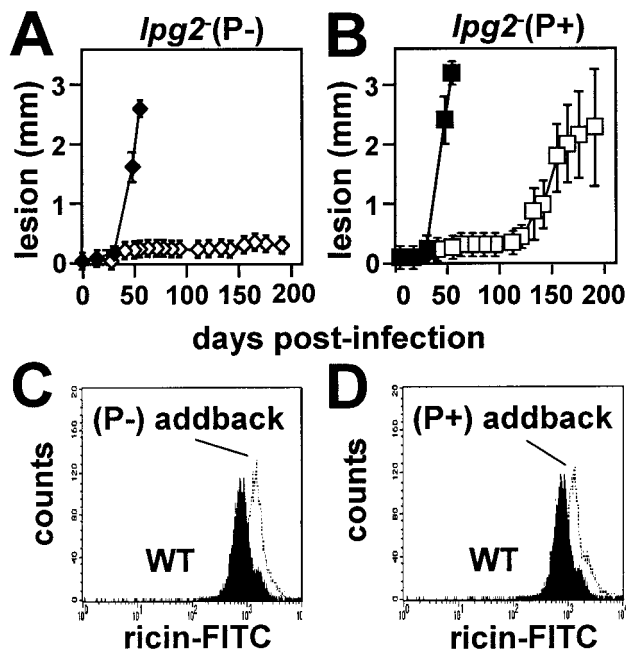


FIG. 3. P+ *lpg2*⁻ population breeds true. (A and B) Mouse infections. A total of 10⁶ purified WT (◆ and ■), *lpg2*⁻ (◇), or *lpg2*⁻ P+ (□) metacyclic parasites were inoculated into the footpads of BALB/c mice, and lesion formation was monitored. Each point indicates the average for three mice inoculated with WT parasites or five mice inoculated with *lpg2*⁻ or *lpg2*⁻ P+ parasites; the error bars indicate standard deviations. (C and D) Restoration of *LPG2* expression fully restores surface *LPG* expression in P+ and P- *lpg2*⁻ lines. *LPG* expression in promastigotes of WT parasites (solid profile) and the *lpg2*⁻ (P-)/+ *LPG2* (panel C, open profile) or *lpg2*⁻ (P+)/+ *LPG2* addback lines was assessed by flow cytometry with fluorescent ricin as described in Materials and Methods. As shown previously, the dominant PG detected by this assay is *LPG* (22). FITC, fluorescein isothiocyanate.

synthesis to WT levels (Fig. 3C) and lesion formation to control levels (Fig. 3A). These results demonstrated that the P- *lpg2*⁻ parasites were not generally and irreversibly avirulent, a potential concern since loss of virulence occurs sporadically during long-term culture or genetic manipulation of *Leishmania* in vitro (5). Thus, the P- *lpg2*⁻ parasites appear to be identical to the original *lpg2*⁻ population described previously (24).

In contrast, the P+ *lpg2*⁻ parasites produced lesions in every infected mouse, again with a delay of ~125 days (Fig. 3B). This showed that the late-lesion or P+ phenotype was a stable trait, and we designated this population *lpg2*⁻*REV*. As in the *lpg2*⁻ parental line, reexpression of *LPG2* in the *lpg2*⁻*REV* population restored LPG and PG synthesis (Fig. 3D).

***lpg2*⁻*REV* parasites survive in macrophages in the amastigote form but not in the promastigote form.** Infection of mouse macrophages with *lpg2*⁻ (P-) or *lpg2*⁻*REV* (P+) metacyclic parasites showed that neither survived, as observed previously (24; data not shown). While *lpg2*⁻ (P-) amastigotes could not be obtained, we were able to recover amastigotes from mouse lesions obtained with *lpg2*⁻*REV* parasites (obtained around day 150). The *lpg2*⁻*REV* amastigotes were capable of surviving and replicating well in macrophages, although the growth rate was about twofold lower than that of WT amastigotes (Fig. 4B). The size of the *lpg2*⁻*REV* parasitophorous vacuole was similar to that seen in other *L. major* infections, and the vacuole was not extended like that seen in *L. mexicana* infections (Fig. 4A).

***lpg2*⁻*REV* parasites do not make PGs.** As described above for promastigotes, it was possible that the *lpg2*⁻*REV* amastigotes had recovered the ability to synthesize PGs through activation of an amastigote-specific pathway that restored Golgi GDP-Man uptake. In WT amastigotes, PGs predominantly localized to the parasite cytoplasm and accumulated in a vesicular compartment, most likely the flagellar pocket, as expected for secreted PGs (Fig. 4A, left panels). However, immunofluorescence analysis of the infected macrophages with anti-PG antisera showed that the *lpg2*⁻*REV* amastigotes failed to synthesize detectable levels of PGs, and only background fluorescence was evident (Fig. 4A, right panels). These data eliminated the possibility that there was activation of an alternative amastigote Golgi GDP-Man transport activity in this line.

DISCUSSION

In previous studies it was shown that LPG was important for the ability of *L. major* to establish infections in macrophages, while *LPG2*-dependent molecules, such as LPG and related PGs, were required for replication of parasites within macrophages in the amastigote form and subsequent pathology (24). The *lpg2*⁻ parasites used in these studies failed to induce any pathology in mice, although they were able to persist indefinitely in the absence of overt lesion formation. In this study we characterized a variant of the *lpg2* line designated the *lpg2*⁻*REV* line, which is able to reproducibly produce pathology in mice, albeit after a considerable delay (Fig. 1A). Notably, neither promastigote nor amastigote forms of the *lpg2*⁻*REV* parasite regained the ability to synthesize PGs, and thus this mutant did not arise through activation of an alternative Golgi GDP-Man transporter activity.

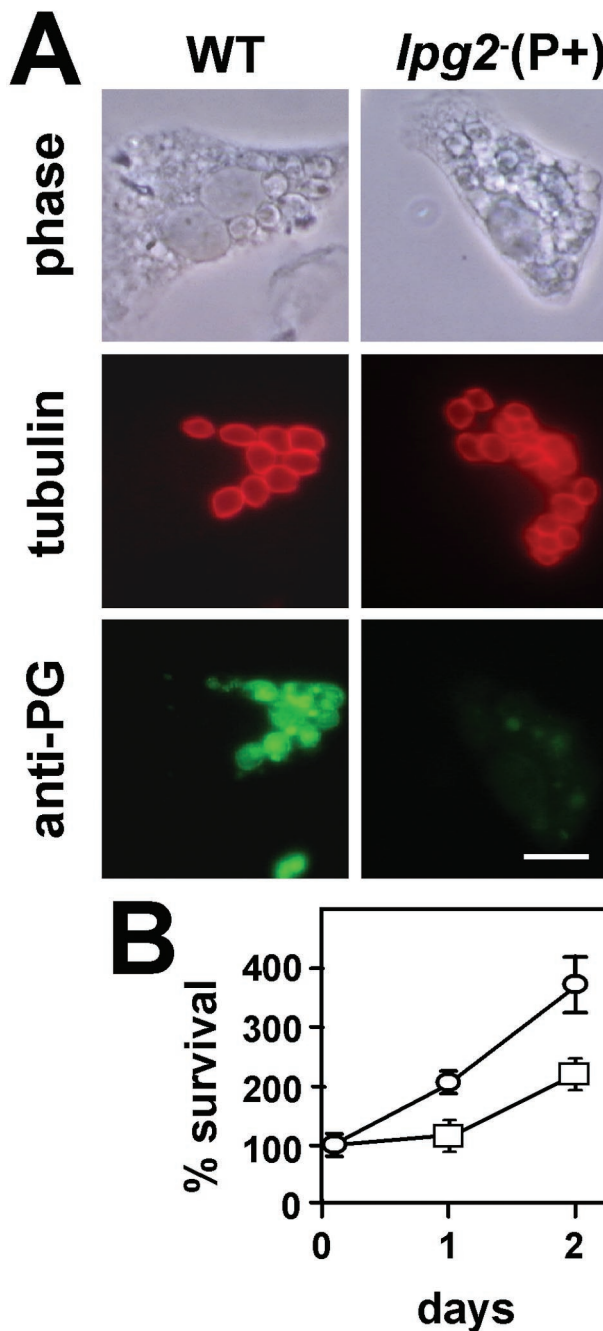


FIG. 4. *lpg2*⁻*REV* parasites replicate in macrophages but do not synthesize PGs. (A) Immunofluorescence analysis of amastigote PGs. PEM were infected with WT and *lpg2*⁻*REV* amastigote parasites and stained 2 days postinfection for reactivity with tubulin or PGs as described in Materials and Methods. Bar = 10 μm. (B) Macrophage replication. Murine macrophages were infected with WT (○) or *lpg2*⁻*REV* (□) amastigotes as described in Materials and Methods, and the numbers of parasites were determined and normalized to the initial infection obtained at 2 h (day zero). The values are averages and standard deviations for a representative experiment performed in triplicate.

In our experience the delayed-lesion phenotype manifested by the *lpg2*⁻*REV* parasite is characteristic of mutants with mutations that affect the ability of *Leishmania* to establish infections in macrophages (e.g., to enter, survive, and differentiate) but not the ability to replicate in the amastigote form. This may be due to defects in macrophage survival (as observed previously with LPG-deficient *LPG1* null mutants or to differentiation to form the infectious metacyclic stage (as observed in sphingolipid synthetic mutants [32; unpublished data]). Consistent with this notion, *lpg2*⁻*REV* parasites were poorly infective in macrophages in the promastigote form, reflecting the absence of PGs, such as LPG. However, in striking contrast to the amastigote form of the *lpg2*⁻ parent, *lpg2*⁻*REV* amastigotes showed good survival and replication in macrophages (Fig. 4B). Thus, these parasites in some manner acquired a compensatory mutation that enables amastigote replication in the absence of PGs or perhaps other molecules dependent on the activity of *LPG2*.

The following hypothetical scenario accounts for the emergence and properties of the *lpg2*⁻*REV* parasites (Fig. 5A) (24). As shown previously, in a normal infection with metacyclic promastigotes most *lpg2*⁻ parasites were phagocytosed by macrophages and destroyed, while a small proportion of the parasites escaped, possibly by residing in a cell type other than macrophages that lacked leishmanicidal potential (3, 22). In this presumptive safe haven the *lpg2*⁻ parasites were able to persist indefinitely (>700 days) at low levels (~1,000 parasites). Little is known about the parasites during this stage, including their differentiation state or whether they replicate. One possibility is that the persistent parasites slowly turn over and constantly leave their safe haven, where they are then challenged by host macrophages. This possibility is consistent with the role of negative host factors, such as NO, which mediate parasite killing by macrophages, thereby keeping persistent *Leishmania* infections in check (25). For the *lpg2*⁻ parasites, entry into macrophages would lead to destruction until the emergence of variant cells that had acquired the ability to survive and replicate in macrophages, such as *lpg2*⁻*REV* cells, which ultimately would lead to disease pathology. Since the *lpg2*⁻*REV* parasites did not regain the ability to synthesize LPG or PGs, they remained attenuated in the ability to establish macrophage infections initially in the promastigote form, as observed here. This model makes several testable predictions regarding the nature of the host cell phenotype, its leishmanicidal potential, and the interaction with the host immune system. However, validating these predictions requires identification of the persistent *lpg2*⁻ parasite population, a challenging task which is currently under way.

The emergence of the *lpg2*⁻*REV* phenotype is relevant to the development of live vaccines in which the *lpg2*⁻ parasite is used (30). To date, numerous protocols in which recombinant leishmanial antigens or heat-killed *Leishmania* have been used have failed to induce long-lasting immunity (15, 18, 19). In contrast, long-term protection is achieved following recovery from a natural infection, suggesting that parasite persistence is a prerequisite for sterile immunity (2, 31). *lpg2*⁻ parasites fulfill the major requirements for a live vaccine candidate (i.e., persistent infection in the absence of disease), and vaccination studies with BALB/c mice and *lpg2*⁻ parasites indeed induced

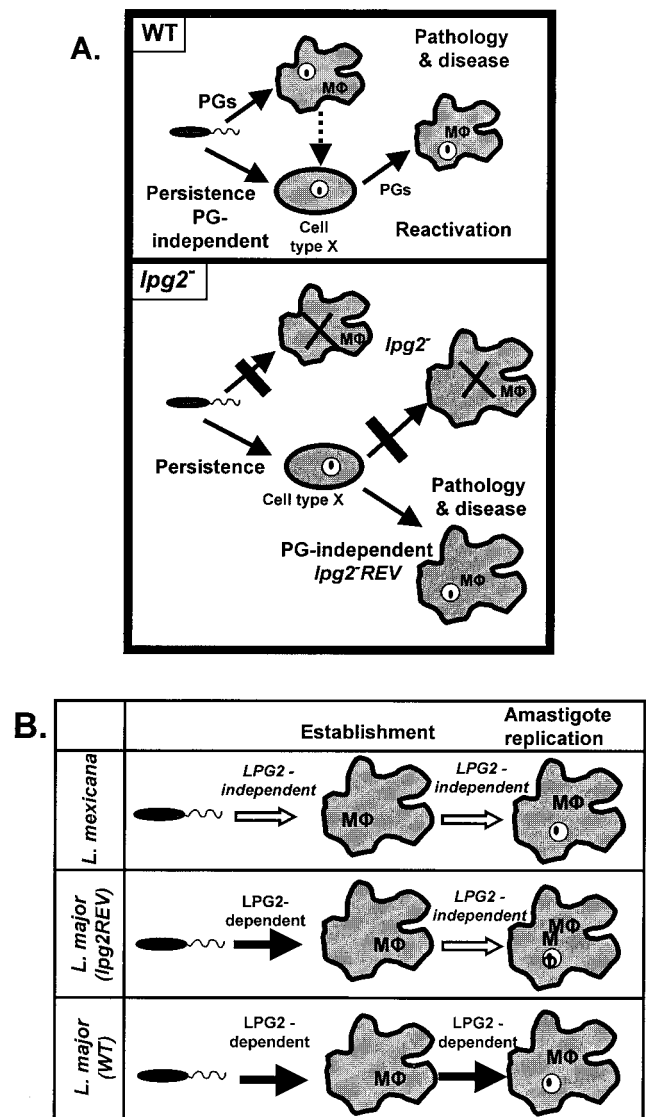


FIG. 5. Summary of comparisons of *lpg2*⁻*REV* *L. major*. In both panels, the known role of *LPG2* is in transport of GDP-mannose into the parasite Golgi apparatus, where it is utilized for the synthesis of PGs of various forms. (A) Proposed working model for pathology and persistence of WT parasites (upper panel) and *lpg2*⁻ or *lpg2*⁻*REV* *L. major* parasites (lower panel) in mouse infections. See Discussion for more information. This figure is modified from that presented by Spáth et al. (24). (B) *L. major lpg2*⁻*REV* parasites have a PG dependence phenotype intermediate between those of WT *L. mexicana* and *L. major*. See Discussion for more information. Mφ, macrophage.

dramatic protection against virulent challenge in the absence of a strong Th1 response (30). An interesting question for future study is the contribution of the host immune response to the delayed emergence of *lpg2*⁻*REV* parasite infections and whether this may provide some further perspective on the nature of the immune response to *lpg2*⁻ parasites. Regardless, it is very clear the *lpg2*⁻*REV* phenotype compromises the potential utility of the *lpg2*⁻ line as a vaccine candidate, and thus preventing its occurrence is important.

In contrast to *L. major*, *L. mexicana* does not require PGs for virulence or amastigote replication, implying that there are

PG-independent mechanisms of macrophage survival (7, 10). In a recent review the possibility that different *Leishmania* species placed different levels of emphasis during evolution on the use of surface glycoconjugates for intracellular survival was discussed (29). The differences among the species are summarized in Fig. 5B, which shows that *L. major* requires LPG2-dependent glycoconjugates, such as LPG and/or PGs, for establishment of macrophage infections by promastigotes, as well as for survival and induction of pathology by amastigotes, while *L. mexicana* does not. Interestingly, the *L. major* lpg2⁻REV line exhibits intermediate behavior; it is deficient in the ability to establish macrophage infections without PGs, like *L. major*, but it retains the ability to survive and replicate in the amastigote form without PGs, like *L. mexicana*. It is tempting to speculate that the gene(s) or pathway(s) altered in the *L. major* lpg2⁻REV line is related to the pathways that enable *L. mexicana* to replicate in macrophages in the absence of PGs. Some interesting glycoconjugates recently implicated in amastigote virulence in *L. mexicana* are the β 1-2 mannans, which are also found in *L. major* (17; unpublished data). However, the cytoplasmic location of mannans makes it unlikely that their synthesis is LPG2 dependent (17).

It may be possible to exploit the differences between the *L. major* lpg2⁻ and lpg2⁻REV lines identified in this study in genetic strategies to identify the relevant genes responsible. Thus, the lpg2⁻REV line provides a unique tool that may facilitate studies to gain important insight into mechanisms of vaccination and virulence.

ACKNOWLEDGMENTS

We thank A. Capul and K. Zhang for reading the manuscript and P. Scott and J. Uzonna for thoughtful discussions about the nature of the immune response to the lpg2⁻ and lpg2⁻REV parasites.

This work was supported by NIH grant AI31078 (to S.J.T. and S.M.B.), by the Deutscher Akademischer Austauschdienst (DAAD), and by the Human Frontiers Science Program (G.F.S.).

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