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Immunology

Effective immunization against cutaneous leishmaniasis with recombinant bacille Calmette–Guérin expressing the *Leishmania* surface proteinase gp63

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ABSTRACT **_Leishmania_ parasites cause a spectrum of diseases that afflict the populations of 86 countries in the world. The parasites can survive within the lysosomal compartments of the host's macrophages, unless those macrophages are appropriately activated. Despite the fact that protective immunity can be induced by vaccination with crude parasite preparations, little progress has been made toward a defined vaccine for humans. In this study the gene encoding the _Leishmania_ surface proteinase gp63 was cloned and expressed as a cytoplasmic protein in a bacille Calmette–Guérin (BCG) vaccine strain. BALB/c and CBA/J mice were inoculated with a single dose of recombinant BCG and challenged with infective _Leishmania major_ or _Leishmania mexicana_ promastigotes. Significant protection was observed in both mouse strains against _L. mexicana_ and in CBA/J against _L. major_, whereas only a delay in _L. major_ growth was seen in BALB/c mice. Recombinant BCG also engendered a strong protective response against challenge with amastigotes of _L. mexicana_, demonstrating that the induced immune response recognized the intracellular form of the parasite. The results support the view that recombinant BCG expressing gp63 may prove a useful vaccine for inducing protective cell-mediated immune responses to _Leishmania_ species causing American cutaneous leishmaniasis.**

Leishmaniasis constitutes a spectrum of diseases that afflict an estimated 12 million people and is endemic in 86 countries around the world. The diseases range in severity from localized cutaneous and mucocutaneous infections to visceral disease which, when symptomatic, is fatal unless treated. There is no prophylactic agent currently available against any of these infections; however, the development of protective immunity following spontaneous resolution of cutaneous leishmaniasis provides considerable impetus for vaccine studies. In fact, in the Middle East, the deliberate infection of individuals with viable *Leishmania* parasites was commonly practiced as a means of avoiding subsequent infections (1).

Results from many laboratories have led to the adoption of experimental murine cutaneous leishmaniasis as a model system for functional analysis of various T-helper cell populations (2–7). Inoculation of mice with crude parasite extracts can lead to protection or exacerbation of the subsequent challenge infection, depending on the route of vaccination (2–4). Protective immunity is mediated by T cells that secrete cytokines capable of activating infected macrophages (8–10). The cytokine profile of these T cells, which secrete interferon γ (IFN-γ) and interleukin 2 (IL-2), is now accepted as characteristic for the T_{H1} arm of the T-helper cell response (8, 9). In contrast, unbalanced expansion of the T_{H2} arm of the T-helper cell response leads to IL-4 and IL-10 production and failure to activate macrophages, leading ultimately to accelerated lesion development (2, 4, 6). Therefore, the rate of lesion development during murine cutaneous leishmaniasis affords a dynamic assessment of the ability of potential vaccine constructs to expand preferentially protective T_{H1} cells.

Recent reports detail successful immunotherapy of patients infected with American cutaneous leishmaniasis with an experimental therapeutic vaccine consisting of killed parasites plus live bacille Calmette–Guérin (BCG) (11, 12). These results suggest that BCG may serve as a potent, and appropriate, adjuvant for induction of protective immune responses to leishmaniasis. In the present study we exploit the recent development of genetic systems in BCG (13, 14) to examine its potential as a vaccine delivery vehicle. We have expressed in BCG the gene encoding the *Leishmania* surface proteinase gp63, which is highly conserved across species (15), is expressed in both the insect and vertebrate forms of the parasite (16–19), and has been shown to induce some level of protection against murine cutaneous leishmaniasis (20–22). Here we report expression of gp63 in recombinant BCG and subsequent induction of protective immunity against cutaneous leishmaniasis following vaccination of BALB/c and CBA/J mice with a single inoculum of live recombinant bacilli.

MATERIALS AND METHODS

Cloning of the _Leishmania_ gp63 Gene into BCG. pMV262 is a 4.5-kb extrachromosomal plasmid designed for expression of polypeptides as protein fusions with the BCG *hsp60* gene product (13). The plasmid contains a mycobacterial origin of replication from pAL5000, the *Escherichia coli* pUC19 origin of replication, and the *aph* gene from Tn*903*, conferring kanamycin resistance. The expression site is under control of the BCG *hsp60* promoter and contains a multiple cloning site at the sixth codon of the BCG *hsp60* gene. The gp63 gene used in these studies was isolated from *Leishmania major* (23). The gene encodes amino acids 101–576 from the full-length protein, following deletion of the pre- and propeptide sequences, and the region encoding the hydrophobic, phosphatidylinositol-glycan addition sequence. In addition, as described previously (23) the four 5′ codons after the ATG start

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Abbreviations: BCG, bacille Calmette–Guérin; IFN, interferon; IL, interleukin; cfu, colony-forming unit(s).

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codons were altered by PCR so that the third base of each codon was T. This sequence alteration was necessary for high-level of expression of gp63 in *E. coli* (23) and BCG. The 1.5-kb fragment was cloned in the correct frame into the *Bam*HI site of the BCG *hsp60* gene in pMV262. The plasmid was electroporated into second-passage BCG (Pasteur strain). Several kanamycin-resistant transformants were isolated and screened for the presence of the plasmid and, by Western blot, for the expression of gp63.

Immunoelectron Microscopy. BCG cells for immunoelectron microscopy were fixed in 1% glutaraldehyde and processed as described (24). Frozen sections were blocked in 1% goat serum/10% fetal bovine serum with BCG sonicate at 1 mg/ml in Hepes-buffered saline, which was filtered through a 0.2-μm-pore filter prior to use. Incubation with anti-gp63 monoclonal antibody 253 and 10-nm gold coated with goat anti-mouse IgG (Amersham) was also carried out in blocking solution. The grids were washed and stained in 0.2% uranyl acetate in 2% polyvinyl alcohol.

Vaccination and Protection Experiments. The initial experiment had five different vaccine conditions: An untreated control group, two groups inoculated intravenously with 10^5 control BCG/pMV261 or 10^5 recombinant BCG/pMV262::gp63 bacteria, and two groups inoculated subcutaneously with 10^6 control BCG/pMV261 or 10^6 recombinant BCG/pMV262::gp63 bacteria. The five different vaccine conditions were duplicated with both BALB/c and CBA/J mice, and each individual group contained 11 females 6–8 weeks in age. Inoculations were given in the tail vein or subcutaneously high on the back. All BCG cells had been isolated from kanamycin agar plates, expanded in Dubos–Middlebrook 7H9 medium with OADC (Difco) plus kanamycin (20 μg/ml), and stored as frozen aliquots. The aliquots were quantified for colony-forming units (cfu) and the expression of gp63 by BCG/pMV262::gp63 transformants was confirmed by Western blot analysis. Colonization of the mice by BCG was assessed by sacrificing 1 mouse from each of the four groups per strain that received live BCG. The spleen was removed and homogenized in 10 ml of medium 199 with 0.1% Tween 20. Aliquots (100 μl) were replica plated on control and kanamycin agar plates.

Leishmania mexicana (MNYZ/BZ/62/M379) and *L. major* (Friedlin strain, clone V1) were maintained in SDM79 medium. Parasites were replenished from frozen isolates transformed from amastigotes isolated from infected mice, to ensure infectivity. Ten weeks after inoculation with BCG, mice were challenged by injection in their shaven rumps with either 5×10^4 *L. mexicana* promastigotes from stationary-phase culture or 10^4 *L. major* metacyclic promastigotes selected by lectin agglutination of the developmentally regulated alterations in the structure of lipophosphoglycan (25). The progression of lesions was assayed by measuring the diameter of the lesion in two directions and plotting the mean lesion diameter for the entire group.

In the follow-up experiment, mice were inoculated intravenously with 10^4 live BCG/pMV261 or BCG/pMV262::gp63 bacteria as described above. After 10 weeks the spleens were removed from six mice that had received live BCG and the efficiency of colonization was evaluated by counting cfu. The mice were challenged with stationary-phase *L. mexicana* promastigotes or *L. mexicana* amastigotes. The amastigotes were isolated from infected CBA/J mice as described (16). Lesion development was assayed as above. The challenge sites were biopsied by removal of the skin tissue, scraping over sterile wire mesh into 10 ml of SDM79. The culture was left overnight to transform into promastigotes, centrifuged, and resuspended in 1 ml, and 250-μl aliquots were plated on four SDM agar plates (10). Colonies were scored 6–8 days after plating.

RESULTS AND DISCUSSION

The DNA sequence encoding the mature gp63 polypeptide from *L. major* was cloned into a multicopy mycobacterial expression vector, pMV262 (13), under control of the BCG *hsp60* promoter, and electroporated into BCG. Following selection of kanamycin-containing agar plates, the expression of gp63 was confirmed by Western blot analysis. Immunoelectron microscopy of the transformed BCG demonstrated that the protein was expressed in the cytoplasm of the bacterium (Fig. 1).

In our initial experiment, detailed in Fig. 2, a variety of different inoculation regimens were explored with two different mouse strains (CBA/J and BALB/c). Mice were inoculated with live BCG/pMV261 with no insert, or with live BCG/pMV262::gp63 via either subcutaneous or intravenous routes, and one group received no treatment. Ten weeks after inoculation, one mouse from each group receiving live BCG was sacrificed, and the spleen was removed, homogenized, and replica plated on nutrient agar plates in the presence and absence of kanamycin. No differences were observed in the number of colonies, indicating that the plasmid had been maintained in the rBCG. The efficiency of colonization at this single time point was $1–5 \times 10^3$ cfu per spleen from both intravenous and subcutaneous inoculations. Western blot analysis of five colonies from the nutrient plates of each mouse inoculated with BCG/pMV262::gp63 confirmed that all colonies assayed continued to express *Leishmania* gp63 (data not shown).

Ten weeks after a single inoculation, the different groups and strains of mice were challenged with either 5×10^4 stationary-phase promastigotes of *L. mexicana*, or 10^4 promastigotes of *L. major*. We had previously ascertained that this level of challenge induced disease in 10 out of 10 control mice. *L. mexicana* induces a progressive disease in both CBA/J and BALB/c mice, whereas *L. major* causes a progressive disease in BALB/c mice but a self-resolving disease in CBA/J mice. The mice were inoculated in a shaved area at the base of the tail, and lesion development was followed by plotting the average lesion diameter (Fig. 2).

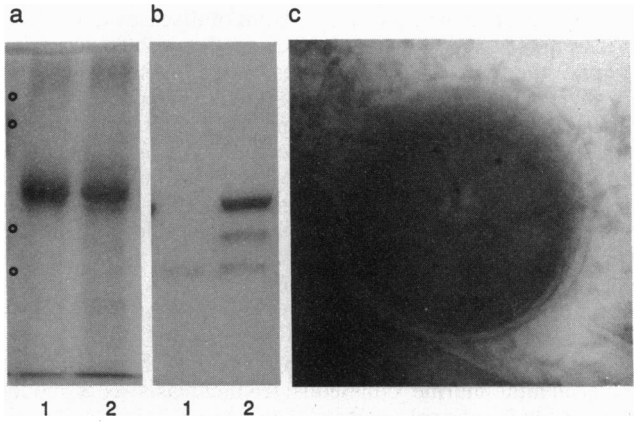

FIG. 1. Coomassie blue stain of SDS/PAGE (*a*) and Western blot analysis (*b*) of BCG clones transformed with pMV261 (no insert) (lane 1) and pMV262::gp63 (lane 2) with a monoclonal antibody specific for *Leishmania* gp63. The major protein in the Coomassie blue-stained lanes (*a*) is the heat shock protein encoded by *hsp60*. The lane containing BCG/pMV262::gp63 shows antibody reactivity with a band of 55-kDa apparent molecular mass, in close agreement with the theoretical mass of the encoding insert. Molecular size markers (small circles) are 106, 80, 49.5, and 32.5 kDa. (*c*) Immunoelectron microscopic analysis with this antibody, followed by 10-nm-gold-conjugated anti-mouse IgG, indicating that the protein is cytosolic. Control electron microscopy performed on BCG transformed with pMV261 was negative (data not shown). (×85,000.)

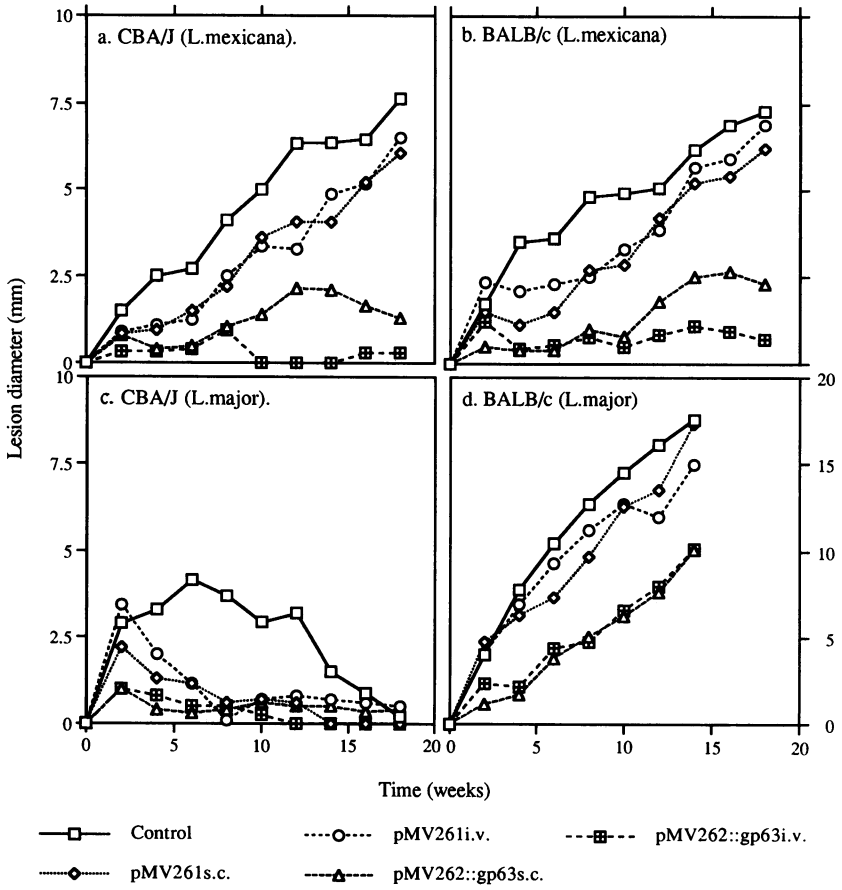

FIG. 2. Lesion development of mice after inoculation with BCG and challenge with *Leishmania* promastigotes. Values represent mean lesion diameter for the groups: The number of mice with lesions in each group at the close of the experiment is given in Table 1. CBA/J (*a*) and BALB/c (*b*) mice show lesion development following challenge with *L. mexicana*, and CBA/J (*c*) and BALB/c (*d*) following challenge with *L. major*. The vertical axes of *a–c* run from 0 to 10 mm; the vertical axis of *d* extends to 20 mm. Inoculation of both CBA/J and BALB/c mice with BCG/pMV262::gp63 induced a strong protective response to infection with *L. mexicana* promastigotes, whereas the controls with no inoculation or inoculation with BCG/pMV261 had little effect on lesion development. In both strains, intravenous (i.v.) inoculation induced a stronger protective response than subcutaneous (s.c.) inoculation. Infection of CBA/J mice with *L. major* resulted in a self-limiting infection even in the control group. However, those inoculated with live BCG, carrying either pMV261 or pMV262::gp63 showed an accelerated development of protective immunity. In addition, the lesion size prior to resolution was appreciably smaller in those mice inoculated with BCG/pMV262::gp63. However, infection of BALB/c mice with *L. major* (*d*) resulted in unimpeded lesion development irrespective of the initial inoculum, although the average lesion size in these groups was smaller.

Lesion development varied considerably between these mouse strains (Fig. 2 and Table 1). The BALB/c and CBA/J mice challenged with *L. mexicana* exhibited a relatively consistent pattern of lesion development. The control mice, receiving no BCG, developed lesions similar in size to those mice that had been inoculated with BCG/pMV261 either subcutaneously or intravenously. In contrast, mice that had been inoculated with BCG/pMV262::gp63 showed a marked reduction in lesion development. The most resistant groups were those inoculated intravenously, in which only 3 (BALB/c) and 1 (CBA/J) mouse out of 10 developed lesions (Table 1). CBA/J mice challenged with *L. major* were able to cure the infection spontaneously, irrespective of whether or not they were immunized with BCG. Interestingly, all the CBA/J mice that received live BCG demonstrated an earlier healing response, as reported previously (26), suggesting that BCG can induce or accelerate expansion of T cells that secrete macrophage-activating cytokines. This accelerated protective response of BCG alone was observed only in the self-healing infections induced by *L. major* infection of CBA/J mice. It was not seen in the progressive infections induced in BALB/c mice challenged with *L. major* or *L. mexicana* or in CBA/J mice challenged with *L. mexicana*. CBA/J mice that had been inoculated with recombinant BCG/pMV262::gp63 showed a further reduction in the lesion size over the BCG/pMV261 control. In contrast to the CBA/J mice, BALB/c mice challenged with *L. major* were unable to limit development of the infection, although the

Table 1. Number of infected mice over the group size at the termination of the experiment for the data in Fig. 2

	L. mexicana		*L. major*	
	CBA/J	BALB/c	CBA/J	BALB/c
Control	10/10	10/10	1/9	10/10
pMV261 s.c.	8/10	10/10	0/10	10/10
pMV261 i.v.	9/10	10/10	2/10	10/10
pMV262::gp63 s.c.	4/10	7/10	1/10	10/10
pMV262::gp63 i.v.	1/9	3/10	0/10	9/10

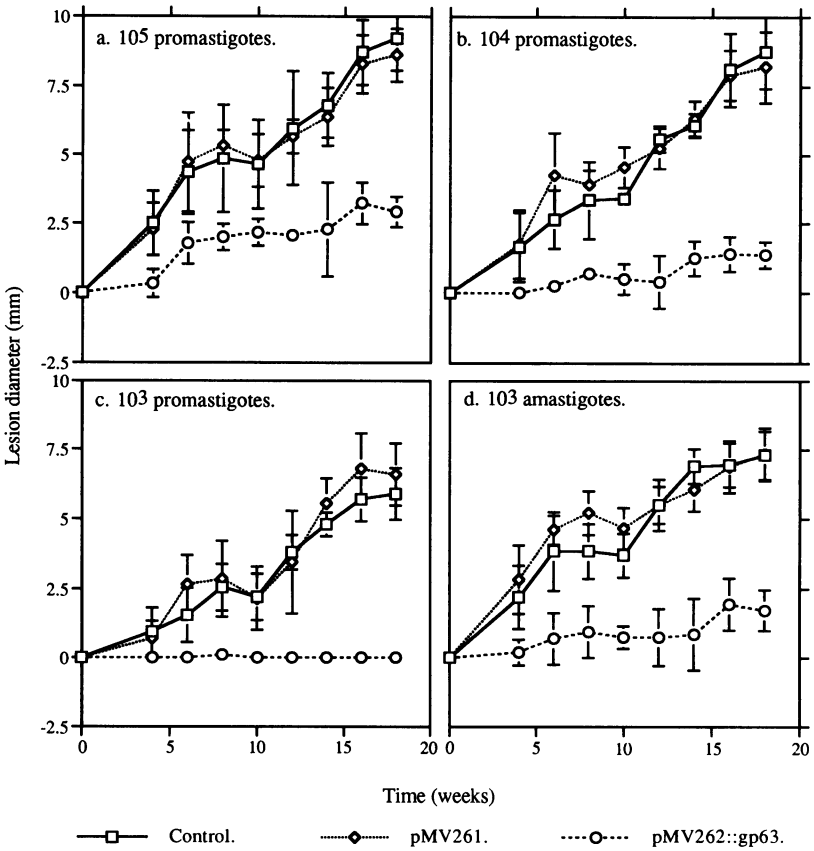

FIG. 3. Lesion development in CBA/J mice challenged with increasing doses of *L. mexicana* promastigotes (*a–c*) and with *L. mexicana* amastigotes (*d*). Mice were untreated (□) or were inoculated with 10^4 live BCG/pMV261 (◇) or BCG/pMV262::gp63 (○) bacteria 10 weeks prior to parasite challenge. The numbers of mice with lesions over the group size at close of experiment are given in Table 2. (*a*) Mice challenged with 10^5 promastigotes. Four of 10 mice in the experimental group inoculated with BCG/pMV262::gp63 developed lesions (Table 2), and the mean lesion diameter was reduced. (*b*) Mice challenged with 10^4 promastigotes. At the close of the experiment only 1 mouse inoculated with BCG/pMV262::gp63 had a lesion (Table 2). (*c*) Mice challenged with 10^3 promastigotes. None of the mice inoculated with BCG/pMV262::gp63 possessed discernible lesions at close of experiment. The challenge site of these mice was biopsied and placed in culture. Parasites were recovered from 2 of the 10 mice, demonstrating that, although no lesion could be detected, the parasites had not been totally eliminated. (*d*) Mice challenged with 10^3 freshly isolated *L. mexicana* amastigotes. Four of the 10 mice inoculated with BCG/pMV262::gp63 in this group had developed lesions following amastigote challenge; however, as in *a* the mean lesion diameter was markedly reduced in these mice.

average lesion size in the groups inoculated with BCG/pMV262::gp63 was, in fact, reduced. Because of the rapid lesion development in the control BALB/c mice, they were sacrificed at week 14.

We extended these observations by examination of lesion development following challenge of CBA/J mice with various doses of *L. mexicana* parasites (Fig. 3 and Table 2). CBA/J mice were inoculated intravenously with 10^4 live BCG bacteria transformed with either pMV261 vector alone or pMV262::gp63. Again, 10 weeks after inoculation, cfu from the spleen revealed levels of colonization comparable to those achieved with the higher inoculum used in the first experiment. Mice were challenged with 10^5, 10^4, or 10^3 *L. mexicana* promastigotes from a stationary-phase culture. At the highest challenge dose, 6 of 9 mice inoculated with BCG/pMV262::gp63 developed lesions, although the mean lesion diameter was considerably reduced. The lower challenge levels of 10^4 and 10^3 promastigotes confirmed the induction of a strong protective response; only 1 and 0 mice, respectively, had developed lesions at the conclusion of the experiment, in marked contrast to the presence of lesions on all control group mice (Table 2). To ascertain whether absence of lesion development signified complete clearance of parasites, we plated homogenized tissue from the sites of inoculation of the group challenged with 10^3 promastigotes, which showed no detectable lesion development. We failed to recover live parasites from 8 of 10 mice. From the remaining 2 mice, we recovered 13 and 21 parasites, respectively. This marked reduction in parasite number at site of challenge in all these mice bears testimony to the effectiveness of the induced immune response to eliminate infecting parasites.

Because immunity to an established leishmanial infection is expressed at the level of the amastigote-infected macrophage, we wished to determine whether or not recombinant BCG/pMV262::gp63 could induce protection against challenge with the amastigote form (Fig. 3*d*). CBA/J mice, immunized as in the preceding experiment, were challenged with 10^3 freshly isolated *L. mexicana* amastigotes. Four of the 10

Table 2. Number of infected mice over the group size at the termination of the experiment for the data in Fig. 3

	10^5 promastigotes	10^4 promastigotes	10^3 promastigotes	10^3 amastigotes
Control	8/8	10/10	10/10	9/9
pMV261	10/10	10/10	9/10	10/10
pMV262::gp63	5/9	4/10	0/10	4/10

mice challenged developed lesions (Table 2). However, lesion development within this group was less than in the untreated and BCG/pMV261 controls. These data are particularly relevant to existing reports that active infections may be resolved by immunotherapy. Convit and colleagues (12) in Venuzuela reported that therapeutic vaccine consisting of killed promastigotes mixed with BCG was as effective as chemotherapy in resolving localized cutaneous leishmanial infections.

It is accepted that protective immunity to leishmaniasis is mediated through the T_{H1} subset of T cells (3, 4, 6, 8, 9) that secrete lymphokines, most notably IFN-γ, that activate host macrophages to kill intracellular *Leishmania*. Although T-cell recognition is highly specific, this final effector mechanism is not, and it is likely that *Leishmania* contains several antigens that could induce varying degrees of protective immunity (27, 28). In the current study BCG expressing *Leishmania* gp63 from *L. major* induced a strong protective response in both CBA/J and BALB/c mice to challenge by either promastigotes or amastigotes of *L. mexicana*. A protective response was not induced in BALB/c mice to challenge with *L. major*, although markedly reduced lesion was observed prior to resolution of the *L. major* infection in CBA/J mice. Previous studies indicate that the inability of BALB/c mice to control *L. major* infections is due to an unbalanced expansion of the T_{H2} subset (2–4, 8, 9). However, BCG alone accelerated resolution of the *L. major* infection in CBA/J mice, and a self-healing infection such as this is the more usual course of the disease in humans (1).

An alternative explanation for the lack of protection of the BALB/c mouse against *L. major* may lie in the expression of gp63 in the different *Leishmania* species. The *Leishmania* surface proteinase gp63 has been extensively characterized in both the promastigote and amastigote stages of *L. mexicana*, as the product of differential expression of structurally divergent gp63 genes (15–19). The levels of expression in the amastigote lie between 10% and 30% of those of the promastigote (16, 18). However, there is some inconsistency in reports detailing expression of gp63 in the amastigotes of *L. major* (29–31), and the failure to protect these mice could be due to low levels of the antigen in the active infection. Obviously the usefulness of gp63 as a vaccine candidate relies on its expression in the amastigote. Since the original study on the efficacy of immunization with gp63-containing liposomes (20), several laboratories have used gp63 to promote antileishmanial immune responses in mice (21, 22) against both *L. mexicana* and *L. major*. And, most significantly, the induction of gp63-specific, IFN-γ-secreting T cells in humans infected with *Leishmania chagasi, L. braziliensis*, and *L. amazonensis* (32) and *L. major*** has also been reported, suggesting that the antigen is present in these amastigote infections.

In conclusion, although it has been possible to induce protective immunity to cutaneous leishmaniasis in the murine model with crude antigen preparations, there remains a need for a defined vaccine suitable for use in humans. The successful induction of a protective immune response to leishmaniasis bears witness to the power of immunization with recombinant antigens in a potent adjuvant delivery system such as BCG. Given the expense and toxicity of available antileishmanial drugs, coupled with the wide use of BCG as a vaccine in man, these results encourage the testing of recombinant BCG–gp63 *Leishmania* vaccines, possibly with additional *Leishmania* antigens, in experimental models for all forms of leishmaniasis and, eventually, in clinical trials of immunotherapy and immunoprophylaxis in humans.

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