Vervet Monkeys Vaccinated with Killed *Leishmania major* Parasites and Interleukin-12 Develop a Type 1 Immune Response but Are Not Protected against Challenge Infection

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Leishmania major is a protozoan parasite that causes chronic cutaneous lesions that often leave disfiguring scars. Infections in mice have demonstrated that leishmanial vaccines that include interleukin-12 (IL-12) as an adjuvant are able to induce protective immunity. In this study, we assessed the safety, immunopotency, and adjuvant potential of two doses of IL-12 when used with a killed *L. major* vaccine in vervet monkeys. The induction of cell-mediated immunity following vaccination was determined by measuring delayed-type hypersensitivity, in vitro lymphocyte proliferation, and gamma interferon (IFN- γ) production. Protection was assessed by challenging the animals with *L. major* parasites and monitoring the course of infection. At low doses of IL-12 (10 μ g), a small increase in the parameters of cell-mediated immunity was observed, relative to those in animals that received antigen without IL-12. However, none of these animals were protected against a challenge infection. At higher doses of IL-12 (30 μ g), a substantial increase in *Leishmania*-specific immune responses was observed, and monkeys immunized with antigen and IL-12 exhibited an IFN- γ response that was as great as that in animals that had resolved a primary infection and were immune. Nevertheless, despite the presence of correlates of protection, the disease course was only slightly altered, and protection was low compared to that in self-cured monkeys. These data suggest that protection against leishmaniasis may require more than the activation of *Leishmania*-specific IFN- γ -producing T cells, which has important implications for designing a vaccine against leishmaniasis.

Cutaneous leishmaniasis is a disease caused by obligate intracellular protozoa of the genus *Leishmania* and is characterized by cutaneous lesions that can be self-resolving with life-long immunity or chronic when accompanied by defective cellular immune responses (27). The disease is prevalent in many tropical and subtropical regions of the world, where it is transmitted via the bite of the sand fly. Treatment for leishmaniasis often involves the use of high doses of pentavalent antimony compounds or various formulations of amphotericin B. However, the increasing prevalence of drug-resistant organisms and the tendency for patients to relapse after an initially successive regimen of chemotherapy underscore the need for an effective prophylactic vaccine (27).

Efforts towards vaccine development have involved both animal model studies and human research. The vervet monkey is an excellent animal model for studying *Leishmania major* infection (4, 11, 17, 23–25, 27). Similar to many human patients with leishmaniasis, the vervet monkey has been shown to undergo spontaneous cure following both natural and experimental infection with *L. major*. Associated with healing was resistance to subsequent challenge with an appropriate dose of homologous parasites. Studies with the vervet monkey model for cutaneous leishmaniasis have demonstrated that resistance

to leishmaniasis in this model is correlated with increased production of gamma interferon (IFN- γ) and strong delayed-type hypersensitivity (DTH) responses (9, 25), similar to those seen in human patients with cutaneous leishmaniasis.

Ongoing human vaccine studies against cutaneous leishmaniasis with newly developed vaccines (killed *Leishmania* parasites plus *Mycobacterium bovis* BCG) have achieved encouraging results, although the protection obtained has not been outstanding (3, 18, 19, 30). Thus, additional studies to optimize protection are required. Work carried out in murine models has demonstrated the adjuvant potential of interleukin-12 (IL-12) in a vaccine against *L. major* (1, 13, 20). IL-12 is a critical component in the development of cell-mediated immunity and stimulates proliferation and the production of IFN- γ from T cells and natural killer cells. More importantly, IL-12 has the ability to promote the development of CD4⁺ Th1 cells, which are necessary for protective immunity in leishmaniasis (15, 32).

The present study was aimed at evaluating the adjuvant potential of recombinant human IL-12 (rhIL-12) for a vaccine against *L. major* in a vervet monkey model of the disease. Monkeys were immunized with autoclaved *L. major* (ALM) with or without rhIL-12. The immune response to *Leishmania* antigen was assessed, and the animals were subsequently challenged with *L. major* parasites.

MATERIALS AND METHODS

Recombinant human IL-12. rhIL-12 was generously provided by Genetics Institute, Cambridge, Mass. The cytokine was reconstituted in 0.1% autologous serum before use.

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Leishmania vaccine. ALM is a World Health Organization/Tropical Disease Research *Leishmania* vaccine (lot 10; courtesy of Yahya Dowlati, Ministry of Health and Medical Education, Center for Research and Education in Skin Diseases and Leprosy, Tehran, Iran). Briefly, promastigotes of *L. major* were grown in volumes of 50 to 200 ml in RPMI 1640 (GIBCO, Paisley, United Kingdom) with 15% fetal calf serum (Sigma) at 25°C in tissue culture flasks. Fresh medium was added gradually to reach 200 ml on different days. Parasites were harvested at stationary phase on day 16 to 20 by centrifugation at $1,800 \times g$ for 30 min, washed five times in pyrogen-free phosphate-buffered saline (PBS) (pH 7.0 to 7.2), and stored at -70°C. Ten to twelve harvests were pooled to constitute a lot. Samples from each lot were assayed for sterility and protein concentration. The sample was diluted in PBS to 11.11 mg/ml, and 2.7 ml was dispersed into vials, autoclaved for 15 min at 121°C (15 lb/in²), and kept at 4°C.

Vervet monkeys and vaccination protocol. Animal acquisition, care, and maintenance have been described (26). Institutional Animal Care and Use and Institutional Scientific Resources and Evaluation Committee guidelines were strictly followed. Adult vervet monkeys with a mean body weight of 3.8 kg were selected and divided into five groups of eight monkeys each as follows: group 1, *L. major*-infected, self-cured monkeys (positive controls); group 2, *Leishmania*-naïve monkeys (negative controls); group 3, monkeys vaccinated with ALM plus rhIL-12; group 4, monkeys vaccinated with ALM alone; and group 5, monkeys vaccinated with rhIL-12 alone. Monkeys in group 1 were selected from other studies (9, 23). These monkeys had been experimentally infected with *L. major* and had healed. Monkeys in group 2 were *Leishmania* naïve and were not subjected to any vaccination protocol; they are referred to as the negative controls.

Two rhIL-12 doses were tested. In one experiment (low-dose experiment), monkeys in group 3 were injected intradermally on the shaven lateral thorax 4 weeks apart with a mixture of 3.3 µg of rhIL-12 (low dose) and 1 mg of ALM in a volume of 200 µl each time. Group 4 animals were subjected to a vaccination protocol similar to that of group 3 but were vaccinated with ALM alone. Group 5 was treated similarly to group 3 but was given rhIL-12 alone. In the second experiment (high dose), the same vaccination protocol was followed except that 10 µg of rhIL-12 was used for the vaccination. Animals were challenged 5 weeks after the second vaccine boost.

Assessment of DTH responses. Animals were assessed for DTH responses 72 h after the third vaccination by measuring skin indurations at the vaccination sites using a metric caliper. A mean induration diameter greater than 5 mm² was considered positive.

***L. major* parasite for challenge and antigen preparation.** *L. major* strain NLB-144 was originally isolated from *Phlebotomus duboscqi* in Baringo District, Kenya, and maintained in BALB/c mice by serial subcutaneous passage. An aspirate from a footpad of an infected BALB/c mouse was cultured in Schneider's *Drosophila* insect medium (GIBCO) supplemented with 20% fetal bovine serum (Flow Laboratories, Irvine, United Kingdom) and 100 µg of gentamicin/ml. Stationary-phase promastigotes were harvested by centrifugation at 3,000 rpm for 15 min at 4°C. The pellet was washed three times in sterile PBS by centrifugation as before, and organisms were enumerated. For in vitro proliferation and cytokine assays, promastigotes were fixed in 1% formal saline for 1 h and then washed three times in sterile PBS as described above. The parasites were then suspended at a concentration of 5×10^8 /ml in sterile PBS and stored at -70°C until used.

PBL preparation and recall proliferative assays. The preparation of peripheral blood lymphocytes (PBL) for recall proliferative responses was as previously described (26). Briefly, the cells were adjusted to 3×10^6 /ml in complete RPMI 1640 medium (GIBCO), which consisted of 10% fetal bovine serum (Flow Laboratories), 2 mM L-glutamine (Sigma Laboratories), 100 µg of gentamicin (GIBCO) per ml, and 0.05 mM 2-mercaptoethanol (Sigma Laboratories). One hundred microliters of cell suspension was distributed to each well of 96-well round-bottomed microtiter plates (Nunc, Roskilde, Denmark). A 100-µl volume of either 5×10^6 formalin-fixed *L. major* promastigotes/ml or 10 µg of concanavalin A (Sigma)/ml was added to the wells. Control wells received 100 µl of complete RPMI 1640 medium. Cultures were prepared in duplicate and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 5 days for *Leishmania* antigen cultures and for 3 days for concanavalin A cultures. The cells were pulsed with 0.5 µCi of [methyl-³H]thymidine (New England Nuclear, Boston, Mass.; 1.85 mBq/ml) over the last 18 h and then harvested on a fiber filter (Whatman International Ltd., Maidstone, United Kingdom). Incorporation of radionuclide into DNA was determined by liquid scintillation spectrometry. Proliferation was expressed as counts per minute in stimulated cultures minus counts per minute in unstimulated cultures.

Production of IFN-γ. Purified PBL were adjusted to 2×10^7 /ml in complete RPMI medium and stimulated with *L. major* as described previously (10, 25).

Culture supernatants were collected from triplicate wells after 72 h of stimulation, and the concentration of IFN-γ in the supernatant was determined by enzyme-linked immunosorbent assay (ELISA). Briefly, polystyrene micro-ELISA plates (Dynatech Laboratories, Sussex, United Kingdom) were coated overnight with 50 µl of a 2-µg/ml concentration of capture monoclonal antibody to human IFN-γ (Chromogenix, Mölndal, Sweden) diluted in bicarbonate buffer, pH 9.6. Excess coating buffer was removed, and nonspecific binding sites were blocked in 3% bovine serum albumin (Sigma) in PBS for 1 h at 37°C. The plates were washed four times with 0.05% Tween 20 in PBS, and 50 µl of culture supernatant was dispensed to appropriate wells. Human IFN-γ (National Institute of Biological Standards and Control, Hertfordshire, United Kingdom) diluted (1 to 600 U/ml) in 1% bovine serum albumin in PBS-Tween was used as a standard. The plate was incubated at 37°C for 1 h and then washed four times. Biotinylated secondary monoclonal antibody to human IFN-γ (50 µl of a 1/2,000 dilution; Chromogenix) was added, followed by incubation at 37°C for 1 h. The plate was washed four times as before, 50 µl of 1/300-diluted alkaline phosphate-conjugated streptavidin (Chromogenix) was added, and the mixtures were incubated for 1 h as described above. The plate was washed 10 times in PBS-Tween, and 50 µl of nitrophenyl phosphate substrate (1 mg/ml) in diethanolamine buffer was added. The plate was incubated at 37°C in the dark for 45 min, and absorbance was read at 405 nm. IFN-γ levels were assessed by comparison with the standard curve generated with human IFN-γ.

Challenges of vaccinated monkeys and controls. Both vaccinated and control monkeys were challenged with a mixture of virulent *L. major* promastigotes and *P. duboscqi* salivary gland lysate as described previously (2). Three-day-old unfed female laboratory-bred *P. duboscqi* sand flies were dissected in 0.15 M NaCl solution. Five pairs of salivary glands were transferred to sterile vials containing 20 µl of PBS. The vials were then vortexed to achieve total disruption. The salivary gland lysate was stored at -70°C until required. Stationary-phase promastigotes were prepared as described above and adjusted to 2×10^6 /ml in PBS. Each monkey was inoculated intradermally with a mixture of 50 µl of promastigote suspension with 20 µl of salivary gland lysate intradermally on the right eyebrow ridge. Lesion development was monitored every 2 weeks, and mean lesion sizes for various groups were compared.

Statistical analysis. Student's *t* test was used in comparative analysis and a *P* value of <0.05 was considered significant.

RESULTS

Induction of antigen-specific responses in vervet monkeys following immunization with ALM and low-dose rhIL-12. Previous attempts to vaccinate vervet monkeys using *L. major* antigen with BCG have been unsuccessful (23). Therefore, in order to determine if IL-12 would boost the immune response to leishmanial antigens in vervet monkeys, animals were injected intradermally with ALM alone (1 mg), IL-12 alone (3.3 µg), or both. Animals were boosted twice 4 weeks apart by injection of the same dose of antigen and IL-12. Following each injection, animals were closely monitored for side effects. There was no noticeable irritation at the injection site, no alteration in leukocyte counts, and no changes in body temperature (data not shown).

Four weeks following the second booster, PBL were collected and restimulated in vitro with leishmanial antigen for assessment of recall proliferative responses and IFN-γ secretion. Positive *L. major* antigen-specific recall proliferative responses were evident in ALM-vaccinated and ALM-plus-IL-12-vaccinated animals (Fig. 1). The data for the two groups were comparable, and there were no significant differences between them or between either of them and the positive controls (Fig. 1). The other groups (IL-12-vaccinated monkeys and naïve controls) gave background responses (Fig. 1). In addition to positive lymphocyte transformation, cells from all monkeys vaccinated with rhIL-12 plus ALM produced IFN-γ after leishmanial antigen stimulation, although the levels were low relative to those in the positive controls. Monkeys in other vaccinated groups had little or no IFN-γ (Fig. 2).

FIG. 1. Recall proliferative responses in vervet monkey PBL to *L. major* antigen after the third immunization with low-dose IL-12. Monkeys received three injections with a total of 10 μ g of rhIL-12 with or without ALM. Purified PBL were adjusted to 3×10^6 /ml in complete RPMI medium and stimulated with *L. major* antigen for 96 h at 37°C in 5% CO₂. Then the cells were pulsed for the last 18 h of incubation with 0.5 μ Ci of [³H]thymidine. Incorporation of [³H]thymidine is expressed as counts per minute in stimulated cells minus counts per minute in unstimulated cells. Each individual point represents a single animal (some points overlap), and the bar represents the mean response. rhIL-12-plus-ALM group, $n = 7$; ALM group, $n = 7$; rhIL-12 group, $n = 8$; naïve control (ctrl) group (*Leishmania*-naïve monkeys), $n = 8$; positive (posit) control group (*L. major*-infected, self-cured monkeys), $n = 6$.

Course of *L. major* in vervet monkeys immunized with ALM and low-dose rhIL-12. Five weeks after the third vaccination, all monkeys, including the positive and negative controls, were challenged, and lesion development was monitored (Fig. 3). By 2 weeks postinfection most of the monkeys had developed measurable nodules in all the groups. Lesions increased in size and peaked by 10 to 12 weeks in all the vaccinated animals and naïve controls. In contrast, the previously infected monkeys

FIG. 2. IFN- γ levels in vervet monkey PBL after the third vaccination with low-dose IL-12. Monkeys received three injections with a total of 10 μ g of rhIL-12 with or without ALM. Purified PBL were collected 3 weeks after the final injection, adjusted to 2×10^7 /ml in complete RPMI medium, and stimulated with *L. major* antigen. Culture supernatants were collected from triplicate wells after 72 h of stimulation, and the concentration of IFN- γ in the supernatant was determined by ELISA. Each individual point represents a single animal (some points overlap), and the bar represents the mean response. Asterisk indicates significant differences between IFN- γ levels in the rhIL-12-plus-ALM group and all the other vaccinated groups. rhIL-12-plus-ALM group, $n = 7$; ALM group, $n = 7$; rhIL-12 group, $n = 8$; naïve control (ctrl) group, $n = 8$; positive (posit) control group, $n = 6$.

FIG. 3. Lesion development in vervet monkeys immunized with low-dose IL-12 (a total of 10 μ g) following challenge with virulent *L. major* promastigotes. Monkeys in all groups were challenged with 10^5 virulent *L. major* promastigotes plus lysate from five pairs of salivary glands from 3-day-old female *P. duboscqi* flies, and monkeys were observed every 2 weeks. Lesion sizes are given as means $\pm$ standard deviations. rhIL-12-plus-ALM group, $n = 7$; ALM group, $n = 7$; rhIL-12 group, $n = 8$; naïve control (ctrl) group, $n = 8$; positive (pos) control group, $n = 6$.

(positive controls) exhibited lesions that were very small and transient (Fig. 3). Similarly, ulceration occurred by the 7th week in all the vaccinated monkeys and in the naïve controls (data not shown). There were no significant differences in lesion development between the ALM-plus-IL-12 group and the ALM, IL-12, and naïve control groups (Fig. 3).

Induction of antigen-specific responses in vervet monkeys following immunization with ALM and high-dose rhIL-12. While we were able to enhance the antigen-specific immune response by inclusion of IL-12 in the vaccine, the levels of IFN- γ produced by cells from the ALM-plus-rhIL-12-vaccinated monkeys were substantially less than that observed from cells taken from monkeys with infection-induced resistance. Therefore, in the next set of experiments the concentration of IL-12 was increased from 3.3 to 10 μ g per injection. This dose of IL-12 was well tolerated, with no appreciable changes in body temperature, irritation at the injection sites, or alteration in leukocyte counts (data not shown).

We measured skin induration (DTH) in all the monkeys at the vaccinated sites 72 h after the second booster. The skin induration was greater in the ALM-plus-IL-12 group and lasted for about 2 weeks (Fig. 4). When these data were compared with those from the low-dose experiment, there was a significant difference in DTH responses between high-dose and low-dose studies. Similarly, there was a significant difference between ALM-plus-IL-12 and ALM groups. In contrast, monkeys vaccinated with rhIL-12 alone were DTH negative (mean score, <5 mm²).

In order to assess antigen-specific IFN- γ production, PBL were collected 4 weeks after the first and second boosters and restimulated in vitro with leishmanial antigen. High levels of IFN- γ were detected after the first and second boosters in all the rhIL-12-plus-ALM-vaccinated monkeys (Fig. 5). There was a significant difference between the first and the second booster vaccinations (Fig. 5). In contrast, cells from ALM-vaccinated and rhIL-12-vaccinated groups produced little or no IFN- γ after the second and third vaccinations.

FIG. 4. DTH responses in vervet monkeys following vaccinations. Monkeys were vaccinated with either low or high (a total of 30 μ g) doses of IL-12 with or without 1 mg of ALM or with ALM alone. The monkeys were given two boosters with the respective vaccines, and 72 h after the second booster, the diameters of skin indurations at the vaccination sites were measured. Positive controls were monkeys self-cured of *L. major*; they were injected intradermally with 1 mg of ALM, and skin induration diameters were measured after 72 h. Asterisk indicates significant differences between DTH responses in the rhIL-12 (30 μ g)-plus-ALM group and all the other vaccinated groups. Data are means plus standard deviations. rhIL-12 (30 μ g)-plus-ALM group, $n = 7$; rhIL-12 (10 μ g)-plus-ALM group, $n = 8$; rhIL-12 group, $n = 8$, ALM group, $n = 15$; positive control (posit ctrl) group, $n = 2$.

Course of *L. major* in vervet monkeys immunized with ALM and high-dose rhIL-12. In order to determine the efficacy of the vaccine, all animals were challenged with *L. major* promastigotes 5 weeks after the second vaccine boost and the lesions

FIG. 5. IFN- γ levels in vervet monkey PBL after the second and third vaccinations with high-dose IL-12 (10 μ g). Monkeys received three injections with a total of 30 μ g of rhIL-12 with or without ALM. Purified PBL were adjusted to 2×10^6 /ml in complete RPMI medium and stimulated with *L. major* antigen. Culture supernatants were collected from triplicate wells after 72 h of stimulation, and the concentration of IFN- γ in the supernatant was determined by ELISA. Asterisk indicates significant differences between IFN- γ levels in the ALM-plus-rhIL-12 group and all the other vaccinated groups and between the second and third boosters in this group. Each individual point represents a single animal (some points overlap), and the bar represents the mean IFN- γ level in each group. rhIL-12-plus-ALM group, $n = 8$; rhIL-12 group, $n = 8$; ALM group, $n = 8$; positive control (posit ctrl) group, $n = 2$.

FIG. 6. Lesion development in vervet monkeys immunized with high-dose IL-12 (total of 30 μ g) following challenge with virulent *L. major* promastigotes. Monkeys in all groups were challenged with 10^5 virulent *L. major* promastigotes plus lysate from five pairs of salivary glands from 3-day-old female *P. duboscqi* flies. Monkeys were observed every 2 weeks, and means $\pm$ standard deviations are shown. rhIL-12-plus-ALM group, $n = 8$; rhIL-12 group, $n = 8$; ALM group, $n = 7$; naïve control (ctrl) group, $n = 8$; positive control (posit ctrl) group, $n = 8$.

were monitored (Fig. 6). Two weeks postinfection, a large number of monkeys had measurable nodules, with the positive control group having a mean measurement of 19.2 mm². The rapid response of the previously infected animals was most likely a DTH reaction, since the lesions regressed with time and never ulcerated, although we would require histological analysis of the lesions to confirm this assumption. The negative control group had the highest mean nodule size (22.6 mm²), followed by the positive control group, the rhIL-12-plus-ALM group (13.1 mm²), the rhIL-12 group (7.6 mm²), and the ALM group (2.4 mm²). Four weeks postinfection, the trend in lesion sizes was reversed, with the negative control group having the largest mean lesion area and the positive control group having the smallest mean lesion area (Fig. 6). Nodules increased in size and eventually ulcerated in all groups except the positive control group, where the lesions were transient. Although the lesion sizes in the ALM-plus-IL-12 group were not significantly different from those in the other vaccinated groups, they were smaller than in all the other vaccinated groups and negative controls. Healing was observed in a majority of the monkeys by the 17th week postinfection.

DISCUSSION

This study assessed the safety, immunopotency, and adjuvant potential of rhIL-12 for a killed *L. major* (ALM) vaccine in the vervet monkey model of cutaneous leishmaniasis. The study was prompted by successful vaccination against *L. major* in murine models using recombinant murine IL-12 as an adjuvant (1). The enhanced protection observed when IL-12 was included in these vaccination protocols has been attributed to its ability to direct *Leishmania*-specific CD4⁺ Th1 cell development, which promotes protection against *Leishmania* infection (14, 28). To assess the development of type 1 responses to leishmanial antigens we measured several immunological parameters, including DTH, in vitro lymphocyte proliferative re-

sponses, and IFN- γ production, and then challenged the monkeys in order to assess the efficacy of the vaccine. Our results demonstrate that IL-12 augments the development of a type 1 response when used as an adjuvant in the vervet monkeys. Surprisingly, however, the enhanced type 1 responses were not associated with high levels of protection.

The first question that we addressed was whether the inclusion of IL-12 in a leishmanial vaccine would enhance cell-mediated immunity. To assess this, we measured DTH responses, in vitro proliferative response, and IFN- γ production. In addition to measuring immunological responses, we examined all vaccinees for side effects, since safety is a major concern in any vaccine formulation and delivery protocol. Injection of the two doses of IL-12 used in this study was not associated with any obvious side effects at the injection site and no appreciable alteration of PBL, suggesting that both doses were well tolerated. This is not surprising, since much higher rhIL-12 doses (2.5 mg/kg of body weight) given every 3 to 4 days for 3 weeks were found to be safe in rhesus monkeys (33). In a similar but unrelated study in *Macaca mulatta*, vaccination with *Leishmania amazonensis* antigen, alum, and 2 μ g of rhIL-12 was found to be safe, although the vaccination was associated with induction of subcutaneous granulomas at the injection site lasting for about 2 months (16). In our study there were no granulomas, suggesting that these granulomas can be attributed to alum rather than rhIL-12 and antigen.

DTH to leishmanial antigens has been widely used in leishmaniasis to indicate exposure to leishmanial infections and to assess the level of host protection to the disease (7, 10). However, the value of DTH as a measure of protection is unclear. In previous studies we found that immunization of monkeys with either killed parasites with BCG (unpublished data) or recombinant *Leishmania* antigen (gp63) plus BCG resulted in enhanced DTH responses but low or no protection (23). In the current study, monkeys vaccinated with either ALM plus rhIL-12 or ALM alone were DTH positive. Nevertheless, monkeys vaccinated with ALM plus 10 μ g of rhIL-12 (high dose) had higher DTH responses than monkeys vaccinated with ALM alone or ALM plus 3.3 μ g of rhIL-12 (low dose). Thus, our results suggest that rhIL-12 amplified the priming of T-cell responses to ALM. Similarly, proliferative responses of PBL to leishmanial antigens have also often been used as a measure of exposure to the parasite, as well as a measure of protection. In this study, monkeys vaccinated with either ALM plus rhIL-12 or ALM alone had enhanced in vitro lymphocyte proliferative responses to leishmanial antigen. Given that neither of these groups of animals was protected against disease, these results confirm that neither DTH nor in vitro lymphocyte proliferation responses are good correlates of protection.

In contrast to DTH and lymphocyte proliferation, the production of IFN- γ after stimulation of lymphocytes with leishmanial antigen has been considered one of the best correlates of resistance. The strong association with IFN- γ and resistance would be expected, since the parasites are killed when macrophages are activated by IFN- γ . In the mouse model of cutaneous leishmaniasis it is clear that IFN- γ , primarily produced by CD4⁺ Th1 cells, is important in the establishment of protective immunity (14, 28). Furthermore, there is strong indirect and direct evidence of the importance of IFN- γ in resistance to human leishmaniasis (27). Several studies done in the vervet

monkey model of leishmaniasis have established that monkeys that have self-cured following *L. major* infection produce high levels of IFN- γ (9, 25). Therefore, in the current study we were particularly interested in assessing IFN- γ as a correlate of protective immunity. We found that PBL from monkeys vaccinated with ALM plus 10 μ g of rhIL-12 secreted amounts of IFN- γ that were comparable to those produced in *L. major*-infected, self-cured monkeys, while PBL from monkeys vaccinated with ALM or IL-12 alone failed to produce IFN- γ in response to leishmanial antigen. Thus, of all the parameters of a type 1 response considered in this study, IFN- γ production was the only one that correlated with inclusion of IL-12 in the vaccine.

Surprisingly, however, while our results clearly demonstrate that IL-12 can augment several immunological parameters of cell-mediated immunity and in particular IFN- γ production, we found that these enhanced immunological responses were not associated with substantial protection when the monkeys were challenged. These results are in contrast to the complete protection achieved in mice vaccinated with parasite extract and IL-12 (1). A notable difference in the two vaccination protocols is that in the murine study, recombinant murine IL-12 (homologous system) was used, while in our study we used rhIL-12 (heterologous system). However, our data indicate that the rhIL-12 was able to enhance specific IFN- γ responses, demonstrating that human IL-12 was active in vervet monkeys. Another factor that can dramatically influence vaccine-induced protection is the time period between the last vaccination and infection. In the murine studies, animals were challenged within 2 weeks of vaccination, while in the present study animals were not challenged until 5 weeks after the last vaccination. Recent studies suggest that long-term memory of Th1 responses may require a prolonged exposure to IL-12 or the antigen (12, 13). Interestingly, protection against *L. amazonensis* parasites in *M. mulatta* was observed when alum and rhIL-12 were included in the vaccine (16). This protection was associated with substantial subcutaneous granulomas at the injection sites and thus may be due to long-term exposure to antigen and possibly continued induction of endogenous IL-12. Interestingly, the animals were not resistant to a secondary challenge, by which time the vaccine-induced granulomas had resolved. While agreeing that longer-term exposure to antigen and/or IL-12 may be critical in maintaining a type 1 response, we do not believe that the lack of protection that we observed was due to a loss of a cell-mediated response. This contention is supported by our findings that *Leishmania*-specific IFN- γ responses were high 1 week prior to challenge, and it seems unlikely that this response was lost in such a short period of time.

Another explanation for our failure to induce protection is that a critical antigen was destroyed during autoclaving or is present only in amastigotes. Our rationale for using autoclaved parasites as the antigen was based on the fact that this was a standardized vaccine currently being used in several clinical trials (3, 30, 31). However, a study comparing immunogenicity of autoclaved and nonautoclaved *Leishmania* antigen demonstrated an obvious change in biochemical profiles associated with decreased immunogenicity in autoclaved antigen as opposed to nonautoclaved antigen (6). Thus, it is quite possible that in order to obtain protection one must induce a type 1

response to a particular set of leishmanial antigens, rather than just any leishmanial antigen.

Finally, the focus of vaccine efforts in leishmaniasis has been primarily on the generation of CD4⁺ Th1 cells. However, there is evidence indicating that after cure of human disease, the number of *Leishmania*-specific CD8⁺ T cells is increased (5). Similarly, in mouse models, resistance to reinfection has been shown to be partially dependent upon CD8⁺ T cells (8, 21, 22). More recently, vaccination against leishmaniasis in mice using DNA was shown to be superior to protein immunization, and one of the major differences was the priming of CD8⁺ T cells in the DNA vaccine (12). We were unable to look at the phenotypes of the cells responding in the vervet monkey vaccine, although given the method of immunization it is unlikely that we induced a strong CD8⁺ response. Determining whether the induction of a *Leishmania*-specific CD8 T-cell response is critical in the development of a successful leishmanial vaccine awaits further experimentation.

It is clear that IL-12 is an effective adjuvant when used in vaccines against several infections, including diseases caused by viruses, bacteria, protozoa, and helminths (29). In each case where IL-12 has been advantageous for enhancing immunity, the induction of IFN- γ played a central role in protection. Certainly, the evidence in experimental murine leishmaniasis supports this notion, and our data in vervet monkeys indicate that IL-12 can augment IFN- γ responses in nonhuman primates. However, the data from the current study suggest that although IL-12 can induce a type 1 response in vervet monkeys, other factors may be required to protect against leishmaniasis. It may be that *Leishmania*-specific CD4⁺ T cells alone are sufficient to mediate protection but that only certain leishmanial antigens are associated with protection. Alternatively, the T cells associated with resistance to reinfection after self-cure may differ from those induced by immunization in ways that we do not yet understand. Finally, successful vaccination may require more than CD4⁺ Th1 cells. It has been argued that the development of a successful vaccine against leishmaniasis should be easier than the development of vaccines against other parasites—*Leishmania* has a relatively simple life cycle, we understand the effector mechanism that eliminates the parasites, and clear resistance to the infection in mice, nonhuman primates, and humans can be generated after self-cure. Nevertheless, our results suggest that a leishmanial vaccine may not be so straightforward and that there remains much to be learned about how resistance to leishmaniasis is mediated after self-cure and, more broadly, about what is required to develop and maintain cell-mediated immunity.

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