Primate Model of Cutaneous Leishmaniasis

Human IL-12 and Alum as Adjuvants in a Protective Immunity

Using Recombinant

Richard T. Kenney, David L. Sacks, Joseph P. Sypek, Luciano Vilela, Albert A. Gam and Kamela Evans-Davis

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Protective Immunity Using Recombinant Human IL-12 and Alum as Adjuvants in a Primate Model of Cutaneous Leishmaniasis

Richard T. Kenney, David L. Sacks, Joseph P. Sypek, Luciano Vilela, Albert A. Gam, and Kamela Evans-Davis

Protection from cutaneous leishmaniasis, a chronic ulcerating skin lesion affecting millions, has been achieved historically using live virulent preparations of the parasite. Killed or recombinant Ags that could be safer as vaccines generally require an adjuvant for induction of a strong Th1 response in murine models. Murine rIL-12 as an adjuvant with soluble Leishmania Ag has been shown to protect susceptible mice. We used 48 rhesus macaques to assess the safety, immunogenicity, and efficacy of a vaccine combining heat-killed Leishmania amazonensis with human rIL-12 (rhIL-12) and alum (aluminum hydroxide gel) as adjuvants. The single s.c. vaccination was found to be safe and immunogenic, although a small transient s.c. nodule developed at the site. Groups receiving rhIL-12 had an augmented in vitro Ag-specific IFN-γ response after vaccination, as well as increased production of IgG. No increase in IL-4 or IL-10 was found in cell culture supernatants from either control or experimental groups. Delayed hypersensitivity reactions were not predictive of protection. Intradermal forehead challenge infection with 10^7 metacyclic L. amazonensis promastigotes at 4 wk demonstrated protective immunity in all 12 monkeys receiving 2 μg rhIL-12 with alum and Ag. Partial efficacy was seen with lower doses of rhIL-12 and in groups lacking either adjuvant. Thus, a single dose vaccine with killed Ag using rhIL-12 and alum as adjuvants was safe and fully effective in this primate model of cutaneous leishmaniasis. This study extends the murine data to primates, and provides a basis for further human trials. The Journal of Immunology, 1999, 163:4481–4488.
reduce the parasite burden in BALB/c mice (20, 21). In addition, both mice vaccinated then treated with anti–IL-12 Abs (12) and genetically resistant mice that lack the IL-12 gene (22) are unable to heal. As an adjuvant combined with a soluble preparation of Leishmania major promastigotes and given s.c., murine rIL-12 affords full immunity to susceptible mice and produces a Th1 instead of the usual Th2 response to infection (23). IL-12 has also been combined with recombinant parasite Ags such as the 24-kDa LACK protein to give protection (12, 24), although this effect may be transient (25). While it remains to be shown in a Leishmania model, adsorption of both Ag and murine rIL-12 onto aluminum hydroxide gel (alum) enhances the immunomodulatory effects to promote both humoral Ab and Th1 cytokine responses to HIV gp120 in mice (26).

Nonhuman primates, used in some of the first studies to establish Leishmania as a pathogen (1), have also been shown to model cutaneous disease (27–29), and may provide a system that more closely approximates human immunity for vaccine development. There is some evidence that immunogenicity in primates requires an adjuvant to be used along with crude Ag (30, 31). A Macaca mulatta model of disease simulates human self-healing cutaneous leishmaniasis with an intradermal infection of a large number of parasites ($10^8$ late log phase promastigotes) to induce lesions in the upper eyelid (28). Apart from the large dose needed to cause a clinical infection, all of the parameters studied parallel natural human disease, including time of lesion onset and healing, histopathology, and humoral and cell-mediated immune responses. Biopsies of lesions were culture positive for parasites at the early phase of developing lesions, but negative later on. We used this model to assess the safety, immunogenicity, and efficacy of a vaccine that combines a heat-killed preparation of Leishmania amazonensis with human rIL-12 and alum as adjuvants.

Materials and Methods

Rhesus monkeys

A total of 48 healthy juvenile thhus macaques (M. mulatta) were obtained, after quarantine, from the Food and Drug Administration colony in South Carolina; 24 for each of two experiments. These studies were conducted and the animals were housed in American Association of Laboratory Animal Care (AALAC)-certified facilities after protocol approval by the Center for Biologics Evaluation and Research (CBER) Animal Care and Use Committee.

Vaccine preparation

Vaccine Ag was produced and provided by Biobrás (Montes Claros, Brazil), and is currently being used in clinical trials in Ecuador and Brazil. It is similar to a polyvalent Ag containing multiple species that has been extensively tested in Brazil (32, 33), but only one was used in this study for simplicity of manufacture and standardization. The vaccine strain of L. amazonensis was originally isolated from a sand fly (IFLA/BR/67/P8), maintained in hamsters until liquid culture became available in the early 1980s, then stored as a master cell bank. Parasites were grown to late log phase, washed several times in saline, pelleted, and frozen for storage. This concentrate was thawed and refrozen three times to break the parasite membranes, diluted to 2.3 mg/ml, then was autoclaved, bottled, and stored frozen. On the day of vaccination, the Ag was mixed with one-half the amount of alum (Rehydragel HPA; Reheis, Berkeley Heights, NJ) based on the protein weight, and the required amount of rIL-12 (Genetics Institute, Cambridge, MA) was added. The alum used was a high protein-absorbing aluminum hydroxide gel specially manufactured to be a homogeneous colloidal suspension that absorbs 2.5 times its own weight.

Vaccination groups and injection

Two experiments were conducted, first to study the dose response to rIL-12 and then to see the effect of decreasing Ag/alum concentration.

Control groups in the first study were vaccinated with Ag alone or adjuvants alone, and in the second study using Ag with either adjuvant, as detailed in Table I. The vaccine dose was given as a single 0.5-ml s.c. injection in the upper arm during ketamine anesthesia.

Experimental infection

The same strain of L. amazonensis used for vaccination (PH8) was obtained from American Type Culture Collection (Manassas, VA) and grown for infection. Promastigotes were grown in medium 199 with 20% FCS, supplemented by 0.1 mM adenine (Life Technologies, Gaithersburg, MD), 25 mM HEPES (Life Technologies), 5 μg/ml hemin (Sigma, St. Louis, MO), 1 μg/ml biotin (Life Technologies), and Pen/Strep/l-glutamine (Life Technologies). To ensure high infectivity, the strain was passed through BALB/c mice once and frozen as amastigotes for storage. These amasti-gotes were freshly transformed in culture to promastigotes, then grown to late log phase for each experiment. After washing the cells, metacyclic promastigotes were purified by negative selection using mAb D5, which recognizes a surface lipophosphoglycan determinant that is differentially expressed by procyclic and other immature stages of L. amazonensis promastigotes (E. Saraiva, unpublished). The promastigotes were incubated for 30 min at room temperature with a 1/200 dilution of D5 ascites, and the agglutinated parasites were pelleted by low-speed centrifugation at 400 × g for 5 min. Metacyclic promastigotes remaining in suspension were pelleted and washed, then resuspended at 1 × 10^7 promastigotes/ml in RPMI. Monkeys were challenged by injection with 1 × 10^5 metacyclic promastigotes in 0.1 ml at 1 mo following vaccination. The forehead, rather than the eyelid, was used for intradermal injection in this study because the base is more stable and the skin is similar. Lesion size was calculated from the average diameter to approximate a circle, measuring length by width.

Safety and immunogenic assays

All animals were examined every 2 to 3 days in the 2 wk following vaccination or challenge and then biweekly. Blood was drawn for complete blood count and chemistries before and at 2, 4, and 28 days following vaccination. Blood was drawn for immunologic assays before vaccination, weekly 2 wk following vaccination until week 8, then every 1–2 mo. PBMCs were obtained by differential centrifugation using Lymphocyte Separation Medium (Litton Bionetics, Kensington, MD), washed in PBS, and plated at 2 × 10^5/well in 24-well tissue culture plates. The cells were then incubated with no additives, 25 ng/ml PMA (Sigma, St. Louis, MO), and 1 μg/ml ionomycin (Sigma) as mitogens, or 5 μg/ml PH8 Ag at 37°C in 5% CO_2. Parasite Ag was prepared by freezing and thawing washed late log phase promastigotes for three cycles, followed by centrifugation at 4000 × g for 5 min to pellet large particulates. Protein concentration of the Ag was determined by BCA assay (Pierce, Rockford, IL).

Cell supernatant cytokine concentrations were determined by adapting standard trapping ELISA assays and as described (34). Cross-reacting hu-

man reagents were used when possible due to a lack of rhesus-specific reagents. For the measurement of IFN-γ, the trapping Ab was a murine monoclonal anti-human IFN-γ clone BMS-107 (BioWhittaker, Walkersville, MD), the detection Ab was biotinylated monoclonal anti-human IFN-γ clone 76-B-1 (DiaPharma, West Chester, OH), while the standard

Table I. Components and doses of vaccination in the rIL-12 dose ranging experiment (Exp. 1) and in the HKLV/alum dose ranging experiment (Exp. 2).

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Components and Doses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>Saline</td>
</tr>
<tr>
<td>1b</td>
<td>Alum (0.5 mg) + rIL-12 (1 μg)</td>
</tr>
<tr>
<td>1c</td>
<td>HKLV (1 mg)</td>
</tr>
<tr>
<td>1d</td>
<td>HKLV (1 mg) + alum (0.5 mg) + rIL-12 (0.04 μg)</td>
</tr>
<tr>
<td>1e</td>
<td>HKLV (1 mg) + alum (0.5 mg) + rIL-12 (0.2 μg)</td>
</tr>
<tr>
<td>1f</td>
<td>HKLV (1 mg) + alum (0.5 mg) + rIL-12 (1 μg)</td>
</tr>
<tr>
<td>2a</td>
<td>Saline</td>
</tr>
<tr>
<td>2b</td>
<td>HKLV (1 mg) + rIL-12 (2 μg)</td>
</tr>
<tr>
<td>2c</td>
<td>HKLV (1 mg) + alum (0.5 mg)</td>
</tr>
<tr>
<td>2d</td>
<td>HKLV (1 mg) + alum (0.5 mg) + rIL-12 (2 μg)</td>
</tr>
<tr>
<td>2e</td>
<td>HKLV (0.5 mg) + alum (0.25 mg) + rIL-12 (2 μg)</td>
</tr>
<tr>
<td>2f</td>
<td>HKLV (0.25 mg) + alum (0.125 mg) + rIL-12 (2 μg)</td>
</tr>
</tbody>
</table>

*Four macaques in each group were injected once s.c. in the arm with the indicated components diluted to 0.5 ml with PBS.*
used was rhesus rIFN-γ CHO cell supernatant (gift of Dr. Francois Vil-
liger, Emory University, Atlanta, GA). IL-4 was measured using a murine monoclonal anti-human IL-4 clone MD4-8 (PharMingen, San Diego, CA) as the trapping Ab, biotinylated rat monoclonal anti-human IL-4 clone MP4-25D2 (PharMingen) as the detecting Ab, and rHL-4 (PharMingen) as a standard. For IL-10, the trapping Ab was murine monoclonal anti-human IL-10 clone 945A5D11 (Biosource International, Camarillo, CA), the detec-
tion Ab was biotinylated murine monoclonal anti-human IL-10 clone 945ASA10 (Biosource International), and rHL-10 (PharMingen) was used as a standard. Duplicate 2-fold dilutions of the standards were made on each plate from 1677 pg/ml to 13.1 pg/ml for IFN-γ and 2000 pg/ml to 15.6 pg/ml for IL-4 and IL-10. Streptavidin-HRP conjugate (Amersham, Ar-
lington Heights, IL) and ABTS peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) were used for detection, as per the man-
ufacturer’s instructions. Quantities of cytokines were determined by inter-
polation from the standard curves at the color development time point with the best fit.

Ag-specific serum Ab concentrations were determined by adapting a standard sandwich ELISA to detect rhesus IgG. A 1/1000 dilution of a goat anti-monkey IgG (whole molecule) conjugated to HRP (Organon Teknika, West Chester, PA), followed by ABTS peroxidase substrate (Kirkegaard & Perry Laboratories), was used. Color reactions were read on an ELISA reader (Dynex, Chantilly, VA) at a wavelength of 410 nm. Titeres were assigned by comparing the adjusted ODs at the predetermined optimal dilution (1/128) to the preimmunization value, and considered positive when that OD was more than twice as great.

Statistical analysis
When appropriate, Student’s unpaired t test was used to determine the statistical significance of the differences between groups.

Results
Vaccine safety
The animals were vaccinated on day 0 in groups, as indicated in Table I. Groups 1a–c and 2a–c served as controls for the two experiments, and groups 1d–f and 2d–f had a dose escalation of rHL-12 or dose reduction of Ag/alum, respectively. There was no local inflammatory reaction upon injection or in the week follow-
ing. Hematologic and serum chemistry parameters were stable with the exception of a mild decrease in the hemoglobin (for ex-
ample, from 11.8 mg/dl to 10.8 mg/dl in experiment 1) due to repeated blood draws.

To study the immediate effect of rHL-12, we measured serum IFN-γ in the days following vaccination. At the lower doses of 40 ng, 200 ng, and 1 μg rHL-12 in experiment 1, only marginal increases in serum IFN-γ were seen (not shown). With 2 μg rHL-
12, the level of serum IFN-γ rose significantly, whether or not alum was included in the formulation for the group (Fig. 1). For most groups, the IFN-γ response peaked on day 4. Serum IFN-γ for all groups returned to baseline by day 14. Control groups that did not receive rHL-12 showed no increase in IFN-γ. There was no increase in temperature, appearance of malaise, or other side effects often seen with higher doses of parental cytokines.

By 2 wk following vaccination, all groups that had received Ag with alum began to develop a s.c. nodule at the site that peaked in size between 3 and 6 wk. The second experiment addressed the size and firmness of these nodules directly using groups with decreasing doses of Ag and alum (Fig. 2). Although group 2b (Ag with 2 μg rHL-12) had a small nodule at 21 days postvaccination, all of the groups that had received Ag with alum (groups 1d–f and 2c–f) had an s.c. nodule that peaked in size between 21 and 28 days postvaccination and resolved over the next 2 mo. Monkeys vaccinated with less Ag and alum (groups 2e and f) had nodules that were qualitatively softer and resolved more quickly. There were no persistent nodules in any animal, and none appeared both-
ered by its presence because there were no excoriations or bite marks on the arm. One-third of the nodule was removed by inci-
sion and dissection in three monkeys (one from each in groups 1d, e, and f). They were white and moderately firm, sitting in the s.c. space, held in place by strands of connective tissue. Histopathol-
ogy showed a central acellular region surrounded by a predomin-
antly mononuclear cell infiltrate typical of a cell-mediated im-
une response-induced caseating granuloma.

Immunogenicity
One of the most predictive in vitro assessments of cellular immu-
nity in human cutaneous leishmaniasis is the ability of PBMCs to secrete IFN-γ in response to Ag stimulation (35). Preinfection im-
mune status was evaluated in experiment 1 (Fig. 3A) and experiment 2 (Fig. 3B) by measuring the amount of IFN-γ in stimulated cell supernatants. The first experiment evaluated increasing doses of rHL-12 and used saline, Ag alone, or adjuvants alone as controls. Groups that had been vaccinated with Ag and any amount of rHL-12 (groups 1d–f) showed substantial IFN-γ responses at 2 wk postvaccination, while none was seen in the control groups. This response reached statistical significance (p < 0.05) in the highest

FIGURE 1. Serum IFN-γ levels in the first week after vaccination. Rhe-
sus IFN-γ was measured in the serum before and after vaccination, as indicated. Results here are from experiment 2 and are shown as the mean ± SEM. The group designation and dose of each component are shown along the horizontal axis for clarity. A dash indicates the component was omitted from the vaccine dose.

FIGURE 2. Subcutaneous vaccination nodule development and resolu-
tion. The second experiment was designed to assess the possibility that dose reduction of the Ag could alleviate the size and firmness of the s.c. granuloma that developed after vaccination. Results are from experiment 2 and are shown as the mean ± SEM. Dips in the trend lines reflect inter-
observer variability.
dose of rhIL-12 (group 1f) when compared with the saline control or its prevaccination response to Ag. This increase was also observed at 4 wk postvaccination, although to a lesser extent (data not shown). There was some IFN-γ in the unstimulated supernatants of two monkeys in group 1e. Presumably, this was due to nonspecific stimulation of circulating cells, although no infection was noted at the time in these two animals.

In the second experiment, we evaluated a higher dose of rhIL-12 plus three Ag alum doses, and as controls, either saline, Ag plus rhIL-12, or Ag plus alum. Groups that had received the combination of Ag and both adjuvants (groups 2d–f) showed a marked increase in supernatant IFN-γ at 2 wk in response to Ag stimulation (Fig. 3B). A marginal response was observed in the groups vaccinated without either adjuvant (groups 2b and c). At 6 wk after vaccination, which was 2 wk after infection, IFN-γ was detected in only 2 of the 24 Ag-stimulated PBMC supernatants. Similarly, no IFN-γ was produced in the PBMCs tested at weeks 12 and 16, or 8 and 12 wk after infection. None of the groups showed an increase in either IL-4 or IL-10 on stimulation of the PBMCs with Leishmania Ag, yet the mitogen-stimulated controls were positive (data not shown).

Delayed-type hypersensitivity (DTH) reactions to skin test Ags are frequently used as in vivo correlates of cellular immunity, but they may not indicate resistance to infection (13, 15, 36). We measured the induration that developed at 48 h after forehead infection in selected groups as a surrogate to skin test DTH (Fig. 4). Vaccination with Ag alone or Ag with rhIL-12 (groups 1c and 2b) induced the largest reaction upon live parasite challenge 1 mo later. This effect was diminished by the inclusion of alum in the vaccine formulation in all cases. All groups had a significantly larger reaction than their respective saline controls (p < 0.05).

The amount of IgG that reacted with crude Leishmania Ag at 2 and 4 wk after vaccination and then at 2 wk after infection was measured by ELISA (Fig. 5). With the exception of the lowest dose Ag group (group 2f), all groups that had received Ag with 200 ng or more rhIL-12 (groups 1e and f, and 2b, d, and e) developed at least a 2-fold increase in Ag-specific IgG titer by 2 wk postvaccination. Most of these had an increased amount of Ab at 4 wk. Groups with no Ag (groups 1a and b, and 2a) failed to develop significant Abs before infection. When little or no rhIL-12 was included in the vaccine (groups 1c and d, and 2c), Ag failed to stimulate Ab production, with or without alum. Two weeks after infection, all groups had Ag-specific IgG, with the exception of the Ag/alum group (group 2c). Measurement of IgG subclasses was not possible due to the lack of rhesus-specific reagents.

Efficacy

A rhIL-12 dose response to vaccination was evident when lesion development was followed over time after intradermal forehead challenge infection (Fig. 6). Ag alone as the vaccine (group 1c) caused the largest lesions (p = 0.005 compared with saline). Increasing amounts of rhIL-12 added to the vaccine as an adjuvant with alum led to decreased lesion size in a dose-dependent fashion (groups 1d–f). At least 1 μg rhIL-12 with Ag and alum was required to produce any protection, with more rapid healing, while the 200 ng dose produced no significant difference compared with saline, and Ag alone or Ag with a very low dose of rhIL-12 (40 ng) actually exacerbated the disease. Groups 1a and c (saline and Ag controls) were available for rechallenge with the same parasite strain injected in the opposite forehead 10 mo after their initial infection. Seven of the eight monkeys developed typical cutaneous lesions, as was seen in the original reinfection studies in this model (28).
In the second experiment, protective immunity was demonstrated in all 12 monkeys that received 2 μg rhIL-12 at all Ag/alum doses (groups 2d–f) because of vaccination (Fig. 7). These monkeys developed minimal or no lesions. This result was significantly better than the saline control group (p < 0.005), in which all of the animals developed a typical cutaneous lesion with a maximum size of 25–50 mm². Interestingly, partial protection (75%) was also observed in the groups receiving high dose Ag plus alum without rhIL-12, or high dose Ag plus rhIL-12 without alum (groups 2b and c), although the mean lesion size was not significantly different from the saline controls due to the development of a large lesion in one of four monkeys in each of these two groups. The groups were too small to determine whether the proportion protected with the single adjuvant vaccines was significantly different from the complete protection observed when both adjuvants were used.

Discussion
Cutaneous leishmaniasis is one of the best targets for modern parasitic vaccine development because long-term immunity is induced in almost all people following natural infection. There is also a long history of successful human vaccination by inoculation with live parasites. Unfortunately, the unpredictable incidence of significant or prolonged vaccine lesions and the increasing prevalence of immunosuppression make the use of a live vaccine less tenable; infection with live parasites is probably sustained throughout life (37, 38). Problems associated with inoculation of live, virulent organisms can best be avoided using either nonliving or live, attenuated vaccines. The safety of crude, killed preparations of Leishmania promastigotes has now been clearly demonstrated in clinical trials (13, 15, 33, 39 – 41). The field efficacy of killed vaccines in the prevention of cutaneous disease, however, is not so convincing. Trials in which Ag alone was used produced marginal protection (13), as did more recent trials using a single dose of killed Ag plus bacillus Calmette-Guérin as adjuvant (40). The recent success using multiple doses of heat-killed Ag plus bacillus Calmette-Guérin has provided the first indication from human studies that forceful immunization using crude killed Ags plus appropriate adjuvant offers a viable approach to vaccination against cutaneous leishmaniasis (15). In the present study, we extend for the first time the use of rhIL-12 as an adjuvant to vaccination in a non-human primate model of disease, and show that the inclusion of alum as an additional adjuvant produces a killed vaccine that is safe, initiates a protective Th1 immune response, and is completely effective in experimental cutaneous leishmaniasis.

Protective immunity seems to require, at least in part, the development of a strong Ag-dependent Th1 response. Subcutaneous administration of crude Ag (42) or even candidate vaccine Ags selected by immune screening (12, 43) actually expands Th2 cell populations and abrogates innate resistance or exacerbates protection in susceptible mice. We found the same to be true in the macaque group vaccinated with Ag alone (group 1c) that produced no IFN-γ and went on to develop the largest cutaneous lesions at challenge, which is indirect evidence of a Th2 response. Even the addition of IFN-γ to a soluble crude Ag vaccine does not have a protective effect in mice and fails to stimulate a Th1 response unless combined with a bacterial adjuvant such as Corynebacterium parvum (44). Murine models of cutaneous disease (23) have

**FIGURE 5.** Serum Leishmania-specific IgG. Ag-specific IgG was measured by ELISA and compared with prevaccination background levels. Results from 2 and 4 wk after vaccination and 2 wk after infection are shown as the mean fold increase ± SEM.

**FIGURE 6.** Dose response of rhIL-12 for protection against L. amazonensis in experiment 1. One month after vaccination, all groups were challenged intradermally on the forehead with 10⁶ L. amazonensis metacyclic promastigotes (vaccine strain). Open symbols show the lesions in the first set of control groups, whereas filled symbols display the effect of increasing amounts of rhIL-12 in the vaccination. The lesions that developed are shown as the mean area (calculated from the length and width of the reaction) ± SEM.
shown the most direct way to potentiate a protective response in mice is with the addition of rIL-12 to protective Ags. We sought to induce and potentially augment a protective response in primates by holding the killed Ags and rhIL-12 together with alum (26).

Vaccination with crude Leishmania Ags using rhIL-12 and alum as adjuvants was safe in this primate model. There were no immediate local or systemic side effects due to any of the three components. Subcutaneous nodules that developed at the site of inoculation were seen in all groups receiving Ag mixed with alum and resembled a caseating granuloma histologically. Reducing the quantity of Ag and alum in the second experiment (groups 2d–f) qualitatively decreased the firmness of the nodule, but it had no effect on its size. A caseating granulomatous collection of immunocompetent cells characteristically forms after injection of vaccines containing alum that eventually resorbs (45, 46). What begins as a lake of Ag-bound aluminum salt in a polymorphonuclear exudate becomes organized with a pseudo-capsule, enclosing plasma cells and macrophages around an acellular core. In this study, all of the s.c. nodules resorbed after several months. We did not see large numbers of multinucleated giant cells or germinal centers that might indicate a more serious or chronic pathology (47).

Aluminum hydroxide gel (26) or water in oil emulsions (48) combined with Ag and murine rIL-12 have been shown to augment a protective immune response to defined HIV Ags in mice, enhancing both humoral Ab production and the type 1 cytokine response. This effect was most efficient when the rhIL-12 was at the site of the alum and Ag, acting to convert a predominant type 2 cytokine response to type 1. As with the murine experience using IL-12 combined with alum and Ag (26), we were able to measure an increase in the serum IFN-γ for up to 7 days after injection, with a peak at 4 days, indicating an extended nonspecific Th1 and/or NK cell activation. The best indicator of specific immunogenicity was the production of IFN-γ by PBMCs in response to Ag in vitro. In the first experiment, only the animals making the highest levels of Ag-specific IFN-γ were protected, while in the second experiment, even the lower levels of IFN-γ detected in this assay correlated with protection. Unlike the typical response in humans, little or no IFN-γ was detected in supernatants after infection, which may indicate a limit of the model and could explain the lack of immunity seen with rechallenge. PBMCs will respond to Ag by producing IFN-γ for several years after infection, and protection is lifelong in human disease. With respect to humoral immunity, an increase in Ag-specific Abs was seen in all groups vaccinated with Ag and 200 ng rhIL-12 or more, with the exception of the lowest Ag dose group, and was not dependent on alum. This response may be similar to the increase in IgG2 and IgG3 isotypes in mice (26) that indicated a polarized Th1 response. Unfortunately, subclass reagents for macaques are not yet available.

Successful vaccination of humans is often related to the development of a DTH reaction to intradermal Ag (13, 15, 36). Surprisingly, even though the monkeys vaccinated with Ag alone (group 1c) had a strong DTH-like reaction to the live promastigote challenge, none were protected. In fact, this group had the largest cutaneous lesions, which parallels the exacerbation of infection seen in BALB/c mice immunized s.c. with similar promastigote Ags (42, 49), and suggests the lack of correlation between DTH and immunity to infection extends to the monkey model. Inclusion of alum in the vaccination inhibited the response, whether rhIL-12 was present or not. Although this DTH-like reaction was probably due to parasites that die and/or release Ag locally just after injection, a formal Ag-based skin test for DTH was not evaluated in these monkeys because no validation data exist for the assay. In mice, both the DTH reaction and the antiproteolytic inhibitory effect could be adoptively transferred by splenic Th cells (50). Separate T cell populations appear to be responsible for cutaneous hypersensitivity and protection in the BALB/c model. The implication of this effect for induction of human protective immunity remains unexplored.

The goal of any vaccination strategy is effective protection against natural challenge. We used this experimental model of infection to assess the immunity afforded by a single dose vaccination using combined adjuvants. Twelve of 12 macaques that had received the highest dose of rhIL-12 (2 μg), or ∼500 ng/kg, in combination with Ag and alum were protected, an outcome that was highly significant compared with the saline controls (p < 0.0001). Partial efficacy was seen in the groups lacking either rhIL-12 or alum, with three of four animals protected in each case. The partial protection in the second experiment using alum and Ag without rhIL-12 was better than the results in the first set of monkeys in which similar concentrations of alum plus Ag clearly did
not protect, even in the presence of low concentrations of IL-12. This apparent difference in response may be due to genetic variability in these outbred animals, although the possibility that either adjuvant alone may be adequate warrants further study.

Primate models can enhance our ability to evaluate immune effects of relatively species-specific compounds such as rHl-12 in complex biologic combinations. Selection of an appropriate dose of rHl-12 for any human vaccine will have to be empirical, although the low doses used in this study may be satisfactory. These experiments were not designed to find a maximal tolerated dose, and there is a 5–10-fold reduced dose effect of rHl-12 in macaque responses compared with those seen in humans (John Ryan, Genetics Institute, unpublished observation). Whether the protective immunity induced by the inclusion of 2 μg rHl-12 in these vaccinations depends on a local response or on total body weight is unknown. The duration of protection was also not evaluated in these studies. A recent study in the mouse indicated the immunity elicited by killed *Leishmania* Ag plus mouse rHl-12 without alum may be relatively short-lived (less than 3 mo) (25).

The extent to which a second dose of vaccine, or even continued without alum may be relatively short-lived (less than 3 mo) (25). 

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