MAJOR ARTICLE

Protection against Cutaneous Leishmaniasis in Outbred Vervet Monkeys, Using a Recombinant Histone H1 Antigen

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Infection with *Leishmania major* parasites results in the development of cutaneous ulcerative lesions on the skin. We investigated the protective potential of a single, recombinant histone H1 antigen against cutaneous leishmaniasis in an outbred population of vervet monkeys, using Montanide adjuvant. Protection was assessed by challenging the animals with a mixture of vector sand fly salivary-gland lysate and a low dose of in vitro–derived parasites, thus more closely mimicking natural infection induced by *L. major*. The course of infection in immunized monkeys was compared with that of animals that had healed from a primary infection and were immune. The monkeys immunized with recombinant histone H1 showed a reduced development of lesion size, compared with controls. Our study therefore illustrates the potential use of histone H1 as a vaccine candidate against cutaneous leishmaniasis in humans.

Leishmaniasis is caused by the protozoan parasites of the genus *Leishmania*, and it affects 12 million people worldwide, with the number of cases rapidly spreading because of *Leishmania* and human immunodeficiency virus (HIV) coinfection in areas where *Leishmania* species are endemic. There is, at present, no vaccine available for the prevention of leishmaniasis. However, vaccination campaigns in humans using material isolated from active lesions, together with numerous experimental animal trials, have provided evidence that vaccination against leishmaniasis is feasible (for a recent review on vaccination against leishmaniasis, refer to [1]). Among the different animal models, the susceptible versus resistant mouse model of cutaneous leishmaniasis caused by infection with *Leishmania major* parasites has provided much information about leishmaniasis; however, the relevance of experiments in mice to the human immune system remains speculative. Therefore, the phylogenetic relationship in an animal model for the study of leishmaniasis is of importance. To this end, several groups have been using nonhuman primates to carry out vaccine trials for human leishmaniasis. There is an increasing consensus that resistance to infection with *Leishmania* requires a complexity of multiple host immune factors. In humans, this involves the production of a strong cellular Th1-type response associated with the production of interferon (IFN)–γ, which leads to lifelong protection after exposure to and cure of natural infection [2]. Delayed-type hypersensitivity (DTH) to leishmanial antigens has been used in human trials to assess the level of host protection to disease, but its significance as a measure of protection is limited, because leishmanin skin-test conversion does not always correlate with protection [3, 4]. We have previously demonstrated, in an experimental infection model of BALB/c mice, the protective capacity of the nuclear protein histone H1 (H1) as a recombinant protein, synthetic peptide, or partially pu-

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rified extract from *Leishmania* parasites [5]. The protection observed from that study provided us with the initiative to carry out a vaccine trial in an outbred population of African green monkeys *(Ceropithecus aethiops),* more commonly known as vervet monkeys. These monkeys are from an Old World primate species [6] and are widely used for biomedical and behavioral research because they share ~92% of their DNA with humans [7]. In the case of cutaneous leishmaniasis infection, vervets produce a self-healing type of infection that closely mimics disease progression in humans. Furthermore, this classic course of disease progression has been successfully established using low numbers of in vitro–derived parasites mixed with a salivary-gland lysate from the sand fly vector, thereby more closely representing natural infection [6].

The choice of antigen and adjuvant are of paramount importance when conducting a vaccine trial for end-point use in humans, particularly for those vaccines based on the use of recombinant polypeptides. A molecularly defined antigen with no homology at the amino-acid level with human antigen and that is inexpensive to produce is advantageous. Furthermore, the antigen and adjuvant complex must be stable without inducing any adverse effects. To this end, we used a single, recombinantly produced H1 antigen coadministered with the Montanide ISA 720 (MISA720) adjuvant. This adjuvant has been shown previously to be safe and to confer immunogenicity (antibody and T cell proliferation and IFN-γ production) in human trials [8–11]. In an attempt to define an animal model and design a molecularly defined vaccine that can be used in humans, we assessed protection against cutaneous leishmaniasis in outbred nonhuman primates using the H1 antigen and MISA720 adjuvant formulation in the absence of stimulating cytokines and with a challenge infection model that closely resembles that of natural infection with phlebotomine sand flies.

MATERIALS AND METHODS

Leishmania *vaccine antigens.* Glutathione-S-transferase (GST) and GST-H1 antigens were expressed in *Escherichia coli* and purified using GST affinity resin (Pharmacia Biotech), as described elsewhere [5].

Adjuvant. MISA720 (Seppic) was used at an adjuvant: solubilized antigen ratio of 7:3 in sterile PBS, as per the manufacturer's instructions.

Vervet monkeys and vaccination protocol. Animal acquisition, care, and maintenance have been described elsewhere [12]. 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 4 groups: group 1, *L. major*–infected and cured monkeys (positive controls) [13]; group 2, monkeys immunized with recombinant GST-H1 and MISA720; group 3, monkeys immunized with recombinant GST and MISA720; and group 4, monkeys immunized with MISA720 adjuvant alone (negative control group). Monkeys in groups 2 and 3 were injected intradermally with 200 μg of antigen and MISA720 for the first immunization and 100 μg of antigen and MISA720 for 2 boosters that were 3 weeks apart. Monkeys in group 4 were treated similarly but were given MISA720 adjuvant mixed in PBS alone. Animals were challenged 6 weeks after the final immunization.

L. major *parasite for challenge and antigen preparation.* *L. major* strain NLB-144 was originally isolated from *Phlebotomus duboscqi* in the Baringo District, Kenya, and maintained in BALB/c mice by serial subcutaneous passage [14]. An aspirate from the footpad of an infected BALB/c mouse was cultured in Schneider's *Drosophila* insect medium (Gibco) supplemented with 20% fetal bovine serum (Flow Laboratories) and 100 μg/mL gentamicin. Stationary-phase promastigotes were harvested by centrifugation at 1500 *g* for 15 min at 4°C. The pellet was washed 3 times in sterile PBS by centrifugation, and organisms were enumerated. For DTH, promastigotes were fixed in 1% formalin saline for 1 h and then washed 3 times in sterile PBS, as described above. The parasites were then resuspended at a concentration of 5×10^8 parasites/mL in sterile PBS and stored at −70°C until use. Freeze-thawed *L. major* antigen for ELISA was prepared as described elsewhere [15].

Assessment of DTH response. After the third vaccination, animals were assessed for DTH responses to respective antigens. A total of 200 μg of respective antigens in 100 μL of PBS was injected intradermally, and skin indurations were measured 48 h later. This amount of recombinant antigen had been demonstrated previously to induce strong DTH responses in positive control monkeys. A mean induration diameter >5 mm was considered to be positive.

ELISA. Polystyrene Micro-ELISA plates (Nunc) were coated overnight with 100 μL of recombinant GST, GST-H1, or freeze/thaw antigen (10 μg/mL each, diluted in bicarbonate buffer [pH 9.6]; Sigma). Excess coating buffer was removed, and nonspecific binding sites were blocked with 4% bovine serum albumin (BSA) in PBS/0.05% Tween 20 buffer for 1 h at 37°C. Unbound BSA was washed off 3 times with PBS/Tween 20 buffer. One hundred microliters of the diluted serum (1:500 in blocking buffer) was dispensed into the appropriate wells and incubated for 2 h at room temperature. Unbound serum was washed off 4 times as described above, and 100 μL of 1:10,000 biotin-conjugated goat anti–monkey IgG (Rockland Immunochemicals), diluted in PBS/Tween 20 buffer plus 1% BSA, was added, followed by an incubation for 2 h at room temperature. Unbound conjugate was washed off as described above, prior to the addition of streptavidin peroxidase conjugate (Sigma) diluted 1:3000 in PBS/

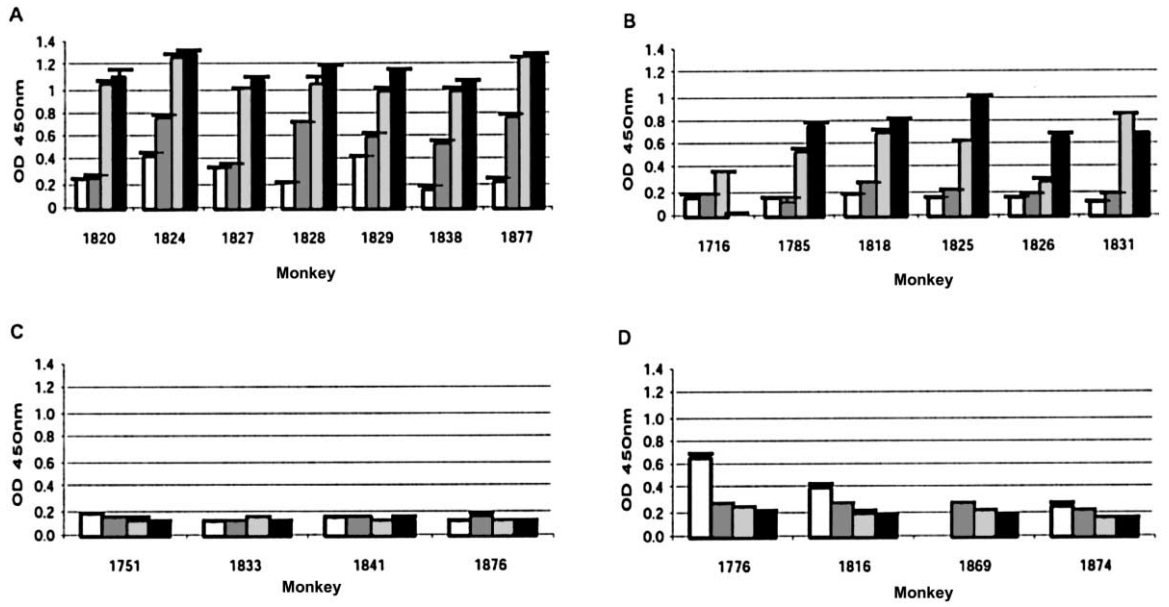

Figure 1. Anti-IgG response in vervet monkeys are shown. Serum from monkeys immunized with recombinant *Leishmania* histone H1 fused to glutathione-S-transferase (rGST-H1) *(A),* recombinant GST (rGST) *(B),* adjuvant control *(C),* and cured monkeys *(D)* was collected and analyzed by ELISA, as described in Materials and Methods. rGST-H1 and rGST were used as coating antigens in the experiments shown in panels *A* and *B,* and a freeze/thaw antigen was used in the experiments shown in panels *C* and *D.* Preimmune serum is shown in the white bars, serum collected after the first immunization in the dark gray bars, after the second immunization in the light gray bars, and after the third immunization in the black bars. Error bars represent the mean ± SD of triplicate ELISA wells.

Tween 20 buffer that contained 1% BSA for 45 min at room temperature. Wells were washed as above, and 100 μL of TMB substrate in citrate buffer (both Sigma) was then added. The plates were incubated, obscured, at 37°C. Optical densities were read at 450 nm in a microplate reader.

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 elsewhere [14]. Three-day-old, unfed, female, laboratory-bred *P. duboscqi* sand flies were dissected in 0.15 mol/L NaCl solution. Five pairs of salivary glands were transferred to sterile vials that contained 20 μL of PBS. The vials were then vortexed, to achieve total disruption. The salivary gland lysate was stored at −70°C until it was required. Stationary-phase promastigotes were prepared as described above and adjusted to 2×10^6 parasites/mL in PBS. Each monkey was inoculated intradermally on the right eyebrow ridge with the mixture of 50 μL of promastigotes and 20 μL of salivary-gland lysate. Lesion development was monitored every 2 weeks, and mean lesion sizes for the various groups were compared.

RESULTS

Specific antibody response of vervet monkeys. We immunized monkeys with a recombinant *Leishmania* histone H1 fused to GST (rGST-H1) or with a recombinant GST (rGST) mixed with MISA720 as the adjuvant. The negative control group was immunized with the adjuvant formulation mixed with PBS. Immunizations were not associated with any obvious local or systemic side effects. Prior to immunization and 3 weeks after the first, second, and third immunizations, antibody responses in all groups were detected by standard ELISA. Figure 1*A* and 1*B* illustrates the antigen-specific IgG response in monkeys immunized with either rGST-H1 or rGST, respectively. All monkeys immunized with the rGST antigen showed an increase in antibody levels that, in most animals, peaked after the third vaccination (serum from monkey 1716 was not available after the third injection) (figure 1*B*). Similarly, monkeys immunized with rGST-H1 antigen had IgG levels that peaked either after the second or third vaccination, with the response, as measured by optical density units, being stronger overall for rGST-H1 than that for rGST. This is likely due to the additional epitopes present in the H1 portion of the fusion protein. The positive response seen in these 2 groups indicates the presence of circulating immunizing antigen in the monkeys, as we had expected. For monkeys immunized with MISA720 adjuvant alone (negative control group) and monkeys in the positive control self-cure group, a freeze/thaw whole-parasite preparation was used as the antigen. All 4 monkeys in the adjuvant-only group (figure 1*C*) showed background levels of antibodies to freeze/thaw antigen. This indicated that they had no prior or subsequent exposure to *Leishmania* parasites or antigen through-

out the course of vaccination. Monkeys in the positive control group were bled at the same time points as the other groups of monkeys that had been vaccinated. These monkeys that had overcome a cutaneous leishmaniasis infection showed a positive response to the freeze/thaw antigen (figure 1*D*) that peaked at the time of the bleed for preimmune serum samples (no preimmune serum samples were available for monkey 1869). This was expected, because the amount of circulating *Leishmania* antigens in these cured monkeys would decrease over time after recovery from disease.

DTH response in immunized vervet monkeys. After the course of immunizations, cell-mediated immunity was determined by measuring DTH in the rGST-H1 and rGST monkey groups. Cured monkeys were used as positive controls for skin induration. Monkeys in groups rGST and rGST-H1 were injected intradermally with 200 μg of the respective antigen. For the rGST-H1 group, this quantity had been selected on the basis of a prior titration and positive DTH reaction to rGST-H1 antigen in the cured monkeys. The rGST antigen did not titrate or titrated poorly in cured monkeys, which suggests that these monkeys had no prior exposure to GST derived from *Schistosoma japonicum* [16]. On the basis of the titration obtained with rGST-H1, 200 μg of rGST was also used for the DTH assay. As a negative control, PBS was injected in the same monkeys but on the side of the lateral thorax opposite of where the immunizing antigens were administered. Forty-eight hours after injection, skin induration was measured on either the left or right lateral thorax. The mean skin induration measurement of 2 diameters is shown in figure 2*A* and 2*B*. Measurements >5 mm were considered to be positive. All monkeys tested for either rGST-H1 or rGST antigen were positive for DTH. This positive response indicates the presence of a specific cellular type of immune reaction [17]. Positive skin induration was not seen at the sites where PBS was injected (data not shown). Cured monkeys that were tested with freeze/thaw antigen had positive skin indurations >15 mm (data not shown).

Protection against cutaneous leishmaniasis challenge in vervet monkeys. Six weeks after the third vaccination, all monkeys, including the positive and negative controls, were challenged, as described in Materials and Methods, and lesion development was monitored every 2 weeks for 18 weeks (figure 3; table 1). Three of 7 monkeys in the rGST-H1 group (1824, 1828, and 1838) developed nodules that slowly progressed and peaked in size 14 weeks after infection. At that point, only 1 of these 3 monkeys had developed a large, wet, ulcerative lesion (monkey 1824) typical of cutaneous leishmaniasis. Of interest, of the remaining 4 monkeys, 3 developed small lesions (<100 mm^2) that did not ulcerate. The remaining monkey did not develop any nodules, aside from a small satellite nodule at the site of initial infection. Sixteen weeks after infection, all monkeys (except for 1824, with the large, wet, ulcerative lesion) showed signs of healing, as was expected. Monkey 1824 also healed (26 weeks after infection).

Monkeys in the rGST-immunized group started to develop nodules 4 weeks after infection (figure 3*B*). Three of 6 monkeys (1785, 1825, and 1831) developed nodules that rapidly progressed into large lesions (>100 mm^2) that were wet and ulcerative. Of the remaining 3 monkeys, 2 (1716 and 1826) had large lesions with a crust starting to form, and 1 (1818) had a small lesion that did not ulcerate. To summarize, 5 of 6 monkeys in the GST group showed the presence of large lesions that progressed rapidly and developed an ulcerative pattern of cutaneous leishmaniasis and were therefore not protected.

Monkeys that were immunized with adjuvant alone (figure 3*C*) developed large, ulcerative lesions that rapidly increased in size. One of the monkeys from this group (1841) died at 12 weeks after infection for unknown reasons. The other 3 monkeys developed very large lesions, 2 of which produced a typical wet

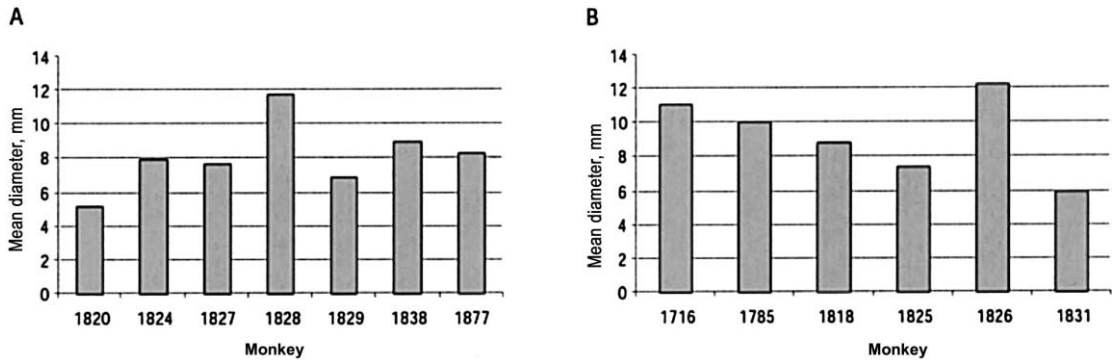

Figure 2. The delayed-type hypersensitivity (DTH) reaction in vervet monkeys is shown. DTH responses were measured 48 h after an intradermal injection in the lateral aspect of the thorax with antigen or PBS. Skin induration diameters were taken, and the mean skin induration is the DTH response. Measurements <5 mm are considered to be negative. *A*, DTH response in monkeys immunized with recombinant *Leishmania* histone H1 fused to glutathione-S-transferase (rGST-H1), as tested with the rGST-H1 antigen, *B*, DTH response in monkeys immunized with recombinant GST (rGST), as tested with the rGST antigen. The response with PBS was negative when tested in these monkeys.

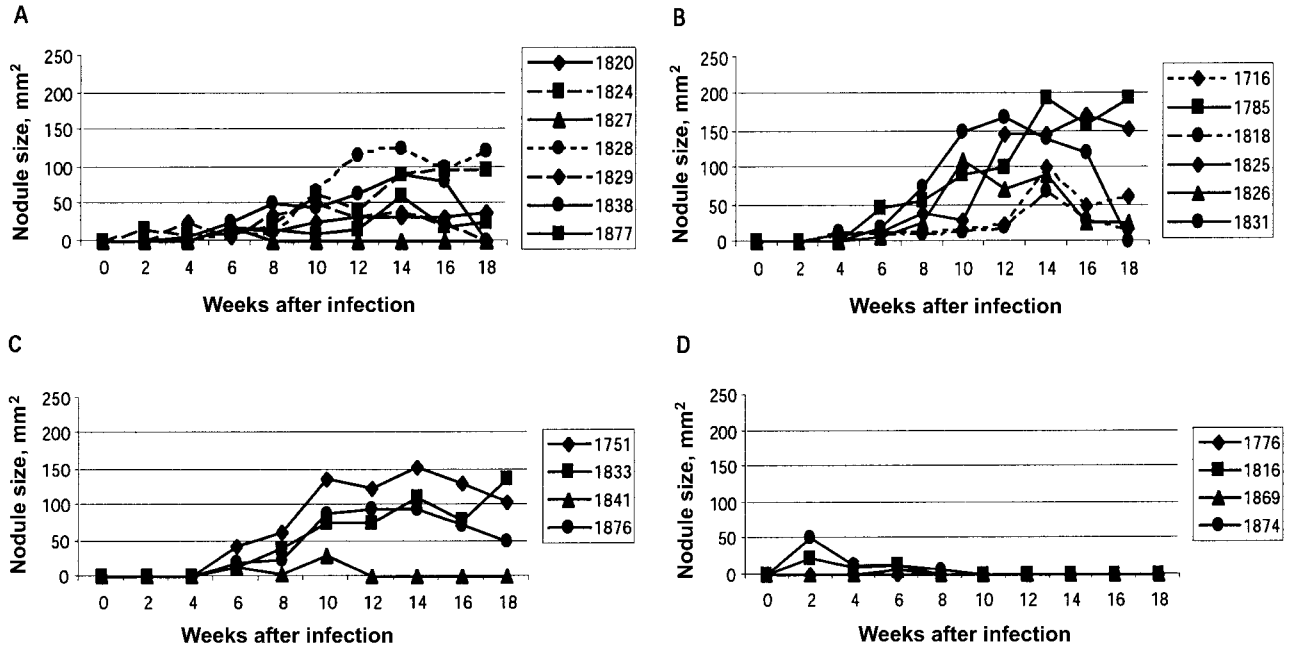

Figure 3. Lesion sizes after the infection of vervet monkeys immunized with recombinant *Leishmania* histone H1 fused to glutathione-S-transferase (rGST-H1) *(A),* recombinant GST (rGST) *(B),* and adjuvant alone *(C)* and cured monkeys *(D)* are shown. Every 2 weeks, after infection with a mixture of *Leishmania major* parasites and salivary-gland lysate from sand flies, lesion sizes in vervet monkeys was measured. The value for each monkey is the mean lesion size in millimeters, as calculated from the average of 2 measured diameters. This value was then divided by 2, squared, and multiplied by π, to represent the whole lesion area. Lesions <100 mm^2 were considered to be small.

ulcer and the other a nodule with a crust at 12–14 weeks after infection. Monkeys in the positive control group (cured monkeys) (figure 1*D*) developed small satellite nodules after infection that were transient and disappeared after week 6 after challenge.

In conclusion, monkeys immunized with rGST-H1 showed a reduced development of lesion size, compared with the control group immunized with adjuvant only and the rGST-immunized group. Furthermore, most monkeys developed only a small nodule type of lesion and not the classic wet, ulcerative cutaneous leishmaniasis lesion that was seen in the group immunized with adjuvant only. Monkeys in the rGST-H1 group, therefore, showed partial protection against leishmaniasis challenge infection in this outbred population of nonhuman primates.

DISCUSSION

In humans, recovery from *Leishmania* infection usually results in long-lasting immunity, thus indicating that vaccines against leishmaniasis are achievable. We used a nonhuman primate model to assess the safety, immunogenicity, and protective capacity of a vaccine that uniquely combines a single defined, recombinant, *L. major*–derived antigen, [5]—namely, the nuclear protein histone H1 together with the adjuvant MISA720.

We have previously shown, in a BALB/c mouse model [5], that the recombinant GST-H1 antigen was protective against cutaneous leishmaniasis infection. This protection was observed in the presence or absence of incomplete Freund's adjuvant or interleukin–12. On the basis of these observations, we decided to perform a vaccine trial in monkeys whose close phylogeny to humans might better predict the human immune response to our candidate vaccine antigen and adjuvant combination.

There is considerable interest in evaluating the safety of adjuvants planned for use in humans. To our knowledge, Montanide has never previously been tested for use in any type of vaccine trial against *Leishmania.* MISA720 has been approved for experimental use in humans as an alternative adjuvant to aluminium hydroxide. To date, there have been several studies that have tested the safety and immune response of MISA720 in humans [8–11]. Furthermore, the reports on Montanide trials done with HIV- and malaria-derived antigens have reached a general consensus that it is a safe and immunogenic adjuvant for use in humans, inducing both Th1-type cellular and humoral immune responses. In the case of *Leishmania* infection, the use of Montanide avoids problems that have been encountered with other adjuvants, such as bacillus Calmette-Guérin, which have batch-to-batch variability and antigenic cross-reactivity *(Mycobacteria)* with *Leishmania* species [18, 19]. We monitored the monkeys in our study subsequent to each immunization and throughout the course of the vaccine trial and observed no local or systemic adverse reactions attributed to the MISA720 and rGST-H1 or rGST vaccine formulation.

We confirmed immune recognition with the detection of an

Table 1. Summary of lesion development and ulceration pattern in vervet monkeys.

Group	No lesion	Small lesion (<100 mm^2)	Large lesion (≥100 mm^2)	Large ulcerative lesion
Cured (positive control)	4	0	0	0
Immunized				
Adjuvant (negative control)	0	0	0	4
GST	0	1	2	3
GST-H1	1	3	2	1

NOTE. Data are no. of monkeys from a single group that had no lesion, a small lesion, a large lesion, or a large ulcerative lesion after infection challenge. GST, glutathione-S-transferase; GST-H1, GST with nuclear protein histone H1.

antigen-specific antibody response. Antibody levels increased over preimmune levels after the first immunization, with most monkeys immunized with rGST-H1 having a peak in antibody levels after the second vaccination. We had previously observed a similar pattern of IgG response in mice immunized with a rGST-H1 antigen and MISA720 adjuvant in which there was no difference on comparison of serum titrations between the second and third immunizations (data not shown). The response seen with the rGST-H1 fusion was generally stronger than that for rGST alone, which demonstrates the antigen-specific nature of the H1 portion of the recombinant protein. This may serve to indicate that 2 immunizations with this antigen and adjuvant combination could be sufficient to induce sustainable antibody levels. A similar pattern of peak antibody response after the second immunization using leishmanial recombinant antigens in monkeys has been reported elsewhere [20].

The rGST sequence we used was based on GST originally derived from *S. japonicum* [16]. *S. japonicum* and *L. major* parasites are not coendemic; therefore, the risk of coinfection would be rare. However *L. major* and *S. mansoni* are coendemic, and a recent report [21] described that mice preinfected with *S. mansoni* delayed the development and resolution of cutaneous *L. major* lesions and parasitemia in coinfected mice, whereas the course of schistosomiasis disease progression was not altered. Furthermore, we tested (by ELISA) whether there was any cross-reaction between our rGST antigen and serum obtained from patients infected with *S. japonicum, S. mansoni,* and *S. haematobium* (20 patients in total). In all cases, only background levels of total IgG in all serums tested was detected (data not shown). Therefore, it is a reasonable approach to use GST as a fusion protein in a human vaccine against leishmaniasis.

A correlation could not be made between individual monkeys and the levels of antibody, DTH, and protection after challenge. A positive DTH response against *Leishmania*-specific antigen is seen in human populations that have been infected with *L. major* but that only develop a localized, self-healing form of the disease. This is indicative of a Th1-type cellular response, because the cells that are seen to infiltrate these lesions are mainly macrophages and $CD4^+$ T cells [17]. Thus, the measurement of DTH response is a parameter of significance in identifying the presence or absence of a cellular immune response. We observed a positive DTH to the rGST and rGST-H1 immunizing antigens, compared with PBS mock antigen as measured after the third vaccination. However, a positive DTH response on its own is not always indicative of protection against cutaneous leishmaniasis infection [3, 4, 13, 22]. Therefore, considering that a Th1-type response characterized by the production of IFN-γ is widely accepted as an indicator of a protective immune response in leishmaniasis, we measured in vitro–lymphocyte proliferation and IFN-γ production in the monkeys prior to challenge. We were able to detect, for some monkeys, an antigen-specific rGST-H1 and IFN-γ response from stimulated polymorphonuclear blood cells after the first immunization only (data not shown). We are uncertain as to why IFN-γ was not detected after subsequent immunizations. Initial studies with vervet monkeys [23, 24] observed positive cell-mediated IFN-γ responses. More recent data in monkey models have suggested that IFN-γ production is not sufficient to confer resistance against cutaneous leishmaniasis challenge [13, 25, 26]. Therefore, we made no additional effort to test this parameter.

The ability to induce a protective immune response is the principal test of a new vaccine and adjuvant combination. We have demonstrated that we were able to generate a durable cellular response that was sufficient to control infection in the majority of monkeys immunized with the combination of rGST-H1 antigen and MISA720 adjuvant. Our challenge infection was based on a natural infection model [6] whereby a low number of in vitro–derived parasites are injected with a mixture of salivary-gland lysate from the vector sand fly. This model produces a course of cutaneous leishmaniasis infection that closely mimics the progression of disease in humans, beginning with satellite nodules at the site of infection that develop central crusts and eventually become ulcerative lesions [6]. In other models, such as rhesus monkeys *(Macacca mulata),* a large number of in vitro–derived parasites are required to achieve a course of infection

that follows that of human disease [20, 25]. Furthermore, our vaccine trial was conducted in an outbred population of monkeys. To our knowledge, this is the first report to describe the immunization of outbred, genetically diverse monkeys with a single defined recombinant antigen and adjuvant coadministration using the challenge model described above.

Our results show that the cutaneous lesions of vervets vaccinated with rGST-H1 were significantly smaller and had slower progression, compared with those of monkeys vaccinated with rGST alone or with MISA720 adjuvant alone. Furthermore, only 1 of 7 monkeys in the rGST-H1–immunized group produced a typical wet, ulcerative, cutaneous leishmaniasis lesion, compared with 3 of 6 monkeys in the rGST group. This further supports the antigen-specific response of the H1 portion of the GST fusion protein and demonstrates its ability to confer protection against cutaneous leishmaniasis in a nonhuman primate model of the disease. The genetic diversity in the monkeys may explain why uniform lesion progression was not observed and is reflective of the clinical situation of human cutaneous leishmaniasis, in which a broad range of disease severity is presented [27].

When considering the production of a vaccine against cutaneous leishmaniasis, it would be desirable to produce a vaccine that produces a low-grade infection and sustainable immune response that would prevent disease development. This subclinical state is commonly presented in humans infected with leishmaniasis and in resistant mice, where, after primary infection and cure, small amounts of parasites remain at the site of infection. These parasites, in combination with regulatory T cells, have a crucial role in sustaining immune memory and preventing disease development on reinfection [28]. The diverse major histocompatibility complex in a heterogeneous human population [29] means that, for a globally widespread disease such as leishmaniasis, there is always a limitation of having nonresponders to a subunit vaccine, especially for small proteins with a limited number of antigenic epitopes. Therefore, we propose that, on the basis of the protection observed in the present study in an outbred population of nonhuman primates, the rGST-H1 antigen may serve as a candidate antigen for a human clinical phase 1 trial in a cocktail vaccine that is possibly composed of several molecularly defined *Leishmania*-derived antigens. We further support the notion that MISA720 is a safe and immunogenic adjuvant that should be considered for use in human trials against leishmaniasis.

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