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Protective Vaccination with Promastigote Surface Antigen 2 from *Leishmania major* Is Mediated by a TH1 Type of Immune Response

EMANUELA HANDMAN,* FIONA M. SYMONS, TRACEY M. BALDWIN, JOAN M. CURTIS, AND JEAN-PIERRE Y. SCHEERLINCK

The Walter and Eliza Hall Institute of Medical Research, Victoria 3050, Australia

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Leishmania major **promastigote surface antigen-2 complex (PSA-2) comprises a family of three similar but distinct polypeptides. The three PSA-2 polypeptides were purified from cultured promastigotes by a combination of detergent phase separation and monoclonal antibody affinity chromatography. Intraperitoneal vaccination of C3H/He mice with PSA-2 with *Corynebacterium parvum* as an adjuvant resulted in complete protection from lesion development after challenge infection with virulent *L. major*. Significant protection was also obtained in the genetically susceptible BALB/c *H-2*^{k} and BALB/c mice. One of the PSA-2 genes was cloned and expressed in both *Escherichia coli* and *Leishmania mexicana* promastigotes. Vaccination with the recombinant PSA-2 purified from *E. coli* did not confer protection, in contrast to the *L. mexicana*-derived recombinant PSA-2, which provided excellent protection. CD4$^+$ T cells isolated from the spleens of vaccinated mice produced large amounts of gamma interferon but no detectable interleukin 4 upon stimulation with PSA-2 in vitro. Limiting dilution analysis showed a marked increase in the precursor frequency of PSA-2-specific gamma interferon-secreting CD4$^+$ T cells. No substantial change in precursor frequency was observed for interleukin 4-secreting T cells in the vaccinated mice. A CD4$^+$ PSA-2 specific T-cell line generated from splenocytes of a vaccinated mouse produces a cytokine pattern consistent with a TH1 phenotype. Intravenous injection of this line into naive mice reduced significantly the parasite burden upon challenge infection. Taken together, the data suggest that vaccination with PSA-2 induces a TH1 type of immune response which protects mice from *L. major* infection. Moreover, a single recombinant PSA-2 polypeptide derived from a genomic clone can also vaccinate, provided that the structural form of the antigen is near native.**

Leishmaniasis, caused by the intracellular protozoan *Leishmania* sp., is a spectrum of diseases varying in severity from the fatal visceral form to the self-healing cutaneous lesion. The useful anti-*Leishmania* drugs are few, and the regimen used is lengthy and not always successful (33). To date, there is no vaccine against any human parasitic disease, including leishmaniasis. Vaccination through controlled infection with live organisms, practiced in the Middle East and Russia for many years for the control of cutaneous leishmaniasis, has been discontinued as a result of unacceptable complications (7, 25).

The rapid development of recombinant DNA technology has prompted the isolation and characterization of potential protein antigens with host-protective capability (4, 14, 21, 36, 37, 41). The availability of an excellent mouse model for leishmaniasis has already allowed a thorough evaluation of several candidate vaccine molecules (reviewed in references 12 and 35).

Data from the mouse model indicate that cure from cutaneous disease is associated with the generation of a TH1 type of immune response. In contrast, TH2-type immune responses have been associated with the disease state (reviewed in reference 2). The type of protective immune response induced by successful vaccination with molecularly defined antigens has been characterized only for gp63 and gp46/M2 (4, 15, 21, 46), and in both cases it was of the TH1 type. To date, it appears that not only healing but also protective vaccination with defined antigens are dependent on the induction of a TH1 response. The ideal vaccine would be a pan-*Leishmania* vaccine including several molecules, preferably conserved among different species.

One of the candidate vaccine antigens fulfilling this ideal has been the *Leishmania mexicana* subsp. *amazonensis* glycoprotein gp46/M-2 shown to protect mice against both *L. mexicana* and *Leishmania major* infections (21). Originally identified as a *Leishmania amazonensis*-specific, promastigote-specific polypeptide (16), it is now clear that glycoprotein gp46/M-2 belongs to a family of genes expressed in most *Leishmania* species (22, 44). We have identified and characterized three polypeptides of *L. major* encoded by genes of the same family as gp46/M2 (19, 44). These polypeptides, promastigote surface antigen-2 complex (PSA-2), are expressed on the promastigote surface and are tethered to the membrane with glycoinositol phospholipid anchors (44). The three polypeptides, with approximate molecular weights of 96,000, 80,000 and 50,000, are products of different genes, with similar, but distinct, amino-terminal sequences (44). The deduced amino acid sequences of the *L. major* PSA-2 genes cloned to date show significant homology throughout the molecule and are identical at the carboxyl-terminal end. Although PSA-2 polypeptides were thought initially to occur in promastigotes only (32), we have obtained clear evidence that some, but not all, PSA-2 polypeptides are also present in amastigotes (unpublished data).

In this study, we show that three PSA-2 polypeptides from promastigotes of *L. major* purified by a combination of Triton X-114 phase separation and affinity chromatography on a monoclonal antibody can protect mice from subsequent infection. This protection can be accounted for by a TH1 type of

* Corresponding author. Mailing address: The Walter and Eliza Hall Institute of Medical Research, Post Office Royal Melbourne Hospital, Victoria 3050, Australia. Phone: 61 3 9345 2555. Fax: 61 3 9347 0852.

T-cell response generated in the vaccinated mice. Moreover, a recombinant DNA-derived PSA-2 polypeptide could also induce protection. However, protection depended on appropriate folding and/or posttranslational modifications of the protein.

MATERIALS AND METHODS

Parasites. The virulent cloned line V121 was derived from the Israeli *L. major* human isolate LRC-L137 obtained from the WHO Reference Centre for Leishmaniasis, Jerusalem, Israel. The *L. mexicana* subsp. *mexicana* strain LRC-L94 was also obtained from the WHO Reference Centre described above. Promastigotes were maintained in vitro in Schneider's *Drosophila* medium supplemented with 10% fetal calf serum (HyClone, Logan, Utah) or in the biphasic blood agar (N.N.N) medium. The parasites and the culture conditions have been described previously (11).

Expression of recombinant PSA-2 in *Escherichia coli* and *L. mexicana*. A *Bst*UI fragment of the full-length genomic clone λTR1 containing the hydrophilic portion of the molecule was expressed in the pGEX3X (Pharmacia Biotech, Melbourne, Australia) vector as a fusion protein with the *Schistosoma japonicum* glutathione *S*-transferase (TR1-GST) (42). The fusion protein was predominantly water soluble when the induction of protein synthesis was done at 20°C. The fusion protein was purified by affinity chromatography on glutathione-Sepharose as described previously (42).

The full-length genomic clone λTR1 including the leader sequence and glycoinositol phospholipid anchor addition sequence was cloned in the pX vector (6, 18) and expressed in *L. mexicana*. Transfected promastigotes produced this PSA-2 polypeptide, which partitioned in the detergent phase during Triton X-114 phase separation, indicating that the molecule is glycoinositol phospholipid anchored (44).

Antibodies. Monoclonal antibody 11E5 was produced by immunization of mice with the recombinant fusion protein TR1-GST (31, 44). Hybridoma supernatants were screened for binding to both TR1-GST and intact promastigotes of *L. major*. 11E5 is an immunoglobulin G1 (IgG1) monoclonal antibody which recognizes PSA-2 on live promastigotes and also recognizes TR1-GST. In addition, the antibody recognizes PSA-2 by immunoprecipitation and immunoblotting. This monoclonal antibody is specific for *L. major* PSA-2, and it does not recognize the *L. mexicana* homolog of PSA-2 gp46/M2. Therefore, it was used to purify the recombinant DNA-derived *L. major* PSA-2 expressed in transfected *L. mexicana* parasites in the absence of gp46/M2. The purity of this preparation was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining.

Protein purification. PSA-2 polypeptides were purified by a combination of Triton X-114 solubilization-phase separation (3), to enrich for membrane proteins, and affinity chromatography on monoclonal antibody 11E5 described above. Briefly, 5 ml of packed promastigotes was solubilized in 100 ml of 0.5% Triton X-114 in phosphate-buffered saline (PBS; pH 7.3) containing the following protease inhibitors at a final concentration of 1 mM: leupeptin, aprotinin, TLCK (*N*α-*p*-tosyl-L-lysine chloromethyl ketone), phenylmethylsulfonyl fluoride, EDTA, and 1,10-phenanthroline. The membrane-enriched detergent phase was separated from the water phase (containing the cytoplasmic proteins) by incubation at 37°C for 7 min and centrifugation at 800 × *g* for 10 min at room temperature. The pelleted detergent phase was reconstituted with ice-cold PBS to a final concentration of 0.5% Triton X-114, vortexed vigorously, and left on ice for 20 min to obtain a homogeneous solution, before being loaded onto a Sepharose 6B precolumn followed by the antibody-containing column. After washing in a high-salt buffer containing 0.5 M NaCl, 50 mM Tris, 0.5% Triton X-100, and 5 mM EDTA (pH 8.0), bound PSA-2 was eluted with 0.1 M glycine HCl (pH 2.3)–0.1% Triton X-100 and immediately neutralized with 1 M Tris (pH 8.0). Purified proteins were electrophoresed in an SDS-PAGE system and stained with silver to determine their purity (29). In early experiments, they were also Western blotted (immunoblotted) and probed with specific antibodies to ascertain that they were PSA-2. Protein quantitation was done by the bicinchoninic acid assay developed by Pierce (Rockford, Ill.). For validation of quantitation, the intensity of the silver-stained PSA-2 bands was compared with the intensity of silver-stained molecular weight standards run under the same conditions at several concentrations between 100 ng and 10 μg (Pharmacia LKB). The gene product of λTR1 expressed in *L. mexicana* was purified from *L. mexicana* promastigote cultures by the same procedure.

Vaccination. C3H/He and BALB/c *H-2*^{k} female mice aged 6 to 8 weeks were obtained from our specific-pathogen-free animal breeding facility and subsequently maintained under conventional conditions. Groups of 8 to 16 mice were injected three times, every 2 weeks, intraperitoneally with 1.5 to 2 μg of PSA-2 purified as described above and mixed with 200 μg of killed *Corynebacterium parvum* as an adjuvant (Wellcome Biotechnology Limited, Beckenham, England) (4, 13). Two weeks after the last injection, all mice were bled individually and divided into groups; one group was used to examine T-cell responses to PSA-2 antigen, and another group was challenged with 10^5 live promastigotes. Antibody titer and isotype were determined with an enzyme-linked immunosorbent assay on intact promastigotes as described previously (28). Lesion development was scored weekly as described before (26–28), and parasite burdens in lymph nodes draining the infection site were estimated 6 and 10 weeks after infection by limiting dilution analysis (26–28, 45).

T-cell assay. CD4$^+$ T cells were enriched from the spleen by negative selection as described previously (27). Rat anti-mouse monoclonal antibody 53-6.7, B220, and GR1 specific for CD8$^+$ T cells, B cells, and macrophages/granulocytes, respectively, were used in conjunction with magnetic beads (Dynal; AS, Oslo, Norway) to remove cells of these lineages, leaving a CD4$^+$ T-cell-enriched population. CD4$^+$ T cells (2×10^5 per well in 200 μl) were incubated with 5×10^5 irradiated (3,000 rads) spleen cells as a source of antigen-presenting cells, with or without PSA-2 for 48 h in Dulbecco modified Eagle (DME) medium containing 5% fetal calf serum (FCS) and 50 μM 2-mercaptoethanol. The culture supernatants were collected and assayed for the presence of gamma interferon (IFN-γ), interleukin 3 (IL-3), and IL-4 by bioassays with the reporter cell lines 32D, CT4.S, and WEHI-279 as described before (17). For each lymphokine, the specificity of the bioassay was validated by use of neutralizing monoclonal antibodies. Prior to its use in the T-cell assays, the detergent in the PSA-2 preparation was removed by acetone precipitation (9). Briefly, the PSA-2 preparation (10 to 100 μg/ml) was supplemented with 10% FCS as a carrier protein and precipitated with 4 volumes of ice-cold acetone. The precipitate was pelleted by centrifugation, and the pellet was washed with cold acetone, dried, and resuspended at a final protein concentration of 1 μg/ml.

Limiting dilution analysis. The precursor frequency of CD4$^+$ T cells secreting different cytokines was estimated by use of a limiting dilution assay described previously (27). Briefly, 5×10^5 irradiated spleen cells were mixed with defined numbers of CD4$^+$ T cells (2,500 to 75 cells per well with 8 wells per dose with PSA-2 and 4 wells per dose without PSA-2) in DME medium supplemented with 5% FCS, 50 μM 2-mercaptoethanol, and 20 U of recombinant IL-2 per ml. After 10 days, the cultures were washed, and 5 μg of concanavalin A per ml was added to restimulate all surviving, antigen-driven T cells (26–28). The supernatants were collected 24 h later and assayed for IFN-γ, IL-3, and IL-4 by the bioassays described above.

PSA-2 T-cell line. A PSA-2-specific T-cell line was generated from splenocytes of a PSA-2-vaccinated C3H/He mouse in the absence of cytokines. A total of 8×10^6 PSA-2-primed CD4$^+$ T cells were incubated with 2×10^7 irradiated spleen cells and 8 μg of purified PSA-2 in 8 ml of DME medium supplemented with 5% FCS and 50 μM 2-mercaptoethanol. After 4 days, the cells were collected, and dead cells were removed by centrifugation on a Ficoll-Paque gradient. Live cells were incubated at a concentration of 10^5 T-cell blasts per ml in the presence of 2.5×10^6 irradiated spleen cells for 7 days. The T-cell line was maintained by alternating cycles of stimulation with antigen and resting periods without antigen. Supernatants were collected after PSA-2 stimulation and assayed for the presence of IL-3, IFN-γ, and IL-4. Cells were examined for activity in vivo after at least three restimulations with antigen.

RESULTS

Purified PSA-2 protects mice from infection with V121 promastigotes. The purification of PSA-2 polypeptides to homogeneity from cultured promastigotes was made possible by a combination of Triton X-114 phase separation and monoclonal antibody affinity chromatography as described previously (44). The purity of the preparation was assessed by SDS-PAGE, followed by silver staining of fractions eluted from the affinity column, and confirmed by protein sequencing of individual polypeptide bands. The identity of the purified proteins was confirmed by probing Western blots of the same material with a rabbit antiserum to the recombinant DNA-derived PSA-2 clone TR1, which recognizes all PSA-2 polypeptides (data not shown). The yield of protein averaged 50 μg per 1 ml of packed promastigotes, but the concentration of individual polypeptides has always been different. The 50,000-M_r polypeptide is the most abundant member of the family. For the purpose of vaccination, the three polypeptides were pooled, and the total protein was quantitated by the Pierce bicinchoninic acid assay.

C3H/He mice with intermediate genetic resistance to disease or BALB/c *H-2*^{k} mice with higher susceptibility to disease (10) were vaccinated with purified PSA-2. One of three similar experiments is presented in Fig. 1; in this experiment, seven C3H/He mice in each group were immunized intraperitoneally with PSA-2 and *C. parvum* or with *C. parvum* alone or were not immunized prior to challenge with 10^5 promastigotes intradermally. None of the mice vaccinated with PSA-2 developed skin lesions at the site of infection during a 10- to 12-week period of observation. During this time, all of the control mice

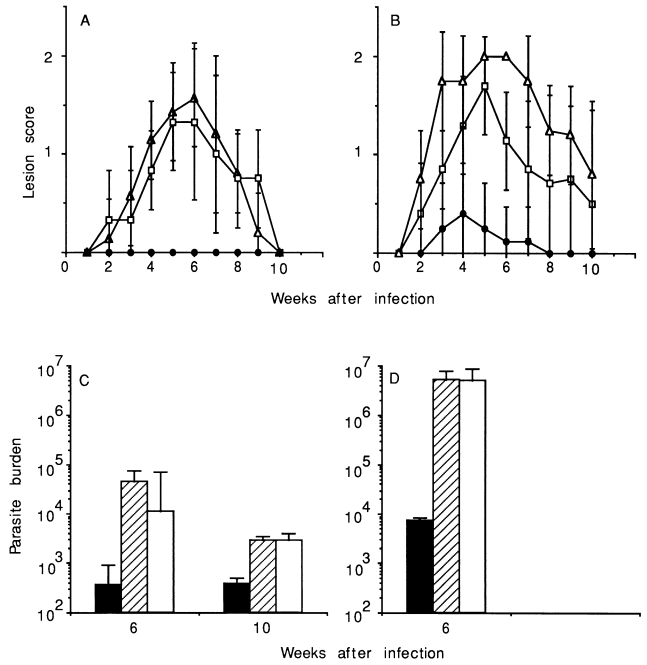

FIG. 1. Protective vaccination of mice with native PSA-2. The lesion scores are shown as a function of time in C3H/He (A) and BALB/c *H-2*^{k} (B) mice either untreated (△; ▲) or injected intraperitoneally with *C. parvum* alone (□) or with PSA-2 and *C. parvum* (●). The parasite burdens of C3H/He (C) and BALB/c *H-2*^{k} (D) mice per draining lymph nodes at 6 and 10 weeks after infection are also shown. PSA-2-vaccinated mice (solid bars), control untreated mice (hatched bars), and mice injected with *C. parvum* alone (empty bars) are compared.

injected with adjuvant or not treated developed lesions which peaked in size at about 6 to 7 weeks and cured by 10 to 12 weeks after infection (Fig. 1A), as expected with mice of intermediate resistance to disease. Similar results were obtained with BALB/c *H-2*^{k} mice (Fig. 1B): some vaccinated mice developed no lesions, and some developed small lesions which healed after 8 weeks. In contrast, the lesions were still present in the controls after 10 weeks of observation (Fig. 1B). Despite the lack of apparent lesions in the vaccinated mice, all animals harbored parasites in their draining lymph nodes. Parasites could be isolated from C3H/He mice at 6 weeks, the peak of lesion development in the control animals, and at 10 weeks after infection, when the control mice had cured their lesions (Fig. 1C). However, 6 weeks after infection, there was a 100-fold-lower parasite burden in the vaccinated mice compared with that in the untreated controls and a 30-fold-lower burden compared with that in the mice injected with *C. parvum* alone. By 10 weeks, the parasite burdens in the draining lymph nodes of the control groups were significantly reduced as expected from the cure of the lesions. However, the parasite burden was still 10-fold larger in the control mice than in the PSA-2-vaccinated ones (Fig. 1C). In this experiment, the parasite burden did not change significantly in the PSA-2-vaccinated mice between 6 and 10 weeks postinfection, although in other experiments, it decreased further. In the BALB/c *H-2*^{k} mice, vaccination produced an almost 1,000-fold reduction in parasite burden at the peak of lesion development (Fig. 1D).

In the highly susceptible BALB/c mice, the level of protection depended on the size of the challenge inoculum. Vaccinated BALB/c mice developed no lesions upon challenge with 10^3 promastigotes, but 50% of mice developed lesions when infected with 10^4 organisms (data not shown). Three of eight vaccinated mice developed lesions upon challenge with 10^5 promastigotes, whereas all controls developed lesions (data not shown).

Vaccination with recombinant PSA-2. A full-length PSA-2 gene, referred to as TR1, was cloned from a genomic library of *L. major* (31) and expressed in *E. coli* as a fusion protein with GST. The purified protein was water soluble as expected because of the lack of the glycoinositol phospholipid anchor in this clone. The TR1-GST fusion protein with *C. parvum* was used to vaccinate C3H/He mice by use of the protocol described above. As shown in Fig. 2A and C, the vaccinated mice were not protected from infection with virulent promastigotes as measured by lesion development and parasite burden. This lack of protection could be attributed to differences in protein folding and/or posttranslational modifications between the native PSA-2 and the polypeptide expressed in *E. coli*. Alternatively, the single product of one of the PSA-2 genes, TR1, which is not expressed in *L. major* promastigotes, may not be able to confer protection. Despite the high degree of similarity between the various PSA-2 genes and their products (31, 44), protective vaccination depends on the presence of cross-reactive T-cell epitopes in the vaccine preparation with amastigote PSA-2. This could be lacking in TR1. To address the role of protein conformation and posttranslational modifications in protection, we used TR1 expressed in a native conformation in *L. mexicana* promastigotes. Using a monoclonal antibody which recognizes only *L. major* PSA-2 and not the *L. mexicana* homolog, we purified the recombinant PSA-2 and vaccinated C3H/He mice as described above. As shown in one of several similar experiments presented in Fig. 2B and D, the PSA-2 produced in *L. mexicana* reduced both the lesion size and the parasite burden. The 3-log reduction in parasite burden in the

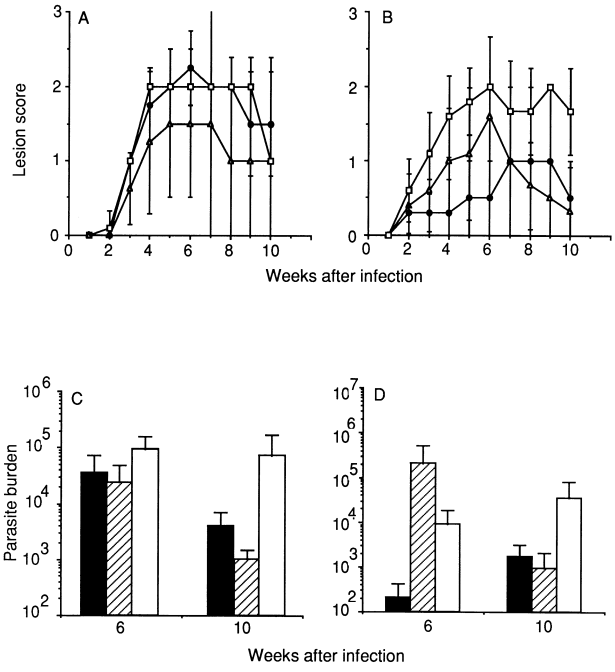

FIG. 2. Protective vaccination of mice with recombinant PSA-2 expressed in *E. coli* (A and C) and *L. mexicana* (B and D). (A and B) The lesion scores are shown as a function of time in mice either untreated (▲) or injected intraperitoneally with *C. parvum* alone (□) or with PSA-2 and *C. parvum* (●). (C and D) The parasite burdens per draining lymph nodes 6 and 10 weeks after infection are shown. PSA-2 vaccinated mice (solid bars), control untreated mice (hatched bars), and mice injected with *C. parvum* alone (empty bars) are compared.

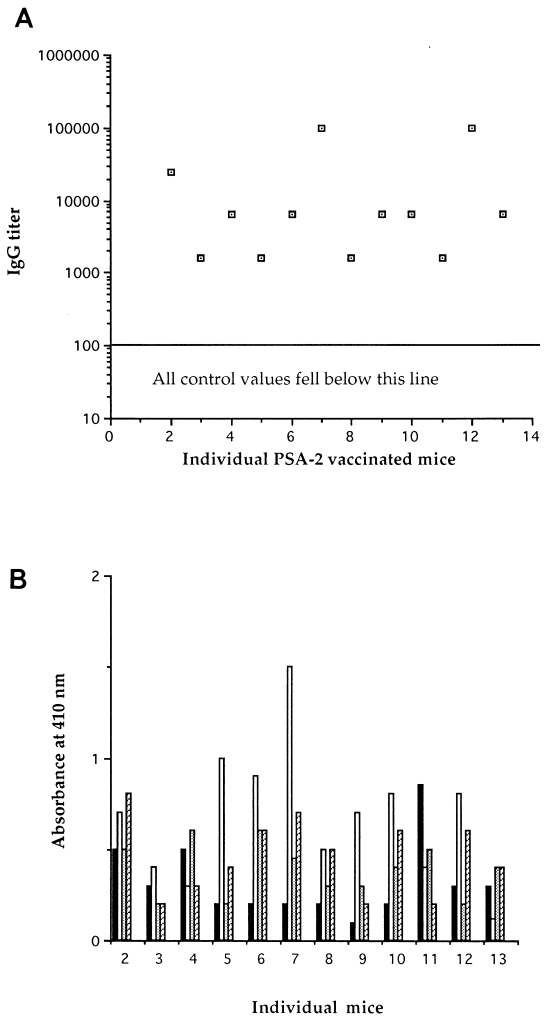

FIG. 3. PSA-2-specific antibody titers and isotype in PSA-2-vaccinated C3H/He mice. (A) Total IgG directed to PSA-2 in vaccinated mice; (B) isotype of the antibodies to PSA-2 in the same sera, namely, IgM (■), IgG1 (□), IgG2a (▤), IgG2b (▨), and IgG3 (▤).

vaccinated mice at the peak of the infection was particularly striking, suggesting that the TR1 gene product has the potential to protect mice provided that it is expressed in a native form.

Immune responses in the vaccinated C3H/He mice. All of the vaccinated mice described above displayed high titers of PSA-2-specific IgG antibodies before challenge with live promastigotes (Fig. 3A). The predominant isotype was IgG1, but significant amounts of IgG2a and IgG2b were also detected (Fig. 3B). There was very little IgG3 directed against PSA-2 in the vaccinated mice. As expected, there was no antibody to PSA-2 in the control mice.

$CD4^+$ T cells purified from the spleens of PSA-2-immunized mice were restimulated in vitro with purified PSA-2 with irradiated syngeneic spleen cells as a source of antigen-presenting cells. After 48 h, these cells were found to produce substantial amounts of IFN-γ, moderate amounts of IL-3, and no detectable IL-4 (Fig. 4). Control cultures without PSA-2 did not produce any detectable IFN-γ or IL-4 and only small amounts of IL-3. The observed response was PSA-2 specific since $CD4^+$ T cells from mice injected with *C. parvum* alone did not produce any detectable lymphokines in the presence or absence of

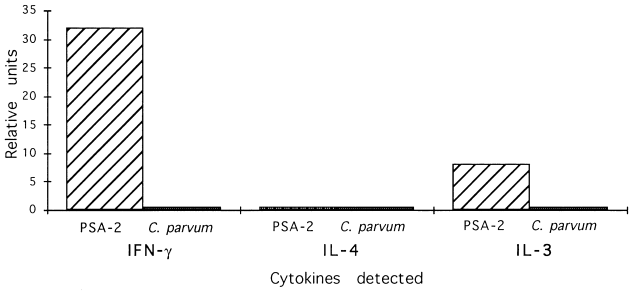

FIG. 4. Profile of cytokine secretion by spleen $CD4^+$ T cells restimulated in vitro with PSA-2. The results are expressed as the dilution of the supernatants at which a half-maximal effect on the reporter cell line was observed. The specificity of the cytokine activity was validated with neutralizing monoclonal antibodies, and the maximal effect was determined with recombinant cytokines.

PSA-2. The lack of detectable IL-4 in the bulk culture may not reflect the real response in vivo (8). To obviate the possibility of an artifact in bulk cultures, we performed limiting dilution analysis of the PSA-2 response.

The limiting dilution analysis supported and extended these results by showing that the precursor frequency of PSA-2-specific $CD4^+$ T cells secreting IFN-γ was much higher in the spleens of immunized mice than in those of controls (Fig. 5a). The precursor frequency of IL-3-secreting cells was higher in the vaccinated mice than in the *C. parvum* controls (Fig. 5b). However, unlike the situation for IFN-γ and IL-4, the background secretion of IL-3 in the absence of antigen was also higher, obscuring the difference between the immunized and the control mice (data not shown). The precursor frequency of IL-4-secreting $CD4^+$ T cells was very low and similar in PSA-2- and *C. parvum*-injected animals (Fig. 5c).

PSA-2-specific T helper cell line. To confirm the mechanism of protection in the vaccinated mice, a PSA-2-specific T helper cell line was established from a vaccinated C3H/He mouse and used to transfer protection to naive mice. The T-cell line secreted IFN-γ and IL-3 but no IL-4 when stimulated with PSA-2 in the presence of syngeneic antigen-presenting cells in vitro, suggesting a TH1 phenotype (data not shown). The T-cell line was established in the absence of additional cytokines to the cultures and was therefore expected to survive for some time after transfer in vivo (5). Naive mice were injected intravenously with 5×10^4 T cells and immediately challenged intradermally with 10^5 live promastigotes. The parasite burden in the draining lymph nodes of these passively vaccinated mice was substantially reduced compared with that in infected controls (Fig. 6). As expected, the differences between the two groups became smaller at later time points, when the mice started to develop their own immune response to the parasites.

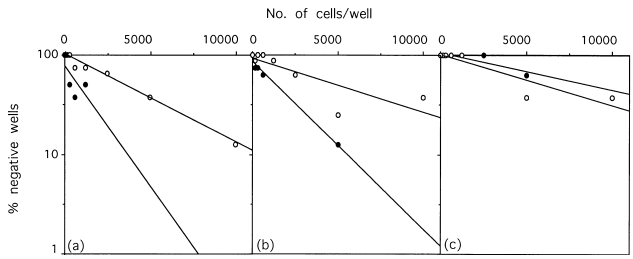

FIG. 5. Precursor frequency of $CD4^+$ T cells secreting IFN-γ (a), IL-3 (b), and IL-4 (c) in response to PSA-2. $CD4^+$ spleen cells isolated from PSA-2-vaccinated animals (●) are compared with cells isolated from *C. parvum*-injected controls (○).

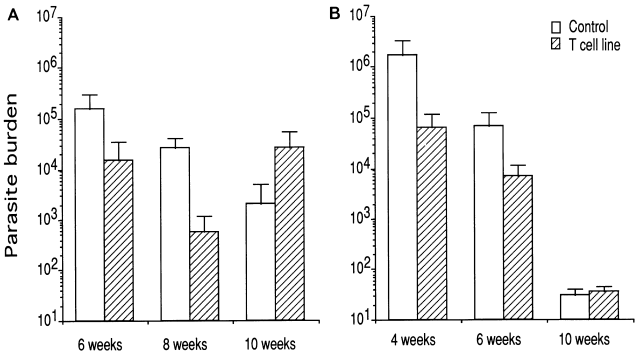

FIG. 6. In vivo effect of a PSA-2-specific T-cell line on the parasite burden in the draining lymph nodes in two independent experiments (A and B). Naive mice were injected intravenously with 5×10^4 PSA-2-specific T helper cells. Simultaneously, these mice and a control group were infected at the base of the tail with 10^5 live promastigotes. The parasite burden was monitored at several time points after infection. The mean parasite burden per mouse and the standard deviation are shown. At least two mice per group per time point were used.

The lesion score was monitored weekly, and no significant difference was found between the groups (data not shown), suggesting that the intravenously injected T cells did not home in on the skin lesions.

DISCUSSION

In the quest for a subunit vaccine against cutaneous leishmaniasis, significant progress has been made with two protein antigens, the major surface protease gp63, common to all *Leishmania* spp., and a surface antigen of unknown function, gp46/M2. First detected and characterized in *L. amazonensis*, the gp46 gene was later shown to be part of a family present in all species except *Leishmania braziliensis* (21). The molecular cloning of several genes of the *L. major* PSA-2 family of polypeptides made it clear that they belonged to the same family that gp46/M2 did (31). Since gp46/M2 was shown to protect mice from disease after infection with *L. amazonensis* (4), it was important to determine if the *L. major* homolog will also protect against *L. major* infection.

The three PSA-2 polypeptides expressed on the surface of *L. major* promastigotes were purified to homogeneity. Since the three polypeptides are very similar in sequence, they could not be separated from each other by immunoaffinity chromatography. These proteins were therefore pooled and used to vaccinate C3H/He and BALB/c *H-2*^{k} mice with *C. parvum* as an adjuvant. No lesions developed in the vaccinated C3H/He mice during the entire 12 weeks of observation after challenge infection, while all controls developed lesions after about 2 weeks. The lesions which developed in the vaccinated, susceptible BALB/c *H-2*^{k} mice were very small and cured rapidly, in contrast to those in the controls. Significant protection was also obtained in the highly susceptible BALB/c mice, although, as described previously, protection was dependent on the size of the challenge inoculum (4, 21). As expected from a number of studies (1, 24, 45), parasites were present and could be isolated from the lymph nodes of all mice, vaccinated ones as well as controls. However, the parasite burden in the vaccinated mice was several orders of magnitude lower compared with that in controls. The fact that so few parasites were present in the lymph nodes suggests that there is an active mechanism which prevents the parasites from expansion. In humans, the breakdown of this active immunological control of parasite growth has been suggested to account for the reactivation of leishmaniasis in immunosuppressed individuals (reviewed in references 20 and 34).

TR1, one of the estimated 14 PSA-2 genes from *L. major* (31), was cloned and expressed in both *E. coli* and *L. mexicana* promastigotes. While *L. mexicana*-derived PSA-2 could protect mice against disease, *E. coli*-produced PSA-2 was ineffective. This suggests that although TR1 is a protective antigen, the native form of the antigen is essential for its vaccinating potential. T cells from mice vaccinated with native PSA-2 did not recognize *E. coli*-derived TR1 and neither did the T-cell line described above, suggesting that processing and presentation of TR1 may be faulty.

Another conclusion stems from the fact that TR1 is a genomic clone which is not expressed in *L. major* or is expressed at very low levels (31). Its ability to vaccinate mice must be due to the antigenic similarity of the various members of the PSA-2 family and suggests that any PSA-2 polypeptide could be used as a vaccine molecule provided that it contains the relevant T-cell epitopes present in amastigotes. Considering that PSA-2 polypeptides are present in all *Leishmania* spp. except *L. braziliensis*, the expectation is that a pan-*Leishmania* vaccine should be feasible.

Although antibodies play no role in leishmaniasis, we examined the isotypes induced by vaccination with PSA-2 as additional markers for the phenotype of T-cell responses induced in these mice. Surprisingly, the serum of vaccinated mice contained antibodies specific for PSA-2 of both IgG2a and IgG1 isotypes, which are thought to be associated with TH1- and TH2-type immune responses, respectively (43). The presence of these antibodies correlates with an earlier observation indicating that an initial mixed TH1-TH2 response occurs early in infection but is followed subsequently by a TH1-dominated response in curing mice (27, 28). A similar mechanism may occur in vaccinated mice. Since antibodies are relatively long lived, TH2-associated isotypes may still be present in the PSA-2-vaccinated mice, long after a TH1-type response has been established.

A TH1-type immune response to PSA-2 in the vaccinated mice was shown both in bulk cultures and by limiting dilution analysis. The correlation between the induction of a TH1 type of immune response and protection in vaccinated mice is consistent with previous work performed with other promastigote vaccine antigens (30, 40, 41). A T-cell line generated from $CD4^+$ splenocytes derived from PSA-2-vaccinated mice was used to confirm the nature of the protective immune response in the PSA-2-vaccinated mice. Indeed, this T-cell line was of the TH1 phenotype. Transfer of 5×10^4 T cells to naive mice significantly lowered the parasite burdens detected in the draining lymph nodes after challenge infection. Interestingly, the number of T cells injected was important, since either too many or too few T cells were unable to provide protection (data not shown). Our results extend previous data showing that protection can be transferred by a specific TH1 cell line (39). This is the only example of a TH1 line derived from mice vaccinated with a molecularly defined antigen which induced protection in naive animals.

Vaccination with intact gp63 of *L. major* (47) and some, but not all, peptides derived from it (15, 47) resulted in the induction of a protective immune response to challenge infection. As in our case, the vaccinated mice mounted a TH1 type of immune response specific for the antigen. Similar observations have been made with gp46/M2, suggesting that TH1 responses not only are responsible for the cure from disease but also are the basis for protective vaccination with subunit vaccines. Therefore, it is essential to ensure that potential vaccine candidates elicit a TH1-type response. In this respect, it was some-

what surprising that recombinant PSA-2 produced in *E. coli* was not protective, but the same gene product derived from a transfected *L. mexicana* was able to confer protection. This lack of protection may be due to faulty antigen processing and presentation, since T cells from infected mice or mice vaccinated with native PSA-2, which recognized native PSA-2, did not recognize the recombinant protein in vitro (data not shown). Posttranslational modifications and/or protein folding may be responsible for the effect.

Because parasites were found in the lymph nodes of vaccinated mice, it is clear that the immune mechanism protecting these mice has to operate at the level of the infected cell. This is in line with data from human studies showing that protection against disease manifests itself in the development of a smaller lesion and faster healing rather than no lesion at all (38). Earlier studies with crude vaccines also suggested that the best vaccine preparation was derived from infected cells rather than promastigotes (23). One would therefore expect that PSA-2-specific T cells would be activated by amastigote PSA-2 peptides in association with class II major histocompatibility complex molecules on the surface of the antigen-presenting cell. Recent data from our laboratory indicate that amastigotes also express members of the PSA-2 family of polypeptides which are distinct from, but very similar to, the promastigote forms (13a).

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