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Strategic evaluation of vaccine candidate antigens for the prevention of Visceral Leishmaniasis

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ABSTRACT

Infection with *Leishmania* parasites results in a range of clinical manifestations and outcomes, the most severe of which is visceral leishmaniasis (VL). Vaccination will likely provide the most effective long-term control strategy, as the large number of vectors and potential infectious reservoirs renders sustained interruption of *Leishmania* parasite transmission extremely difficult. Selection of the best vaccine is complicated because, although several vaccine antigen candidates have been proposed, they have emerged following production in different platforms. To consolidate the information that has been generated into a single vaccine platform, we expressed seven candidates as recombinant proteins in *E. coli*. After verifying that each recombinant protein could be recognized by VL patients, we evaluated their protective efficacy against experimental *L. donovani* infection of mice. Administration in formulation with the Th1-potentiating adjuvant GLA-SE indicated that each antigen could elicit antigen-specific Th1 responses that were protective. Considering the ability to reduce parasite burden along with additional factors such as sequence identity across *Leishmania* species, we then generated a chimeric fusion protein comprising a combination of the 8E, p21 and SMT proteins. This *E. coli* –expressed fusion protein was also demonstrated to protect against *L. donovani* infection. These data indicate a novel recombinant vaccine antigen with the potential for use in VL control programs.

1. Introduction

Leishmaniases cause human suffering on a global scale. Due to their residency in endemic areas approximately 350 million people are at risk of infection with *Leishmania* parasites and therefore development of a form of leishmanaisis. Approximately, 12 million-diseased individuals are currently under management and an estimated 2 million additional new cases emerge each year. The geographic distribution of particular *Leishmania* species affects the type, and severity, of disease that manifests in each area. Visceral leishmaniasis (VL) is the most severe form and is fatal if left untreated. The largest VL burden is focused in the cross-border regions of Bangladesh, India and Nepal, where, as in east Africa, the disease is caused by infection with *Leishmania donovani*. VL cases in the Mediterranean, the Middle East, Latin America and some parts of Asia manifest from infection with *Leishmania infantum* [1]. *L. infantum* also infects canids and in addition to canine VL being a significant veterinary concern, infected dogs represent a major reservoir for potential transmission to humans in endemic regions [2].

To date, VL control strategies have predominantly centered on limiting the vectors and reservoirs or on improving the drug treatment regimen. Both strategies have limitations. Transient eradication of the sandfly vectors provides only temporary abatement in the transmission cycle and the culling of diseased dogs has had little impact on the longer term VL situation in *L. infantum*-endemic regions. For *L. donovani*, some animals have been indicated to be infected but humans are believed to be the dominant source of transmission. It is now clear that a large number of individuals can be asymptomatically infected and, although improved drug

Abbreviations: CL, cutaneous leishmaniasis; GLA-SE, glucopyranosyl lipid adjuvant in stable emulsion; H2B, histone H2B; MDH, malate dehydrogenase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SMT, sterol methyltransferase; TLR, Toll-like receptor; VL, visceral leishmaniasis.

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regimens are becoming available for the treatment of VL, their toxicity or expense has meant that these can only be administered to disease individuals and chemoprophylaxis through mass drug administration does not appear feasible [3–9]. Vaccination has the potential to provide not only long-term protection against infection/disease but could also impact infectious reservoirs to reduce transmission. Thus, widespread vaccination programs are the most likely way in which VL elimination can be achieved. An efficacious vaccine has, however, not yet been advanced to large-scale phase II/III trials.

The use of whole parasites and crude antigens with appropriate adjuvants has demonstrated the potential for vaccination to protect from leishmaniasis [10–13]. The incredible difficulty in standardizing and optimally formulating crude preparations to selectively induce appropriate immune responses, however, are likely major factors in the inconsistent results that have been obtained in clinical trials of such vaccines. Furthermore, current regulations and release criteria appear prohibitive for the widespread administration of crude antigen vaccines. A more feasible option for mass vaccination campaigns would appear to be the use of defined products, such as proteins produced by recombinant methods, used in conjunction with appropriate adjuvants. The recombinant nature of such vaccine antigens render them accessible to large scale, reproducible and cost-effective production. Several antigens have been proposed and evaluated as vaccine candidates for various forms of leishmaniasis [14]. Varying levels of protection in mouse models of *L. donovani* infection have been reported for several of these antigens, including KMP-11 (~45% reduction in parasite burden) [15], rORFF (45–80%) [16], A2 (89%) [17], and hemoglobin receptor (100%; sterility) [18].

The criteria by which we chose to initially select potential vaccine antigens include sequence conservation among *Leishmania* species but a lack of sequence identity with human genes, as well as practical considerations such as the ability to express by recombinant methods and to purify at high levels. Extending upon early promise observed with single antigens, we then typically incorporate two to four components into single chimeric fusion molecules for further evaluation and production. Incorporating multiple antigens has the potential to increase the number of individuals who can respond to the fusion protein, enhancing vaccine uptake and efficacy. To consolidate the data generated by many laboratories and across many vaccine platforms, we expressed multiple *Leishmania* genes via recombinant expression in *E. coli* to permit their evaluation in a defined subunit antigen plus adjuvant platform. Given that VL is the most severe presentation of leishmaniasis, and that *L. donovani* infection causes the majority of VL cases, we decided to use a mouse model of *L. donovani* infection as the evaluation system. We first verified that each recombinant protein retained protective efficacy when formulated with synthetic TLR4 ligand glucopyranosyl lipid adjuvant in stable emulsion (GLA-SE) [19]. As a step toward a multivalent vaccine antigen we then assessed the interaction of selected proteins within mixtures. Finally, we constructed a chimeric fusion protein incorporating the 8E, p21 and SMT proteins and evaluated if it could protect against infection with *L. donovani*. Our data identify a chimeric fusion protein that could potentially be used in targeted vaccination campaigns in *L. donovani*-endemic regions.

2. Materials and methods

2.1. Recombinant proteins

Recombinant proteins were cloned and expressed in *E. coli* as previously described [20]. The fusion proteins were constructed by aligning the individual gene sequences as a single product for cloning and recombinant expression in *E. coli*. Affinity-purified protein fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and quantified using the BCA protein assay (Pierce, Rockford, IL, USA). Endotoxin levels were measured by Limulus Amebocyte Lysate QCL-1000 assay (Lonza Inc., Basel, Switzerland) and were all <100 EU/mg protein.

2.2. Antigen-specific antibody responses of VL patients

Sera were obtained from residents of a *Leishmania*-endemic area of Bangladesh (VL patients) or the United States (non-endemic normal). Written informed consent for study participation was obtained from each participant prior to screening. Subjects were defined according to parameters set by the Kala-Azar Elimination Program as follows: VL, a person with clinical symptoms of VL (fever for more than 2 weeks duration and splenomegaly) and a positive rK39 rapid diagnostic test result. ELISA was conducted by coating high binding 384-well ELISA plates (Corning, MA, USA) overnight at 4 °C with 50 μl/well of recombinant protein at a concentration of 1 μg/ml diluted in carbonate buffer. The next day, plates were washed with 0.1% Tween-20 in PBS and 200 μl of blocking buffer (PBS + 1% BSA) added to each well for 2 h at room temperature. After blocking, plates are washed five times before serum samples diluted 1:400 dilution in 0.1% Tween-20 + 0.1% BSA in PBS was added at 50 μl/well and incubated for 30 min at room temperature. After incubation, plates were washed and 50 μl/well of peroxidase labeled-anti-human IgG (Life Technologies, CA, USA) in serum diluent was added. Plates were incubated for 30 min at room temperature, and then washed as previously described. To reveal reactions, 100 μl of TMB SureBlue Peroxidase Substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was added to each well for 15 min at room temperature, after which the reaction was stopped by adding 1 N H_2SO_4. Plates were read within 10 min of reaction stoppage at OD 450 nm, using 570 nm as the reference wavelength on a SpectraMax plate reader (Molecular Devices, CA, USA).

2.3. Mice and immunizations

Female C57BL/6 mice (purchased from Charles River Laboratories, Wilmington, MA, USA) were maintained in specific pathogen-free conditions and in accordance with animal procedures approved by the IDRI institutional animal care and use committee. Mice entered experiments at 6–8 weeks of age and were immunized by subcutaneous injection of recombinant protein formulated with adjuvant at the base of the tail. Vaccines were prepared to provide a total of 5 μg/dose protein and 5 μg/dose GLA-SE in a total volume of 0.1 ml. When multiple proteins were used simultaneously, they were mixed prior to use to provide molar equivalence of each within a total dose of 5 μg. Mice were injected a total of three times at three weeks intervals.

2.4. Analyses of mouse antibodies

Blood was collected from five mice per group, serum prepared and antigen-specific antibody responses were analyzed by ELISA for total IgG, as well as IgG2 and IgG1 isotypes. Briefly, ELISA plates (Nunc, Rochester, NY, USA) were coated with 1 μg/ml antigen in 0.1 M bicarbonate buffer and blocked with 0.1% BSA-PBS. Then, in consecutive order and following washes in PBS/Tween, serially diluted serum samples, anti-mouse IgG-HRP, anti-mouse IgG1-HRP or anti-mouse IgG2a-HRP (Southern Biotech, Birmingham, AL, USA) and ABTS-H_2O_2 (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) were added to the plates. Plates were analyzed at 405 nm (EL_X808, Bio-Tek Instruments Inc, Winooski, VT, USA). Endpoint