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Randomized, placebo-controlled, double-blind trial of the *Na*-ASP-2 Hookworm Vaccine in unexposed adults

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Summary *Necator americanus* Ancylostoma Secreted Protein-2 (*Na*-ASP-2) is a leading larval-stage hookworm vaccine candidate. Recombinant *Na*-ASP-2 was expressed in *Pichia pastoris* and formulated with Alhydrogel®. In a phase 1 trial, 36 healthy adults without history of hookworm infection were enrolled into 1 of 3 dose cohorts ($n=12$ per cohort) and randomized to receive intramuscular injections of either *Na*-ASP-2 or saline placebo. Nine participants in the first, second and third cohorts were assigned to receive 10, 50 and 100 μg of *Na*-ASP-2, respectively, on study days 0, 56 and 112, while 3 participants in each cohort received placebo. The most frequent adverse events were mild-to-moderate injection site reactions; in 8 participants these were delayed and occurred up to 10 days after immunization. No serious adverse events occurred. Anti-*Na*-ASP-2 IgG endpoint titers as determined by ELISA increased from baseline in all vaccine groups and peaked 14 days after the third injection, with geometric mean titers of 1:7066, 1:7611 and 1:11,593 for the 10, 50 and 100 μg doses, respectively, compared to <1:100 for saline controls ($p<0.001$). Antibody titers remained significantly elevated in all vaccine groups until the end of the study, approximately 8 months after the third vaccination. *In vitro* stimulation of PBMCs collected from participants with *Na*-ASP-2 resulted in robust proliferative responses in those who received vaccine,

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which increased with successive immunizations and remained high in the 50 and 100 μg dose groups through the end of the study. This first trial of a human hookworm vaccine demonstrates that the Na-ASP-2 vaccine is well-tolerated and induces a prolonged immune response in adults not exposed to hookworm, justifying further testing of this vaccine in an endemic area.
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An estimated 740 million people are infected with the hookworms *Necator americanus* and *Ancylostoma duodenale*, mostly in rural areas of the tropics [1]. Although mortality due to infection is rare, the global burden of hookworm disease is high, with an estimated annual loss of 22 million disability-adjusted life years [2]. Hookworm infection is primarily acquired after skin contact with infective larvae (L3) in fecally contaminated soil. Following penetration of the host's skin, larvae enter the vasculature and pass through the heart to the lungs, where they become trapped in pulmonary capillaries. After migrating into the alveolae the larvae ascend the bronchial tree and are swallowed into the gastrointestinal tract where they develop into adult worms. As adult worms, they attach to intestinal microvilli, mate, and feed on host blood and mucosa [3,4]. Chronic infection with hookworm, often lasting between 5 and 7 years, results in long-term pathological consequences to the host. The intestinal blood loss caused by hookworm infection over years can result in iron-deficiency anemia, which is the major clinical manifestation of infection [3]. In children and adults living in resource-poor countries, where iron stores are often lower than those in developed countries due to dietary deficiencies, there is a well-established relationship between the intensity of hookworm infection, intestinal blood loss, and anemia [5–7].

While treatment with the benzimidazole class of anthelmintic drugs is highly effective against established hookworm infection [8], sustained chemotherapy programs have proven difficult to implement, especially in developing countries where there is often rapid reversion (e.g., within 12 months) to high levels of transmission after mass treatment. Therefore, development of a vaccine against hookworm has become a public health priority in the tropical and subtropical regions of the world [9]. Since it is by far the most prevalent hookworm worldwide, *N. americanus* is the primary target of hookworm vaccine development.

The development of a human hookworm vaccine is considered feasible due to past production of a safe and efficacious vaccine against the canine hookworm (*Ancylostoma caninum*), which was commercially available in the 1970s [10]. Since the canine vaccine consisted of irradiated L3, development of a hookworm vaccine for humans has focused on identifying antigens produced by the invading L3 and expressing them as recombinant proteins. As such, *Ancylostoma* Secreted Protein-2 from *N. americanus* (Na-ASP-2) was selected as the lead human hookworm vaccine candidate. Na-ASP-2 is a 21.3 kDa excretory/secretory (ES) product and is the most abundant ES product released by *N. americanus* L3 upon entry into the host [11,12]. Encouraging preclinical results showed that vaccination of canines and hamsters with recombinant ASP-2 reduced adult worm burden, fecundity (as measured by fecal egg counts), and *in vitro* migration of larvae through tissue [13–15]. Studies of populations living in hookworm-endemic areas also showed that anti-ASP-2 antibodies were associated with a reduced

risk of acquiring heavy hookworm infection in areas of high transmission [14]. Based on this evidence, recombinant Na-ASP-2 was manufactured and tested in a phase 1 trial in healthy, hookworm-naïve adults, the results of which are reported herein.

Materials and methods

Vaccine preparation

The Na-asp-2 gene was cloned from an L3 cDNA library, amplified by polymerase chain reaction (PCR) using specific Na-asp-2 primers, and then transferred into the expression vector pPICZaA. A positive pPICZaA-Na-asp-2 clone was further characterized and transformed into *Pichia pastoris* for expression [16]. Clinical lots of vaccine were produced at the Walter Reed Army Institute of Research Pilot Bioproduction Facility (Silver Spring, Maryland, USA) according to current Good Manufacturing Practice. The 21.3 kDa recombinant protein was expressed, purified using a series of chromatography steps followed by diafiltration in phosphate-buffered saline (PBS), and then adsorbed to Alhydrogel® (Biosector, Denmark).

The Na-ASP-2 Hookworm Vaccine was supplied as an off-white suspension in vials containing 500 μg of Na-ASP-2 and 3000 μg of Alhydrogel® in 1.0 mL PBS, without stabilizers or preservatives. Potency studies of vaccine stored at 2–8 °C were conducted in mice every 6 months and confirmed that the lot used in the trial was stable and fully potent throughout the course of the study.

Prior to injection, the vaccine was diluted under aseptic conditions with Alhydrogel® diluent (3000 $\mu\text{g}/\text{mL}$) to reach the appropriate concentration of Na-ASP-2 while maintaining a constant concentration of Alhydrogel® (3000 $\mu\text{g}/\text{mL}$) such that each 0.5 mL dose of vaccine contained 1500 μg Alhydrogel®. The placebo was sterile, preservative-free saline solution (0.9% NaCl, Abbott Laboratories, Abbott Park, Illinois, USA). Individual doses of vaccine or placebo were supplied to the clinic in syringes that were masked with a label to obscure the contents and ensure that study personnel administering the vaccine remained blinded.

Study design

A randomized, double-blind, placebo-controlled dose-escalating phase 1 clinical trial in healthy adult volunteers was conducted to evaluate the safety, reactogenicity, and immunogenicity of the Na-ASP-2 vaccine formulated on Alhydrogel®. This study was performed under an Investigational New Drug application (BB-IND-12166) to the U.S. Food and Drug Administration and was conducted at the George Washington University Medical Center. The protocol, amendments to the protocol, informed consent

form, advertisements, and other study-related documents were approved by the Western Institutional Review Board (Olympia, Washington, USA).

Volunteers

Thirty-six healthy volunteers between the ages of 18 and 45 years, inclusive, were recruited from the metropolitan Washington area. Written informed consent was obtained from all volunteers. Individuals were excluded if they had any of the following: evidence of clinically significant systemic disease; were pregnant or breast feeding; had serological evidence of HIV, chronic hepatitis B, or hepatitis C infection; current medication with corticosteroids or immunosuppressive drugs; immunization with a killed vaccine within the previous 2 weeks or a live vaccine within the previous 4 weeks; or, past or current hookworm infection. Three cohorts of 12 volunteers each were enrolled successively, with 9 participants in each cohort randomized to receive the *Na*-ASP-2 vaccine and 3 to receive saline placebo. In the first, second, and third cohort, those randomized to receive *Na*-ASP-2 were administered 10, 50 and 100 μ g, respectively; each dose contained 1500 μ g Alhydrogel®. Participants received a 0.5 mL injection of either vaccine or placebo intramuscularly in the deltoid on study days 1, 56 and 112, with successive vaccinations being administered in alternating arms.

All volunteers in a lower dose cohort had to have completed up to study day 14 prior to initial vaccination of the next dose cohort. Escalation to the next higher dose required approval by an independent safety monitoring committee. All females had a serum or urine β human chorionic gonadotrophin test at screening and immediately prior to each vaccination.

Assessment of safety and tolerability

Following each vaccination, volunteers were observed for between 15 and 60 min and then evaluated 3 and 14 days after vaccination for evidence of local and systemic reactivity. Participants were also evaluated in the study clinic 2 and 8 months after the final injection. Local adverse events that were assessed included erythema, swelling, warmth, pruritis and pain at the site of injection. Solicited systemic adverse events included fever (oral temperature $\geq 37.5^{\circ}\text{C}$), headache, nausea, myalgia, and arthralgia. Volunteers recorded local and systemic reactivity (including oral temperature) daily on diary cards for 14 days following each vaccination. An abbreviated history and physical examination was performed at each follow-up visit. All abnormal signs and symptoms were considered adverse events and were graded for severity and assigned causality relative to vaccination. Severity was graded as either mild (easily tolerated), moderate (interfered with activities of daily living), or severe (prevented activities of daily living). Erythema or swelling at the injection site was graded as follows: mild (>0 to ≤ 5 cm in diameter), moderate (>5 to ≤ 15 cm), or severe (>15 cm). A complete blood count and white blood cell differential, as well as serum creatinine, blood urea nitrogen, electrolytes (potassium, chloride, sodium and calcium), total protein,

albumin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, bilirubin, glucose, creatine phosphokinase (CPK), prothrombin time, partial thromboplastin time, and urinalysis were performed at screening and 14 days following each vaccination. An electrocardiogram was performed at screening and 2 weeks following the third injection.

Anti-*Na*-ASP-2 antibody responses

Measurement of anti-*Na*-ASP-2 IgG, IgM, and IgA were performed according to an indirect ELISA method. Briefly, 96-well microplates (PolySorp™, Nalge, Nunc Intl., Rochester, NY, USA) were coated with 100 ng/well of *Na*-ASP-2 protein overnight at 4°C in PBS (pH 7.2), washed 3 times with PBS (pH 7.2) and then blocked with 10% bovine serum albumin (BSA) diluted in PBS (pH 7.2) with 0.05% Tween 20 (PBST) for 2 h at 37°C . Sera were diluted in 5% BSA with PBST and added at a starting dilution of 1:100 followed by twofold serial dilutions and incubated overnight at 4°C . Wells were then washed 10 times (400 μ L/well) with PBST and peroxidase-conjugated anti-human IgG (KPL Laboratories Inc., Maryland, USA) was added at a dilution of 1:2500 for 1 h at room temperature (RT) in the dark. After incubation, plates were washed as before, 100 μ L of the chromogen *ortho*-phenylenediamine (Sigma–Aldrich Co., St. Louis, MO, USA) with 3% hydrogen peroxide (Sigma–Aldrich Co., St. Louis, MO, USA) were added to each well, and the plates were incubated in the dark for 30 min at RT. Fifty microliters of H_2SO_4 were added to each well to stop the reaction and the optical density at 492 nm (OD_{492}) was read using an automated microplate reader (SpectraMax 340 PC reader, Molecular Devices, Sunnyvale, CA, USA) with SOFTmax Pro software (Molecular Devices, USA). A pool of sera from 25 Brazilian hookworm-infected volunteers with high levels of IgG antibodies against *Na*-ASP-2 ($\text{OD}_{492} \geq 1.000$) was used as a positive control on each plate. Also used on each plate was a negative control consisting of a pool of sera from 5 healthy hookworm-uninfected volunteers living in the US, all of whom had an OD_{492} of ≤ 0.100 when tested in the assay. Endpoint titers were assigned by taking the lowest dilution with an OD that was higher than the negative control plus 0.100.

Antigen-specific IgG subclasses (IgG1, IgG2, IgG3 and IgG4) were also determined by indirect ELISA using the same conditions as described above. The following dilutions of horseradish peroxidase-conjugated anti-human antibodies (Zymed Laboratories, California, USA) were added to each well: 1:5000 of anti-IgG1, and 1:1000 of anti-IgG2, -IgG3 and -IgG4. Plates were washed and developed for 30 min with *ortho*-phenylenediamine (Sigma–Aldrich Co., St. Louis, MO, USA) containing 3% hydrogen peroxide.

Measurement of anti-*Na*-ASP-2 IgE was also performed according to a modification of the indirect ELISA method described above. Briefly, 96-well microplates (MaxiSorp™, Nalge Nunc Intl., Rochester, NY, USA) were coated with *Na*-ASP-2 and blocked using the same procedure as described above. Sera were diluted in 3% BSA with PBST, added at a dilution of 1:50, and incubated overnight at 4°C . After washing with PBST, biotin-conjugated anti-human IgE (KPL

Laboratories Inc., Maryland, USA) was added at a dilution of 1:1000 for 1 h at RT in the dark. After incubation, plates were washed as before, 100 μ L of streptavidin horseradish peroxidase diluted 1:1000 in 3% BSA with PBST was added to each well and plates were incubated for 1 h at RT in the dark. After incubation, plates were washed, developed, and read as above. A pool of sera from 20 Brazilian hookworm-infected volunteers with high levels of IgE antibodies against Na-ASP-2 ($OD_{492} \geq 1.000$) was used as a positive control on each plate. Each plate also had a negative control consisting of a pool of sera from 5 healthy hookworm-uninfected volunteers living in the US, all of whom had an OD_{492} of ≤ 0.100 when tested in the assay. For measurement of IgE, pre-adsorption to remove the other isotypes was not performed because initial studies showed that this did not affect the detection of IgE in this ELISA (data not shown). Endpoint titers were calculated as described above.

Hookworm antigen preparation

Larval (L3) extracts from *N. americanus* were prepared by maceration of L3 recovered from pre-treatment fecal cultures of infected Brazilian volunteers. L3 were homogenized and sonicated in PBS to generate soluble protein extract. Since L3 extracts were obtained from human coproculture, a cleaning procedure was performed to prevent non-specific recognition of products such as human light chains or albumin in the immunoassays. Briefly, 100 μ L of protein A-agarose beads (Invitrogen, Grand Island, NY, USA) were washed with 0.5% PBST and rotated overnight at 4°C with 100 μ L of neat human sera pooled from 9 adult non-vaccinated healthy volunteers. After 3 washes with PBST, 400 μ L of human-derived *N. americanus* L3 extract were rotated overnight at 4°C. Beads were then precipitated by centrifugation and the supernatant containing clean L3 extract (CLE) was collected for use in the Western blot and immunoprecipitation assays.

Western blot assay

Western blotting was performed using recombinant Na-ASP-2 or CLE. Briefly, antigen preparations were diluted in reducing SDS-PAGE loading dye (Invitrogen, Grand Island, NY, USA), mixed and boiled for 5 min. After centrifugation, 15 μ L of supernatant containing approximately 2 μ g Na-ASP-2 or approximately 5 μ g CLE were applied on a 4–20% gradient SDS-PAGE gel (Invitrogen, Grand Island, NY, USA) and electrotransferred onto a nitrocellulose membrane (Schleicher & Schuell BioScience Inc., Keene, NH, USA). Blots were blocked with 3% BSA diluted in PBS for 1.5 h and then probed with serum samples from study participants and controls (positive and negative pools) at a dilution of 1:5000. Horseradish peroxidase-conjugated anti-human IgG (KPL Laboratories, Maryland, USA) was used as a secondary antibody at a dilution of 1:5000. Visualization of bands was obtained by incubating blots with ECL reagent (Amersham Biosciences, Buckinghamshire, England) for 1 min according to the manufacturer's instructions.

Immunoprecipitation

Sera from vaccinated study participants (1:100 dilutions) were rotated overnight at 4°C with 1 μ g of CLE in 500 μ L PBS for formation of immunocomplexes. Protein A-agarose beads (100 μ L, 50% slurry; Invitrogen, Grand Island, NY, USA) were added to remove antibody-antigen complexes, which were recovered after 3 washes followed by centrifugation at 14,000 rpm. Supernatants were retained as unbound sample. Protein A-agarose with bound antibody-antigen complex was re-suspended in 50 μ L reducing SDS-PAGE loading dye, mixed and boiled for 5 min. Beads were pelleted and the supernatants collected as bound fraction. Fifteen microliters of each sample were run on a 4–20% gradient SDS-PAGE gel (Invitrogen, Grand Island, NY, USA) and electrotransferred onto a nitrocellulose membrane (Schleicher & Schuell BioScience Inc., Keene, NH, USA). After blocking with 3% BSA in PBS, blots were incubated with rabbit polyclonal anti-Na-ASP-2 sera (1:10,000 in 3% BSA/PBST). Goat anti-rabbit horseradish peroxidase-conjugated IgG (1:10,000 in 3% BSA/PBST) (Sigma, St. Louis, MO, USA) was used as a secondary antibody at a dilution 1:5000. Development was performed with ECL reagent (Amersham Biosciences, Buckinghamshire, England) according to the manufacturer's instructions.

Lymphoproliferation assay

Lymphoproliferation assays were performed using peripheral mononuclear blood cells (PBMCs) obtained after separation from heparinized blood using a Ficoll-Hypaque density gradient (Histopaque®, Sigma, St. Louis, MO, USA). PBMCs were purified from blood and washed twice in RPMI 1640 medium (GIBCO, Grand Island, NY, USA), counted and re-suspended in RPMI 1640 supplemented with 3% Antibiotic/Antimycotic solution (GIBCO, Grand Island, NY, USA) and 10% heat inactivated normal human serum (Sigma-Aldrich Co., St. Louis, MO, USA).

Cells were incubated in 96-well flat-bottomed tissue culture plates (Corning, Acton, Massachusetts, USA) in the presence of the mitogen phytohemagglutinin (PHA; Sigma-Aldrich Co., St. Louis, MO, USA) at a concentration of 2.5 μ g/well or crude extract of *Candida albicans* (Greer Laboratories, Lenoir, North Carolina, USA) at a concentration of 3.125 μ g/well to assess cell culture viability. Specific proliferative responses were evaluated by incubation of PBMCs (250,000 per well) with Na-ASP-2 at 3.125 μ g/well. Incubation was carried out in a humidified 5% CO₂ incubator at 37°C for 2 days for PHA-stimulated cultures and 5 days for antigen-stimulated cultures. Cells were pulsed for the last 6 h of incubation with 1 μ Ci of [³H] methyl thymidine (PerkinElmer LAS, Shelton, CT, USA) and harvested onto glass fiber filters (Printed Filtermat A, Wallac, Finland). Radioactive incorporation was determined by liquid scintillation spectrometry (MicroBeta JET, PerkinElmer Inc., USA). Proliferation responses were expressed by the stimulation index (SI = mean proliferation of stimulated cultures/mean proliferation of unstimulated cultures).

Statistical analysis

Differences in the proportion of individuals experiencing each adverse event (of any severity) between randomization status (placebo vs. 1 of the 3 dose concentrations of *Na*-ASP-2) and between vaccinations (first vs. second, etc.) within each dose group were not analyzed systematically due to the small numbers of participants in each group.

For the primary immunogenicity outcome measure, the number of participants with a detectable anti-*Na*-ASP-2 antibody titer (i.e., a titer ≥ 100) was tabulated. Statistical comparison of antibody titers between groups was made using the analysis of variance (ANOVA) test on geometric mean titers. For calculation of geometric mean titers, those below the limit of detection of the assay (i.e., <100) were arbitrarily assigned a value of 50. Differences in lymphocyte proliferation SIs were compared between groups using the Kruskal–Wallis test. All statistical tests were two-sided with a 5% Type I error rate, and 95% confidence intervals were calculated. No formal adjustment was made for multiple comparisons. The SAS (version 9.1; SAS) software package was used for all analyses.

Results

Study population

Forty-one volunteers were screened of which 36 were considered eligible and were enrolled and randomized to receive either the *Na*-ASP-2 Hookworm Vaccine or saline placebo. Reasons for exclusion included: abnormal screening laboratory tests ($n=2$), withdrawal of consent prior to randomization ($n=1$), abnormal screening physical exam ($n=1$) and significant history of asthma ($n=1$). Study participants ranged in age from 18 to 33 years and were mostly white. All randomized participants received the first scheduled injection. Four were lost to follow-up after receiving the first injection and did not receive any further injections. The third injection was not administered to 2 participants due to the occurrence of adverse events after the second injection (1 who received 10 μ g *Na*-ASP-2 and 1 who received 100 μ g *Na*-ASP-2; see below).

Safety

All vaccinations were well-tolerated. Injection site pain, swelling, erythema and pruritus were more common in those who received the *Na*-ASP-2 vaccine compared to those who received placebo, although there were no apparent differences between the *Na*-ASP-2 dose groups (i.e., no dose response) or between successive vaccinations within each dose group (Table 1). All reported local injection site and solicited systemic adverse events were graded as either mild or moderate in severity. Late-onset injection site erythema (diameter ranging from 1 to 12 cm) was observed after the second vaccination in 3 participants who received 10 μ g *Na*-ASP-2, in 3 participants who received 50 μ g *Na*-ASP-2, and in 2 participants who received 100 μ g *Na*-ASP-2. These reactions began between 5 and 10 days after vaccination but were short-lived, lasting for less than 5 days in all partici-

Table 1 Solicited local injection site and systemic adverse events after vaccination with the *Na*-ASP-2 Hookworm Vaccine or saline placebo

	Vaccination #1			Vaccination #2			Vaccination #3		
	<i>Na</i> -ASP-2			<i>Na</i> -ASP-2			<i>Na</i> -ASP-2		
	10 μ g ($n=9$)	50 μ g ($n=9$)	100 μ g ($n=9$)	10 μ g ($n=8$)	50 μ g ($n=8$)	100 μ g ($n=8$)	10 μ g ($n=7$)	50 μ g ($n=8$)	100 μ g ($n=7$)
Local ^a									
Pain	8	7	6	4	7	6	2	7	5
Swelling	3	1	0	4	3	1	1	3	2
Erythema	1	1	0	4	3	2	2	3	2
Pruritus	1	0	0	2	3	1	1	3	3
Systemic									
Fever	0	0	0	1	0	0	2	0	0
Headache	0	1	0	1	0	3	0	1	2
Nausea	0	1	1	1	1	0	0	1	1
Myalgia	0	0	0	0	0	0	0	0	0
Arthralgia	0	0	1	0	0	0	0	0	0

^a Data are number of study participants. Reactogenicity is listed by vaccine allocation and vaccination number. All reactions were mild or moderate in intensity.

pants. Erythema was accompanied by injection site swelling, pain, pruritus, and in one case (10 μ g dose group), by fever (38.2 °C). All reactions resolved spontaneously, and in all participants except 1 from the 10 μ g dose group who was not re-vaccinated (see below), this reaction did not recur after administration of the third vaccination.

Solicited systemic reactions were uncommon, and consisted primarily of mild headache, fever and nausea (Table 1). No differences between *Na*-ASP-2 and placebo or between successive vaccinations within dose groups were seen for these events, and there was no obvious dose response among the groups vaccinated with *Na*-ASP-2. Similarly, there was only 1 abnormal laboratory adverse event that was considered possibly related to receipt of the *Na*-ASP-2 vaccine, due to its occurrence 2 weeks following the first dose of vaccine. This was a case of a mild elevation in blood glucose (120 mg/dL) in a participant in the 50 μ g dose group that was asymptomatic, resolved spontaneously, and did not recur following subsequent vaccinations in the same individual. No serious adverse events occurred during the study.

Two participants were suspended from receiving the third vaccination due to the occurrence of adverse events. The participant in the 10 μ g dose group who had moderate delayed-onset injection site erythema that started 10 days after the second injection was not given the third injection. One male participant who received 100 μ g *Na*-ASP-2 had repeatedly positive tests for microscopic hematuria by both dipstick and microscopy and did not receive the third injection; hematuria had not been present at screening. Although the episodic hematuria was on one occasion graded as moderate (20–40 red blood cells per high-power field on microscopy) and was initially considered to be possibly related to vaccination, it was asymptomatic, all serum creatinine and blood urea nitrogen levels were normal, and subsequent consultation with a nephrologist suggested that it was likely exercise-induced in etiology.

Anti-*Na*-ASP-2 IgG responses

Immunization with *Na*-ASP-2 elicited significant antigen-specific IgG responses compared to placebo (Fig. 1). Although significant anti-*Na*-ASP-2 IgG titers were not observed after the first injection in any of the *Na*-ASP-2 groups, an increase in antibody responses was seen in each *Na*-ASP-2 dose group beginning 2 weeks following the second vaccination and which was still increasing by the time of the third vaccination 2 months after the second. Antibody levels peaked 2 weeks following the third injection, with geometric mean titers of 1:7066, 1:7611 and 1:11,593 in the 10, 50 and 100 μ g dose groups, respectively, compared to 1:86 in the placebo group. Although no dose response was seen between the 3 *Na*-ASP-2 dose groups at this time point, all had IgG levels that were significantly higher than placebo ($p < 0.001$; overall ANOVA).

Geometric mean titers decreased from the peak observed after the third injection, but were still significantly elevated compared to placebo at the end of the study, with levels of 1:1600, 1:4935 and 1:4307 in the 10, 50 and 100 μ g dose groups, respectively, on day 336 (7.5 months after the third injection), compared to 1:71 in the placebo group. West-

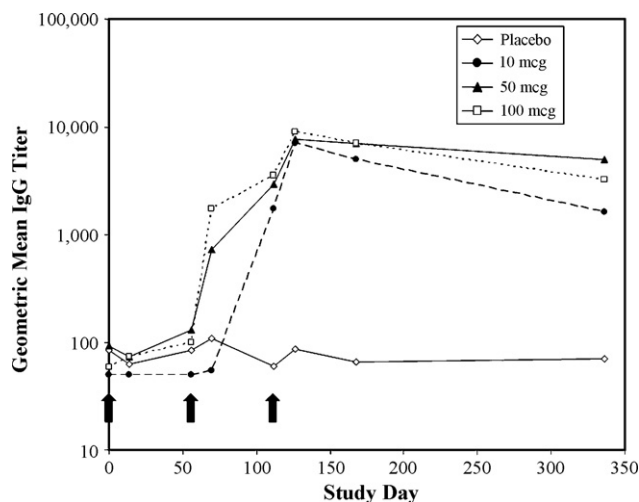


Figure 1 Geometric mean anti-*Na*-ASP-2 IgG antibody responses in volunteers vaccinated with the *Na*-ASP-2 Hookworm Vaccine, as determined by ELISA. Arrows indicate vaccination time points.

ern blots confirmed the specificity of the anti-*Na*-ASP-2 IgG detected by ELISA.

Anti-*Na*-ASP-2 isotype and IgG subclass responses

Anti-*Na*-ASP-2 IgM, IgE, IgA isotypes and IgG subclasses (IgG1, IgG2, IgG3 and IgG4) were measured from serum samples at baseline, 14 days after each injection, and 56 days after the third injection. Of the IgG subclasses, only antigen-specific IgG1 and IgG4 were detectable. No detectable IgG1 or IgG4 was observed in the sera of those receiving placebo at any time point (data not shown). Anti-*Na*-ASP-2 IgG1 and IgG4 were detected after the second and/or third injections in all dose groups. No IgM or IgA was detected against *Na*-ASP-2 at any time point. However, antigen-specific IgE antibodies were detected throughout the trial and seemed to increase following the third vaccination in all *Na*-ASP-2 groups (Fig. 2), although at no point were levels significantly different from baseline in any group.

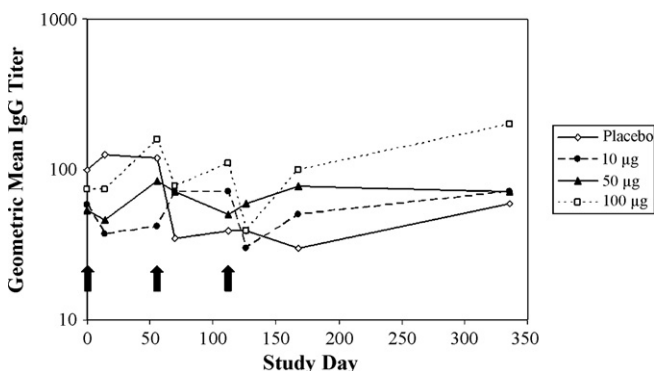


Figure 2 Geometric mean anti-*Na*-ASP-2 IgE antibody responses in volunteers vaccinated with the *Na*-ASP-2 Hookworm Vaccine, as determined by ELISA. Arrows indicate vaccination time points.

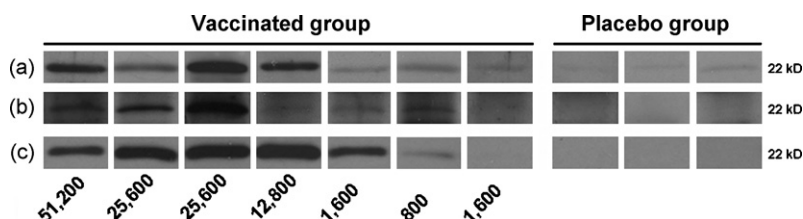


Figure 3 Western blot against recombinant *Na*-ASP-2 (a) and against L3 extracts of *Necator americanus* (b) were performed using day 126 (i.e., 2 weeks post-immunization #3) sera from 7 volunteers vaccinated with 100 μ g of the *Na*-ASP-2 Hookworm Vaccine, and three injected with saline placebo. Immunoprecipitation using sera from the same groups was performed with L3 extract (c) and developed with polyclonal rabbit sera against *Na*-ASP-2. Reciprocal values of IgG endpoint ELISA antibody titers against recombinant *Na*-ASP-2 are indicated for each individual. Sera from placebo group had no detectable titers.

Western blots and immunoprecipitation (*Na*-ASP-2 and *Na*-L3)

Fig. 3a and b shows that antibodies induced by the *Na*-ASP-2 vaccine recognized both recombinant *Na*-ASP-2 and the native protein in *N. americanus* L3 extract, respectively, in Western blots. In addition, immunoprecipitation was performed with sera from participants who were vaccinated with either the *Na*-ASP-2 vaccine or with saline placebo; only sera from those who received the *Na*-ASP-2 vaccine recognized the native structure of the natural protein in crude *N. americanus* L3 antigen extracts (Fig. 3c). Furthermore, the 22 kDa band corresponding to *Na*-ASP-2 was absent in unbound fractions from L3 extracts incubated with sera from individuals vaccinated with *Na*-ASP-2 but not from those receiving placebo (data not shown).

Lymphocyte proliferative response after vaccination with *Na*-ASP-2

As shown in Fig. 4, proliferative responses of PBMCs to *Na*-ASP-2 were observed in all 3 groups receiving the vaccine starting 2 weeks after the first injection. Vaccination with *Na*-ASP-2 resulted in significant cell proliferation, with SI values as high as 575 observed, which is comparable to what is observed with the mitogen PHA. Statistically significant differences were observed between the SI values obtained from vaccinated and placebo groups after each injection and at the end of the study ($p < 0.01$ for all time points). The proliferative responses increased after each subsequent dose of the vaccine and remained high in all 3 vaccine groups until the end of the study (day 336). Proliferative responses to *Na*-ASP-2 in those receiving placebo were negligible ($SI \leq 3.6$) throughout the study. Participants had robust responses to PHA at all time points (SIs ranged from 2.5 to 1215.3) as well as to *C. albicans* crude antigen (SIs ranging from 0.6 to 288.4), indicating that all cell cultures were viable.

Discussion

The major approach to hookworm control currently relies on administration of benzimidazole anthelmintic drugs [8]. However, rapid re-infection after treatment [17], the diminishing efficacy of benzimidazoles with repeated use [18], and a growing concern that the drug resistance that has been seen in veterinary medicine may also develop in

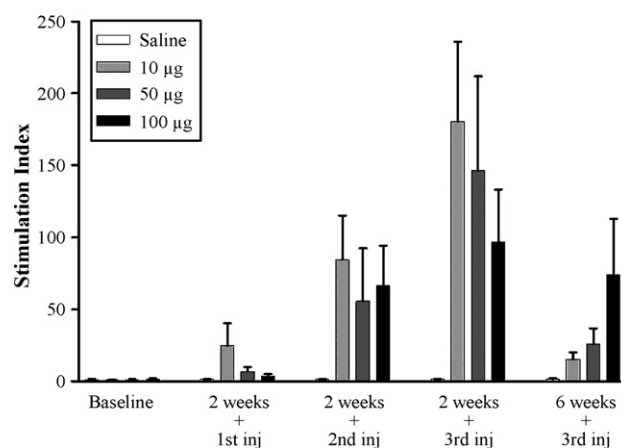


Figure 4 Cellular proliferative response of 2.5×10^5 PBMCs/well after stimulation with 3.125 μ g/well of *Na*-ASP-2 for 5 days at 37°C with 5% CO₂. Proliferation was measured by the incorporation of [³H]-methyl-thymidine for the last 6 h of culture. Stimulation indices (SIs) were calculated by dividing the mean of the counts in triplicate for each sample by the mean of the counts for triplicates of cells incubated with culture media alone. Mean SIs are plotted with error bars representing the standard errors (inj = injection).

human hookworms [19] have made the successful development of an anti-hookworm vaccine an important public health objective [20]. The results of this first trial in humans of a nematode vaccine indicate that *Na*-ASP-2 adjuvanted with Alhydrogel® is well-tolerated when administered to hookworm-naïve adults from a non-endemic area and does not result in significant vaccine-related adverse events. Furthermore, the vaccine proved to be highly immunogenic, inducing both humoral and cellular responses to all 3 antigen concentrations that were tested.

Although most of the adverse events that were observed in the trial were mild-to-moderate injection site reactions, some of these were delayed in onset up to 10 days following the second vaccination. These were observed in individuals who had both detectable IgG and lymphoproliferative responses to *Na*-ASP-2, although there was no evident correlation between the strength of the immune response and the occurrence of these delayed reactions (data not shown). Although the mechanism of these delayed reactions is unknown, it is possible that they represented Arthus-

type reactions caused by immune complex formation at the injection site, after induction of significant levels of antigen-specific IgG. However, arguing against this possibility is the fact that similar reactions were not seen after the third vaccination when even higher antibody responses were induced, even in those who had the delayed reactions after the second vaccination.

Although significant anti-Na-ASP-2 IgG titers were observed after the second immunization, there were no significant differences in antibody responses between dose groups. Even higher antibody responses were seen after the third immunization in all 3 dose groups, which encouragingly, remained elevated until the end of the study, approximately 8 months after the last vaccination. Of note, antibody levels were higher at the time of the third vaccination in all dose groups than they were 2 weeks following the second vaccination, suggesting that the timing of the third vaccination could be delayed, or may not even be necessary; this will need to be addressed in future clinical trials.

In addition to inducing significant levels of anti-Na-ASP-2 IgG (primarily IgG1 and IgG4), vaccination also induced measurable levels of antigen-specific IgE, although on an individual level, titers of IgE were far below those of antigen-specific IgG. Furthermore, although anti-Na-ASP-2 IgE was detected after the second vaccination in some volunteers, administration of a third vaccination did not result in any hypersensitivity reactions. Interestingly, antigen-specific IgE was also detected in some participants at baseline, which could be related to non-specific binding in the assay; it remains possible that there was cross reactivity with as yet unknown antigens.

Antigens expressed during the larval stage of *N. americanus* infection are attractive targets for a human hookworm vaccine for several reasons. During invasion of the human host, infective L3 encounter host-specific signals that induce a programmed chain of developmental events that result in the successful establishment of a parasitic relationship [21]. These events include completion of the interrupted second molt, expression of genes encoding proteins required for development, and the release of ES products [21]. ES products include molecules involved in exsheathment [22], molting [23,24], tissue invasion [25], and modulation of the host immune response to infection [26–29]. Hookworm L3 that are activated *in vitro* release 3 major ES products: a metalloprotease involved in tissue invasion [30,31] and 2 cysteine-rich secretory proteins termed *Ancylostoma* Secreted Protein (ASP)-1 and ASP-2 [11,12]. The ASPs belong to the pathogenesis-related (PR) protein superfamily which are produced by invertebrates, vertebrates, and plants, and are typically secreted in response to pathogens and other stressors [11,12,32]. ASPs have been found in all parasitic nematodes studied to date [33].

Two major types of ASPs have been isolated from adult and larval nematodes, each containing either a single or double PR-1 domain. Previous attempts to express ASP-2 in bacteria failed to produce soluble protein, most likely because the high cysteine content causes improper protein folding secondary to aberrant disulfide bond formation, with Na-ASP-2 containing 11 cysteine residues [16]. However, expression of Na-ASP-2 in *P. pastoris* resulted in a secreted product that was soluble and did not require

refolding [16]. Encouragingly, in our study, evidence from both Western blots and immunoprecipitation-Western blots indicates that sera from hookworm-unexposed participants vaccinated with recombinant Na-ASP-2 recognize the native structure of the natural protein in crude *N. americanus* L3 antigen extracts.

Hookworm infection offers a unique set of immunological challenges for the development of a vaccine [34]. These challenges include a robust but largely ineffective immune response to natural infection, dominated by high levels of T helper type-2 (Th2) cytokines and ablation of T cell proliferative responses to parasite antigens; successive developmental stages of the parasite within the host, each bearing stage-specific antigens, which may allow earlier stages to determine the character of the response to subsequent stages and vice versa; and invasion and occupation of a range of internal tissues during development, including the skin, vasculature, and intestine, with immune evasion likely to be finely tuned to each environment. However, there is strong evidence that vaccine-induced protection requires an antibody-mediated response as shown in the successful X-irradiated larval vaccine developed commercially in the 1970s for use against the canine hookworm *A. caninum* [10]. It has been postulated that interference with the migration of larvae in the lungs was the key factor in the high efficacy seen with the irradiated larval vaccine. This hypothesis was based on the observation that three quarters of the irradiated larvae became “arrested” before reaching the intestine, with their demise probably occurring in the lungs, a hypothesis similar to the mechanism proposed for protection in experimental irradiated schistosome vaccines. The canine hookworm vaccine was removed from the market due to the concerns of veterinarians and pet-owners that sterilizing immunity was not induced despite attaining up to 90% protection [35], a level of protection sufficient to prevent serious complications of anemia or fatal hemorrhage [10].

Subsequent studies of the irradiated larval vaccine have indicated that ASP-2 is the major determinant of protection, as evidenced by the fact that sera from canines immunized with this vaccine and subsequently relatively protected against challenge infection preferentially recognize this antigen [36]. Furthermore, vaccination of dogs [14] and hamsters [13,15] with recombinant ASP-2 resulted in a marked reduction in adult worm burden and fecundity (as determined by fecal egg counts) following larval challenge. Additionally, sera from ASP-2 vaccinated laboratory animals are able to inhibit the *in vitro* migration of larvae through tissue [14,16]. Finally, studies of populations living in hookworm-endemic areas have shown that anti-ASP-2 antibodies are associated with a reduced risk of acquiring heavy hookworm infection in areas of high transmission [14]. These observations suggest that the humoral immune response to vaccination with ASP-2 might interfere with the early stages of L3 invasion, thereby decreasing the number of hookworms that reach the gastrointestinal tract and develop into adult worms, leading to a reduction in host blood loss and its clinical sequelae [20,37].

Although identification of larval antigens such as Na-ASP-2 has been the initial goal of hookworm vaccine antigen discovery, evidence from prior work conducted on the canine irradiated larval vaccine demonstrates that it is unlikely that vaccination with a single antigen will induce complete

protection against hookworm infection. In this event, some L3 might still reach the gastrointestinal tract and develop into blood-feeding adult hookworms. Therefore, an eventual hookworm vaccine will likely contain at least 2 components, one that targets the larval stage and the other targeting the adult stage of the hookworm life cycle. To date, several proteins have been identified that are required by adult hookworms to utilize human blood as a food source and that are essential to worm survival and reproduction and which could be combined with a larval antigen such as *Na*-ASP-2 [38].

In the first clinical trial of a hookworm vaccine, the *Na*-ASP-2 vaccine was shown to be safe when administered to adults living in a non-endemic area and was able to induce significant humoral and cellular immune responses against the antigen. *Na*-ASP-2 is therefore a promising larval-stage vaccine candidate and should undergo continued clinical development, including trials conducted in individuals living in hookworm-endemic areas.

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