

Generalized urticaria induced by the *Na*-ASP-2 hookworm vaccine: Implications for the development of vaccines against helminths

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Background: *Necator americanus* Ancylostoma-secreted protein 2 (*Na*-ASP-2) is secreted by infective hookworm larvae on entry into human hosts. Vaccination of laboratory animals with recombinant *Na*-ASP-2 provides significant protection against challenge infections. In endemic areas antibodies to *Na*-ASP-2 are associated with reduced risk of heavy *N americanus* infections.

Objective: To assess the safety and immunogenicity of recombinant *Na*-ASP-2 adjuvanted with Alhydrogel in healthy Brazilian adults previously infected with *N americanus*. **Methods:** Participants were randomized to receive *Na*-ASP-2 or hepatitis B vaccine. Major IgG and IgE epitopes of the *Na*-ASP-2 molecule were mapped by using sera from these same subjects. Seroepidemiologic studies in adults and children residing in hookworm-endemic areas were conducted to assess the prevalence of IgE responses to *Na*-ASP-2.

Results: Vaccination with a single dose of *Na*-ASP-2 resulted in generalized urticarial reactions in several volunteers. These reactions were associated with pre-existing *Na*-ASP-2-specific IgE likely induced by previous hookworm infection. Surveys revealed that a significant proportion of the population in hookworm-endemic areas had increased levels of IgE to *Na*-ASP-2. Epitope mapping demonstrated sites on the

Na-ASP-2 molecule that are uniquely or jointly recognized by IgG and IgE antibodies.

Conclusion: Infection with *N americanus* induces increased levels of total and specific IgE to *Na*-ASP-2 that result in generalized urticaria on vaccination with recombinant *Na*-ASP-2. These data advance knowledge of vaccine development for helminths given their propensity to induce strong T_H2 responses. Study data highlight the important differences between the immune responses to natural helminth infection and to vaccination with a recombinant helminth antigen. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Hookworm, *Necator americanus*, urticaria, vaccine, *Na*-ASP-2, IgE, Brazil

More than 500 million persons worldwide are infected with hookworm, predominantly in impoverished tropical regions of the world.¹ Hookworm is a soil-transmitted nematode that is acquired after skin contact with infective larvae in fecally contaminated soil. After penetration of the skin, larvae migrate through tissues before entering the gastrointestinal tract, where they develop into adult worms that attach to the intestinal mucosa and feed on the host's blood.² Chronic infection lasting for years leads to long-term pathology caused by intestinal blood loss that can result in iron-deficiency anemia, which impairs physical and intellectual development in children and has a significant effect on future economic productivity.³

Current control measures consist of regular mass administration of an anthelmintic, such as albendazole, in endemic areas.⁴ However, this strategy has several limitations, including rapid reinfection after treatment caused by continued exposure to larvae and the potential development of drug resistance. A vaccine that would prevent moderate- or heavy-intensity infections would be a major advance in reducing hookworm-related morbidity.^{5,6}

Necator americanus Ancylostoma-secreted protein 2 (*Na*-ASP-2) is a 21.3-kDa protein that is secreted by infective hookworm larvae on entry into the host.⁷⁻¹⁰ Vaccination of hamsters and dogs with recombinant ASP-2 results in reduced worm burdens and fecundity after challenge infection.¹ Furthermore, sera from vaccinated animals inhibit migration of infective hookworm larvae through tissue in an *in vitro* assay. Studies of populations living in hookworm-endemic areas have also shown that anti-ASP-2 antibodies are associated with a reduced risk of having a heavy hookworm infection.¹ On the basis of these results, *Na*-ASP-2 was chosen as the lead hookworm vaccine candidate.⁶ In a phase I study conducted in hookworm-naïve adults living in the United States, *Na*-ASP-2 adjuvanted with Alhydrogel (Biosector, Copenhagen, Denmark) was well tolerated and

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Abbreviations used

FIOCRUZ: Fundação Oswaldo Cruz

IDST: Intradermal skin test

ITH: Immediate-type hypersensitivity

MAP: Multiple-antigen peptide

Na-ASP-2: *Necator americanus* Ancylostoma-secreted protein 2

PST: Puncture skin test

immunogenic.¹¹ As reported herein, a phase I trial of the vaccine was therefore conducted in adults living in a hookworm-endemic area of Brazil.

METHODS

Study site and population

All studies reported herein were conducted in Americaninhas, Minas Gerais, Brazil, and the surrounding rural areas. The epidemiology of *N americanus* and other helminth infections in this area has previously been extensively studied.¹²⁻¹⁵

Phase I trial of *Na*-ASP-2

A randomized, double-blind, controlled phase I trial of the *Na*-ASP-2 hookworm vaccine was conducted at a study clinic operated by the Fundação Oswaldo Cruz (FIOCRUZ). Written informed consent was obtained from healthy adults between the ages of 18 and 45 years who had been treated with albendazole for *N americanus* infection within the previous 3 months. Volunteers were excluded if they had evidence of clinically significant systemic disease, were pregnant or breast-feeding, had serologic evidence of chronic hepatitis B or C infection, were receiving corticosteroids or immunosuppressive drugs, or had been immunized with a live vaccine within the previous month. The study was approved by the institutional review boards of FIOCRUZ, the George Washington University Medical Center, and the Brazilian Ministry of Health. It was conducted under investigational new drug applications to the US Food and Drug Administration and the Agência Nacional de Vigilância Sanitária of Brazil.

The study was designed to enroll 48 participants into 1 of 3 cohorts. Within each cohort, participants were to be randomized to receive either the *Na*-ASP-2 or hepatitis B vaccines. Participants randomized to *Na*-ASP-2 were to receive 10, 50, and 100 μ g per vaccination in the first, second, and third cohorts, respectively, through intramuscular injection in the deltoid muscle on study days 0, 56, and 112. Syringe barrels were masked with opaque tape to disguise the contents, and injections were administered by physicians who were not involved in postvaccination safety assessments or study analysis. Participant safety was monitored by an independent physician and a safety-monitoring committee that reviewed data at intervals throughout the study.

Study vaccines

Recombinant *Na*-ASP-2 was manufactured and adsorbed to Alhydrogel, as described previously.¹¹ Each 0.5-mL vaccine dose contained either 10, 50, or 100 μ g *Na*-ASP-2 and 800 μ g of Alhydrogel in 50 mmol/L sodium acetate buffer. Studies of vaccine stored at 2°C to 8°C were conducted in mice every 6 months to confirm stability and potency. Hepatitis B vaccine (Instituto Butantan, São Paulo, Brazil) was supplied in multidose vials containing recombinant hepatitis B surface antigen adsorbed to amorphous aluminum hydroxyphosphate sulfate; each 1.0-mL dose of vaccine contained 10 μ g of antigen.

Assessment of safety and tolerability

After vaccinations, volunteers were observed for 60 minutes and then evaluated 1, 2, 3, 7, 14, and 28 days after vaccination for evidence of local and systemic reactogenicity and then at regular intervals until study day 290. Injection sites were examined for erythema, swelling, and tenderness.

Solicited systemic adverse events included fever, headache, nausea, malaise, myalgia, arthralgia, and urticaria. Adverse events were graded as mild (easily tolerated), moderate (interfered with activities of daily living), or severe (prevented activities of daily living) and assigned causality relative to the study vaccine. Injection-site erythema and swelling were graded as mild (>0 to $\leq$ 20 mm in diameter), moderate (>20 to $\leq$ 50 mm), or severe (>50 mm); oral temperature was graded as mild (>37.5°C to $\leq$ 38.0°C), moderate (>38.0°C to $\leq$ 39.0°C), or severe (>39.0°C); and urticaria was graded as mild (not requiring medication), moderate (requiring oral or topical noncorticosteroid treatment or intravenous medication or corticosteroids for <24 hours), or severe (requiring intravenous medication or corticosteroids for >24 hours). A complete blood count and serum creatinine, alanine aminotransferase, and total IgE measurements were performed immediately before vaccination, as well as on the second and fourteenth days after vaccination.

Immediate hypersensitivity skin testing for *Na*-ASP-2

Because of urticarial reactions observed in 3 subjects vaccinated with 10 μ g of *Na*-ASP-2 (see below), a skin test was developed for immediate-type hypersensitivity (ITH) to the antigen. Puncture skin tests (PSTs) were performed by inserting a bifurcated needle into the skin of the forearm through drops of the following solutions: (1) sterile albumin saline negative control solution (ALO Laboratories, Columbus, Ohio); (2) 2.75 mg/mL histamine phosphate positive control solution (ALO Laboratories); (3) *Na*-ASP-2 solution (10 μ g/mL); (4) *Na*-ASP-2 solution (100 μ g/mL); and (5) *Na*-ASP-2 solution (1000 μ g/mL). For intradermal skin tests (IDSTs), approximately 0.02 mL of *Na*-ASP-2 or control solutions were injected intracutaneously on the volar surface of the forearm by using a 1.0-mL tuberculin syringe. The following solutions were tested by using IDSTs: (1) sterile albumin saline solution; (2) 0.275 mg/mL histamine phosphate (ALO Laboratories); and (3) *Na*-ASP-2 solution (100 μ g/mL).

Na-ASP-2 skin test reagent was supplied in vials containing 1.0 mg/mL *Na*-ASP-2 in 50 mmol/L sodium acetate buffer. Dilutions of *Na*-ASP-2 were prepared immediately before skin testing by using sterile albumin saline solution. Participants were tested first by using PSTs and then by using IDSTs provided that none of the *Na*-ASP-2 PSTs resulted in a wheal greater than 15 mm in diameter. Wheal-and-flare responses were measured 15 minutes after skin tests were applied. A positive *Na*-ASP-2 PST or IDST result was defined as one producing a mean wheal diameter equal to or greater than that of the histamine control.

Seroepidemiologic surveys

Serum and fecal samples were collected from participants of 3 separate epidemiologic studies conducted in and around Americaninhas.¹⁶ Participants ranged in age between 1 and 85 years. Approvals for all studies were obtained from the ethics committees of FIOCRUZ, George Washington University Medical Center, and the Brazilian Ministry of Health. Written informed consent was obtained from adult subjects or from parents or guardians of minors.

For these studies, serum was separated from blood samples and tested for *Na*-ASP-2-specific IgG and IgE antibodies by using the ELISA methods described below. Fecal samples were examined microscopically by using the formalin-ether sedimentation technique. If positive for hookworm eggs, samples were then examined by using the Kato-Katz method.¹⁷ The intensity of infection was estimated indirectly by counting the mean number of eggs per gram of feces.

Antibody measurement

For serum samples collected from participants of the phase I trial, *Na*-ASP-2-specific IgG and IgE antibody levels were measured by using the fluoroenzyme immunoassay (ImmunoCAP 250; Phadia, Uppsala Sweden) at the Johns Hopkins Dermatology, Allergy and Clinical Immunology Reference Laboratory. Levels of greater than 0.1 kU/L (0.24 ng/dL) for IgE and 2000 ng/mL for IgG were considered seropositive. For the seroepidemiologic studies, *Na*-ASP-2-specific IgE and IgG antibody levels were determined by using a qualified ELISA^{1,11} with a standard calibration curve to convert absorbance levels

TABLE I. Solicited local injection-site and systemic adverse events after vaccination with 10 μ g of *Na*-ASP-2/Alhydrogel or hepatitis B vaccines

	10 μ g of <i>Na</i> -ASP-2 (n = 7)			Hepatitis B vaccine (n = 2)		
	Mild, no. (%)	Moderate, no. (%)	Severe, no. (%)	Mild, no. (%)	Moderate, no. (%)	Severe, no. (%)
Local						
Pain	3 (43)	—	—	2 (100)	—	—
Swelling	5 (71)	2 (29)	—	—	—	—
Erythema	1 (14)	—	2 (29)	—	—	—
Systemic						
Fever	—	—	—	1 (50)	—	1 (50)
Headache	1 (14)	4 (57)	—	—	—	1 (50)
Nausea	5 (71)	—	—	—	1 (50)	—
Myalgia	2 (29)	—	—	1 (50)	—	—
Arthralgia	1 (14)	1 (14)	—	—	—	—
Malaise	1 (14)	1 (14)	—	—	—	1 (50)
Urticaria	—	3 (43)	—	—	—	—

Data are shown as numbers (percentages) of participants.

produced by individual sera into arbitrary antibody units (SOFTmax PRO version 4; Molecular Devices Co, Sunnyvale, Calif).

IgE and IgG epitope mapping

ELISAs were used to identify reactive epitopes recognized by anti-ASP-2 IgG and IgE in sera from participants who had urticaria after *Na*-ASP-2 vaccination. IgG epitopes were assessed by using untreated sera, whereas IgE epitope mapping was performed with sera that had been depleted of IgG by Protein G sepharose (GE Biosciences, Fairfield, Conn), according to the manufacturer's instructions. Ninety-six-well flat-bottom, polystyrene microtiter plates containing streptavidin were coated with biotinylated sequential linear 13-mer peptides from *Na*-ASP-2 (with an overlap of 2 amino acids) synthesized by using mild Fmoc chemistry (Mimotopes, Victoria, Australia). Coated plates were blocked with BSA. Serum was diluted 1:20 (IgE assay) or 1:200 (IgG assay) with 2% BSA/PBS, and 50 μ L was then applied to the respective wells. Plates were incubated at 4°C overnight and washed with PBS/0.1% Tween-20. Bound antibody was detected by reaction for 2 hours at room temperature with the following secondary antibodies: goat anti-human IgG (heavy and light chain specific) horseradish peroxidase-conjugated antibody (Chemicon International, Temecula, Calif) or murine anti-human IgE (heavy chain specific) horseradish peroxidase-conjugated antibody (Invitrogen, San Diego, Calif). Plates were washed as described above, and the OD was determined at 405 nm by using a microplate spectrophotometer (Benchmark Plus; Bio-Rad Laboratories, Hercules, Calif). Structural depictions of *Na*-ASP-2 were produced with PyMOL (<http://www.pymol.org>) and VMD¹⁸ based on the x-ray crystal structure of *Na*-ASP-2 (PDB code 1U53). Animated depictions were constructed with PyMOL and Mencoder (<http://www.mplayerhq.hu>).

Statistical methods

Proportions of participants experiencing each adverse event were tabulated by vaccine allocation but were not analyzed statistically because of the small number of participants vaccinated. Differences in antibody levels between groups were compared by using the Mann-Whitney *U* test, and correlations between anti-*Na*-ASP-2 IgE levels and age or hookworm infection intensity were assessed by using Pearson correlation tests. STATA software (version 8.0; StataCorp, College Station, Tex) was used for all analyses. *P* values of .05 or less were considered significant.

RESULTS

Phase I trial participant safety

In June 2007, 9 eligible volunteers were enrolled in the first cohort and vaccinated with either *Na*-ASP-2 (10 μ g) or hepatitis

B vaccine (see the Methods section in this article's Online Repository at www.jacionline.org for a description of the participants' baseline characteristics). All had been treated for hookworm 35 days before vaccination; their fecal egg counts ranged from 78 to 13,992 eggs per gram before treatment. Between 1 and 2 hours after vaccination, 3 had an acute generalized urticarial rash involving predominantly the head, neck, and upper torso. None had bronchospasm, laryngeal edema, or hemodynamic instability. All 3 participants with urticaria responded to administration of an oral antihistamine: in 2 cases (participants A07 and A09) the urticaria completely resolved within 6 hours of receiving 1 dose of an antihistamine, whereas in the remaining participant (A02) treatment was required for 2 days because of persistent lesions. Because of the allergic reactions, further vaccinations were suspended, and after consultation with the safety-monitoring committee, the study was unblinded. Of the 9 vaccinated subjects, 7 had received *Na*-ASP-2, including all 3 with urticaria.

In addition to urticaria, mild pain and swelling were the most commonly observed injection-site reactions (Table I). Severe injection-site erythema was observed in 2 participants who received *Na*-ASP-2: 1 was a subject with urticaria. The other was a participant who did not have urticaria but who, 13 days after vaccination, had injection-site erythema (9 $\times$ 6 cm) and induration that was not associated with pain and that resolved spontaneously 6 days after onset. Mild nausea and mild-to-moderate headache were the most frequently observed solicited systemic reactions. No serious adverse events or clinical laboratory abnormalities occurred that were related to vaccination.

IgE and IgG responses to *Na*-ASP-2 before vaccination

IgE to *Na*-ASP-2 was detected in the prevaccination sera of the 3 subjects who had generalized urticaria immediately after vaccination with a single dose of *Na*-ASP-2 (Fig 1, A). Of those who were vaccinated with *Na*-ASP-2 but did not have urticaria, IgE to *Na*-ASP-2 was detected in 2 subjects. Of note, median levels of IgE to *Na*-ASP-2 were 21 times higher in the subjects with urticaria compared with levels seen in those who received the vaccine but did not have this reaction (26.5 vs 1.25 kU/L, *P* = .03).

The 3 subjects with urticaria also had the highest levels of total IgE (Fig 1, B), with a median level 8 times that of subjects who

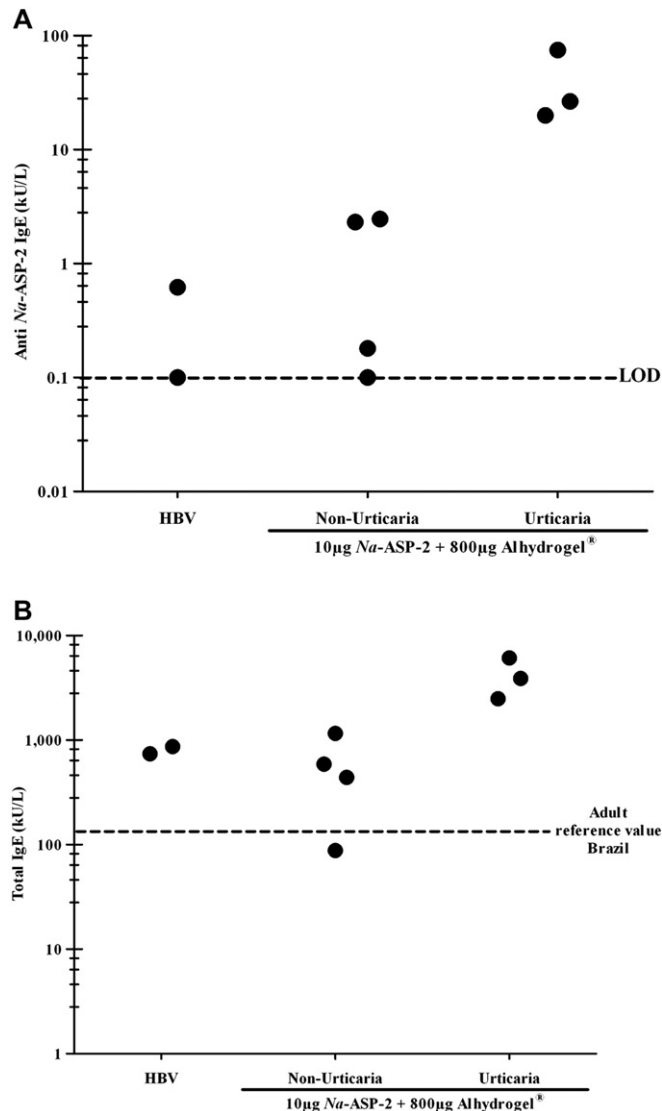


FIG 1. Levels of IgE against *Na*-ASP-2 (**A**) and total IgE (**B**) in participants before immunization with *Na*-ASP-2/Alhydrogel, as measured by using ImmunoCAP. *HBV*, Subjects who received hepatitis B vaccine; *LOD*, limit of detection; *Non-Urticaria*, subjects who did not have generalized urticaria after vaccination with *Na*-ASP-2; *Urticaria*, subjects who had generalized urticaria after a single vaccination (10 μ g) with the *Na*-ASP-2 vaccine.

received *Na*-ASP-2 but did not have urticaria (3878 vs 514 kU/L, $P = .03$). However, with the exception of 1 subject, all participants had total IgE levels much higher than the Brazilian upper reference limit of 140 kU/L (median, 1867 kU/L; range, 87.7–6120 kU/L). Subjects who received the vaccine without having urticaria had a median *Na*-ASP-2–specific IgG/IgE ratio that was 85 times higher (2664 vs 31.4, $P = .03$) than those who received the vaccine and had urticaria (see Table E1 in this article’s Online Repository at www.jacionline.org).

Anti-*Na*-ASP-2–specific IgG antibodies were detectable in all 9 participants at baseline (Fig 2). No marked differences were seen between those who received *Na*-ASP-2 and had urticarial responses and those who received *Na*-ASP-2 but did not have urticaria (data not shown).

IgE and IgG responses to *Na*-ASP-2 after vaccination

Anti-*Na*-ASP-2 IgE levels in subjects vaccinated with *Na*-ASP-2 peaked at day 28 after vaccination and remained increased for the duration of the study (see Fig E1, A, in this article’s Online Repository at www.jacionline.org). This postvaccination increase occurred exclusively in those who did not experience urticaria. One participant (A03) who received the *Na*-ASP-2 vaccine and did not have urticaria showed a marked increase in specific IgE levels after vaccination, starting at day 28. There was no apparent change in either IgE or IgG levels to *Na*-ASP-2 in the 2 volunteers vaccinated with the hepatitis B vaccine. Levels of total IgE remained unchanged in most volunteers throughout the study (see Fig E1, B).

Na-ASP-2–specific IgG antibody levels increased after vaccination with *Na*-ASP-2, peaking at day 28 (Fig 2). *Na*-ASP-2–specific IgG antibody levels were significantly higher at this time point in the *Na*-ASP-2 versus hepatitis B vaccine groups (median, 4570 vs <2000 ng/mL, respectively; $P = .04$). Levels of *Na*-ASP-2–specific IgG decreased over the remainder of the study, although they remained increased in the *Na*-ASP-2 group compared with those in the hepatitis B vaccine recipients.

Skin testing of vaccinated subjects

Of the 9 participants who were vaccinated in June 2007, 7 subsequently underwent immediate hypersensitivity skin testing for *Na*-ASP-2 18 months after vaccination. Of the 2 who were not skin tested, 1 had received the hepatitis B vaccine, whereas the other had received *Na*-ASP-2 and was 1 of the 3 who had urticaria.

Three of the 7 had wheal reactions to either the puncture or intradermal injections of *Na*-ASP-2 that were equal to or greater than reactions to the histamine control (Table II). These 3 included 2 subjects who had urticarial reactions after immunization with *Na*-ASP-2 in June 2007, as well as 1 subject (A03) who did not have urticaria when vaccinated but who subsequently had a significant *Na*-ASP-2–specific IgE antibody response after vaccination. Serum levels of *Na*-ASP-2–specific IgE, as measured by using ImmunoCAP on the same day as skin testing, revealed increased levels in the 3 participants with positive skin test reactions but also 1 with a negative skin test reaction.

Serologic survey of adults and children living in a hookworm-endemic area

Fig 3¹² shows the levels of IgE antibodies to *Na*-ASP-2 from 826 subjects between the ages of 1 and 85 years living in a hookworm-endemic area of Brazil. Levels of *Na*-ASP-2–specific IgE antibodies were significantly correlated with age (Pearson $r = 0.15$, $P < .001$, see Fig E2 in this article’s Online Repository at www.jacionline.org), although levels greater than 10,000 AU were nevertheless observed in 108 of 243 children between the ages of 1 and 9 years. *Na*-ASP-2–specific IgE antibody levels were also correlated with hookworm infection status (Pearson $r = 0.17$, $P < .001$) and infection intensity (Pearson $r = 0.16$, $P < .001$, see Fig E3 in this article’s Online Repository at www.jacionline.org). As reported previously, negligible levels of IgE to *Na*-ASP-2 were detected in a phase I trial of *Na*-ASP-2 conducted in hookworm-naïve adults (Fig 3).¹¹

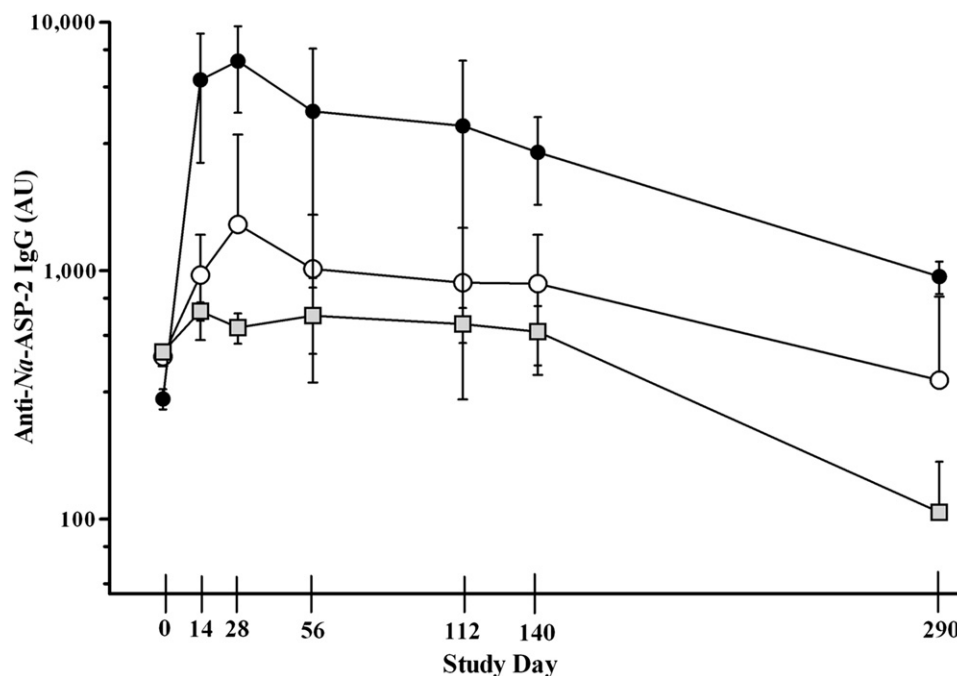


FIG 2. Anti-*Na*-ASP-2 IgG levels by study day, as measured by using ImmunoCAP. Data are arithmetic mean antibody levels, with bars showing SEs. Solid circles represent subjects who had a generalized urticarial response after a single vaccination (10 μ g) with the *Na*-ASP-2 vaccine. Open circles represent subjects who did not have generalized urticaria after a single vaccination with *Na*-ASP-2/Alhydrogel. Gray boxes represent subjects who received hepatitis B vaccine.

TABLE II. Results of PSTs and IDSTs for ITH to *Na*-ASP-2

Participant	Vaccine	Skin test result		IgE	
		PST	IDST	Anti- <i>Na</i> -ASP-2 (kU/L)	Total (kU/L)
A01	<i>Na</i> -ASP-2	—	—	0.47	57
A02*	<i>Na</i> -ASP-2	+	+	42.00	2137
A03	<i>Na</i> -ASP-2	+	+	30.00	272
A04	HBV	—	—	0.11	135
A05	<i>Na</i> -ASP-2	—	—	0.88	931
A06	HBV	ND	ND	ND	ND
A07*	<i>Na</i> -ASP-2	+	+	4.39	1087
A08	<i>Na</i> -ASP-2	—	—	4.67	422
A09*	<i>Na</i> -ASP-2	ND	ND	ND	ND

Serum antibodies to *Na*-ASP-2 were measured by using ImmunoCAP in blood collected immediately before administering the skin tests to determine levels of *Na*-ASP-2-specific IgE antibody.

HBV, Hepatitis B vaccine; ND, not available for follow-up skin testing.

*Developed urticaria after vaccination with *Na*-ASP-2.

Mapping of *Na*-ASP-2 epitopes bound by IgE and IgG

Table E2 shows the epitopes recognized by circulating anti-ASP-2 IgG and IgE in sera collected from participants in the phase I trial. A total of 14 epitopes were detected (see this article's Online Repository at www.jacionline.org). The most widely recognized epitope was W135, which was bound by IgE from 8 and IgG from 8 participants. Peptide S149 was recognized by IgE from the same 8 persons but was not a target of IgG from any of the subjects. Peptide F123 was recognized by IgG from 7 subjects and IgE from only 2 subjects. A total of 4 distinct epitopes were demonstrated for IgE binding and 6 for IgG binding; in

addition to these distinct sites, 5 bound both IgE and IgG. Fig 4 depicts a 3-dimensional representation of *Na*-ASP-2 based on its crystalline structure and highlighting epitopes that are uniquely or jointly recognized by IgG and IgE antibodies. See Video E1 in this article's Online Repository at www.jacionline.org for a 3-dimensional video of the protein's conformation and antibody-binding epitopes.

DISCUSSION

In the first phase I clinical trial of a vaccine targeting a nematode infection in human subjects living in a hookworm-endemic area, the development of generalized urticarial reactions after a single (10 μ g) immunization with the *Na*-ASP-2 hookworm vaccine led to the study being halted. Subsequent analysis showed that the urticarial reactions were associated with increased prevaccination levels of IgE antibodies specific for *Na*-ASP-2, which were most likely present as a result of previous hookworm infection. A survey of adults and children from the same hookworm-endemic area revealed that a significant proportion had increased levels of IgE to *Na*-ASP-2. Our findings have major implications for the development of vaccines against not only hookworm but indeed all helminths given their common propensity to induce strong T_H2 immune responses.

It is accepted that T_H2 responses are elicited during natural hookworm infection similarly to most other helminth infections, such as schistosomiasis, onchocerciasis, and filariasis.^{19,20} As part of this T_H2 response, subjects have increased levels of total and parasite-specific IgE, as well as increased levels of IL-4, IL-5, and IL-13, with concomitant increases in eosinophil and mast cell numbers. Parasite-specific IgE might have a partially protective effect against helminth infections, especially *Schistosoma*

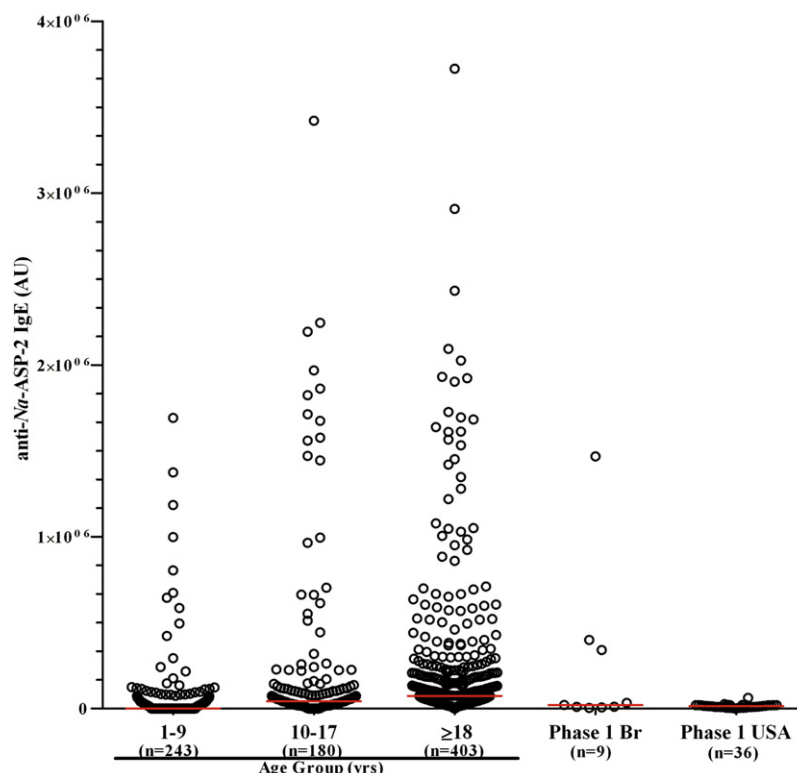


FIG 3. Levels of *Na*-ASP-2-specific IgE by age and study. Data are from 3 studies of adults and children between 1 and 85 years of age residing in hookworm-endemic areas in and around Americaninhas (site of the phase I trial), Minas Gerais state, Brazil. Serum samples were collected from both hookworm-positive and hookworm-negative subjects and reflect IgE levels to *Na*-ASP-2 before treatment of soil-transmitted helminth infections with albendazole. Also presented are data for the 9 volunteers in the phase I study of the *Na*-ASP-2 hookworm vaccine in healthy Brazilian adults previously infected with *N americanus*, as well as a phase I study of the vaccine conducted in healthy American hookworm-naïve adults.¹² IgE to *Na*-ASP-2 is expressed in arbitrary units of antibody, as determined by using a qualified indirect ELISA.

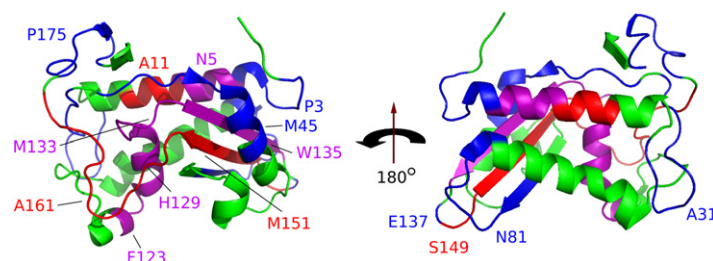


FIG 4. A 3-dimensional representation of *Na*-ASP-2 based on its crystalline structure highlighting the epitopes that are uniquely or jointly recognized by IgG and IgE antibodies, as determined by using sera collected from the 9 participants in the phase I trial of *Na*-ASP-2 in Brazilian adults. Epitopes in blue are those recognized by IgG, those in red are recognized by IgE, and those in purple are recognized by both IgG and IgE.

species,^{21,22} but also *Trichuris*,²³ *Ascaris*,²⁴ and *Echinococcus* species.²⁵ High levels of induced parasite-specific IgE are associated with efficacy of vaccines targeting *Haemonchus contortus*,²⁶ *Onchocerca volvulus*,²⁷ and *Ancylostoma caninum*.²⁸ Our own studies have shown that increased levels of IgE to recombinant *Ac*-ASP-2, the *Ancylostoma caninum* homologue of *Na*-ASP-2, are associated with a decreased risk of heavy hookworm infection.¹

The T_H2 response during helminth infection is induced against a background of potent parasite-induced immunoregulation, which is referred to as a modified T_H2 response.^{19,20} This

modified T_H2 response can consist of alternatively activated macrophages, forkhead box protein 3-positive CD4 regulatory T cells, and CD4⁺ T_R1 IL-10-producing T cells. The effect of this response is to create an immune environment so downregulated that it can protect the host not only from the strong inflammatory effects of helminth infections but also from IgE-mediated disorders, such as atopy, asthma, and anaphylaxis.²⁹ Reduced allergic responses have been shown in studies of infection of mice with various helminths.³⁰ Moreover, epidemiologic evidence suggests that hookworm infection is associated with reduced skin

reactivity to common allergens and a decreased risk of extrinsic asthma.³¹

Two specific mechanisms are often mentioned in the context of the protection from IgE-mediated disorders associated with helminth infection. The first is that IL-10 or TGF- β secreted by antigen-presenting or regulatory T cells in response to chronic helminth infection directly interferes with allergic effector mechanisms by inhibiting mast cell degranulation or T_H2 cell proliferation.²⁹ Alternatively, nonspecific IgE induced by helminth infection might prevent mast cell or basophil degranulation by saturating IgE-binding sites on these cells, thereby interfering with the binding of allergen-specific IgE.³⁰ Clearly, neither mechanism protected the 3 participants in the phase I study from having urticaria after immunization with *Na*-ASP-2, despite high total IgE levels.

It is possible that intrinsic structural or biological properties of the *Na*-ASP-2 molecule were responsible for the observed ITH reactions.^{32,33} An intriguing possibility is that human subjects have targeted a restricted range of helminth antigens to enable the host to defend itself without exposing itself to self-harm or inducing tolerance.³⁴ *Na*-ASP-2 would be an attractive target for antibody-mediated defense against hookworm infection. During penetration of host tissue, infective *N americanus* L3 encounter host-specific signals that induce a programmed chain of developmental events that result in the establishment of the parasitic relationship. Among the most abundant proteins released by hookworm larvae during this transition to parasitism are ASP-1 and ASP-2, both of which belong to the pathogenesis-related protein superfamily.⁷⁻¹⁰ Vaccination with recombinant *Na*-ASP-2 induced significant protection in laboratory animal models, and sera from animals immunized with ASP-2 inhibited the migration of infective larvae *in vitro* through host tissue.^{1,2} The latter observation suggests that antibodies against *Na*-ASP-2 might attenuate larvae during tissue migration and prevent them from becoming healthy adult hookworms in the host intestine.^{1,6}

Subsequent seroepidemiologic studies in Brazil showed that a significant proportion of subjects had high levels of IgE to *Na*-ASP-2, which led us to halt development of this vaccine. Systemic allergic reactions are rare for licensed vaccines, averaging 0.65 cases per million doses.³⁵ Few of these reactions implicate the immunizing agent itself, as with *Na*-ASP-2. More common are allergic reactions to components such as animal protein (gelatin), antibiotics (neomycin), or latex (vial stoppers).³⁶ However, a comparable situation was reported for the clinical testing of the experimental circumsporozoite multiple-antigen peptide (MAP) vaccine against *Plasmodium falciparum*.³⁷ During phase I trials in unexposed subjects, vaccinees experienced ITH reactions associated with increased levels of IgE to MAP. However, in contrast to the results presented here, vaccinees had MAP-specific IgE only after repeated vaccinations with MAP and were not sensitized by a natural parasite infection, such as was the case with *Na*-ASP-2. In contrast, vaccination of Brazilian volunteers with *Na*-ASP-2 likely cross-linked IgE on mast cells armed during years of chronic infection, triggering substantial histamine release.

Several alternatives were considered to permit continued development of the *Na*-ASP-2 vaccine. Young children might not have the same degree of sensitization to *Na*-ASP-2 as chronically exposed adults. However, our survey in *N americanus*-endemic areas in Brazil indicates that children have IgE responses to *Na*-ASP-2 at as young as 12 months of age. It is possible that infants might not have IgE, but designing a development plan based on this assumption is risky. Other options considered

were coadministration with T_H1-driving immunostimulants, such as CpG oligodeoxynucleotide sequences, or skin testing before vaccination to screen out sensitized subjects. However, this would be operationally difficult for a licensed vaccine. The only feasible alternatives would be to either re-engineer the *Na*-ASP-2 antigen to remove or mutate epitopes recognized by IgE or identify new vaccine antigens that are protective but not recognized by IgE antibodies induced by natural infection. The latter option has been pursued to the point that 2 adult worm antigens that are involved in hemoglobin digestion are being advanced to clinical trials.³⁸

The results of our studies have several implications for helminth vaccine development. First, although helminth infections are known to induce strong T_H2 responses during natural infection, screening for pre-existing levels of antigen-specific IgE in endemic areas should be used as a critical step in the selection and development of potential vaccine antigens. Not only should this overcome a significant safety barrier to vaccine development, it will also avert losses in cost and time.

Second, contrary to the results from an endemic area, the *Na*-ASP-2 vaccine was well tolerated in a phase I trial in nonexposed subjects in the United States.¹¹ This contradiction adds to a growing consensus that vaccine trials in nonendemic countries often do not yield information that can readily be generalized to disease-endemic areas.³⁹

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Clinical implications: Hookworm infection might induce IgE antibodies to vaccine antigens that result in hypersensitivity reactions on immunization. Screening endemic populations for antigen-specific IgE should precede selection of potential helminth vaccine antigens.

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METHODS

Phase I trial participant flow and baseline data

Of 28 adults screened for inclusion in the phase I trial of *Na*-ASP-2, 25 were deemed eligible. Of these, 9 (6 male and 3 female subjects) were enrolled in the first cohort. Reasons for exclusion were prior receipt of hepatitis B vaccine

($n = 1$) and intent to move from the study area during the study period ($n = 2$). Thirteen volunteers were eligible but not enrolled because of suspension of the trial, as described in the text. The median age of enrolled participants was 34 years (range, 23–45 years).

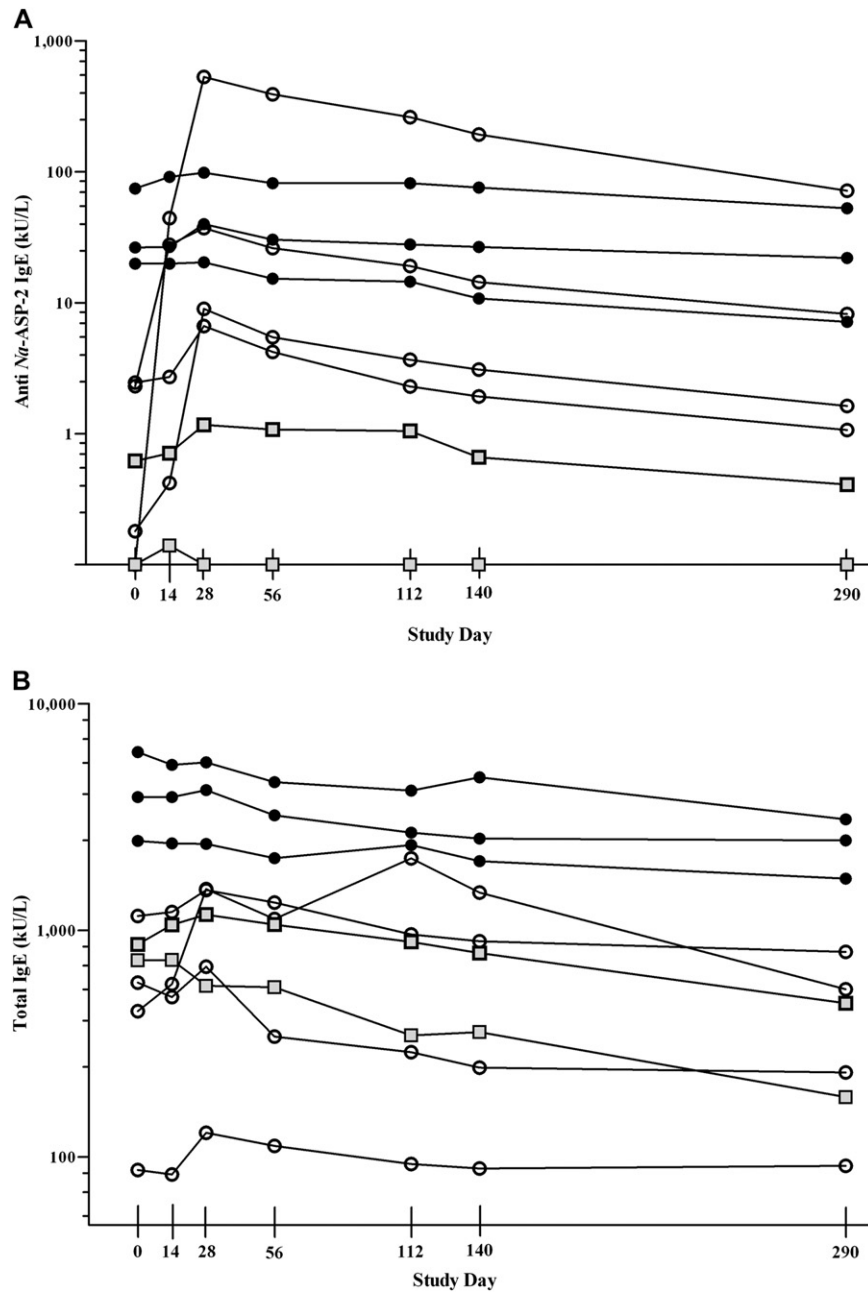


FIG E1. Levels of IgE to Na-ASP-2 (**A**) and total IgE levels (**B**) by study day, as measured by using ImmunoCAP. *Solid circles* represent subjects who had generalized urticaria after a single vaccination (10 μ g) with the Na-ASP-2 vaccine. *Open circles* represent subjects who did not have generalized urticaria after vaccination with Na-ASP-2. *Gray boxes* represent subjects who received hepatitis B vaccine.

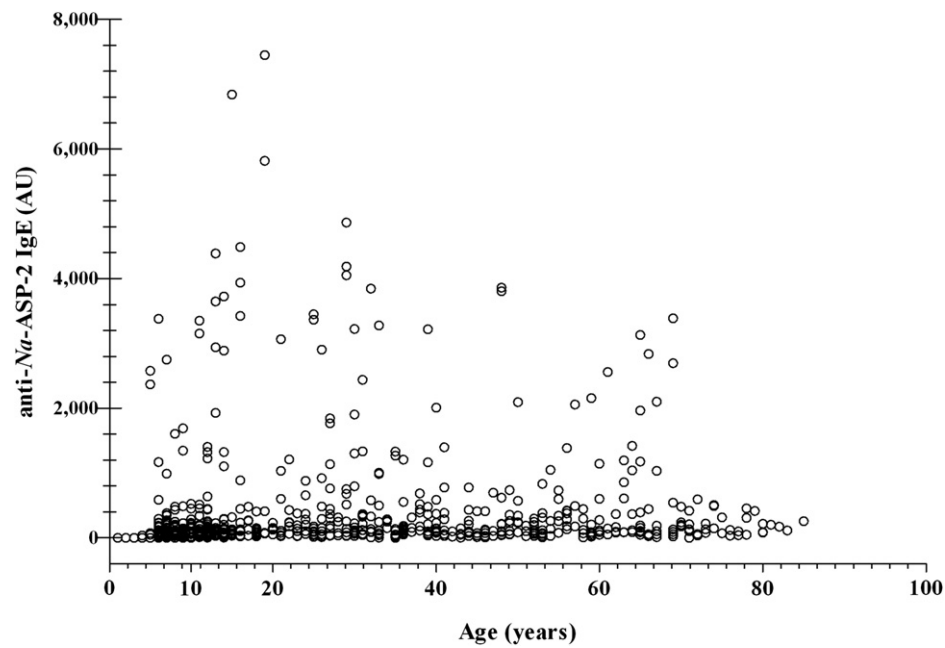


FIG E2. Relationship between levels of IgE to *Na*-ASP-2 (expressed in arbitrary units) and age in years for subjects living in and around the phase I study site of Americaninhas before treatment with albendazole.

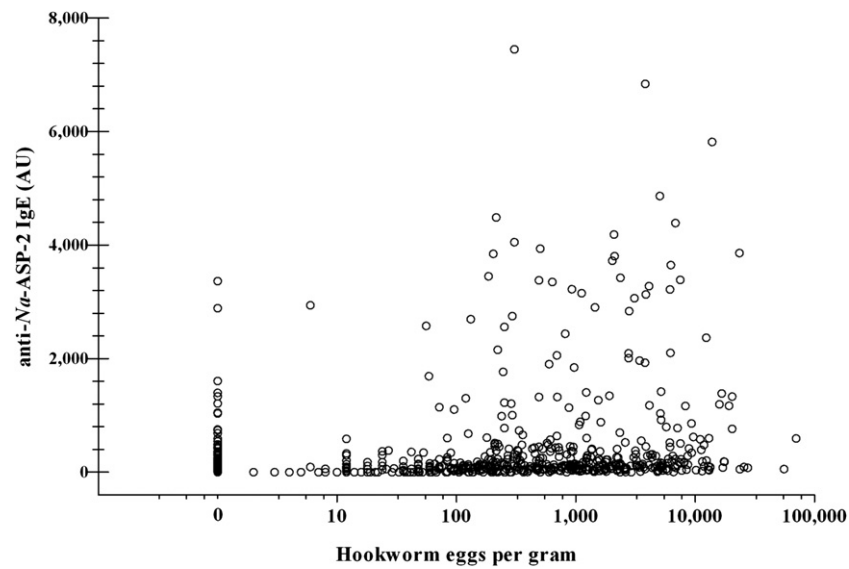


FIG E3. Relationship between levels of IgE to *Na*-ASP-2 (expressed in arbitrary units) and the intensity of *N. americanus* infection (expressed as eggs per gram of feces) for subjects residing in and around the phase I study site of Americaninhas before treatment with albendazole.

TABLE E1. Summary of quantitative total and *Na*-ASP-2-specific IgE responses in participants of the phase I trial of the *Na*-ASP-2 vaccine before vaccination

Vaccine	Urticaria	Anti-ASP-2			Total IgE	
		IgG (ng/mL)	IgE (ng/mL)	IgG/IgE ratio	ng/mL	% ASP-2 IgE
<i>Na</i> -ASP-2	Positive	2,300	179	13	9,307	1.92
<i>Na</i> -ASP-2	Positive	<2,000	48	<42	5,962	0.39
<i>Na</i> -ASP-2	Positive	<2,000	64	<31	14,688	0.21
<i>Na</i> -ASP-2	Negative	2,080	0.43	4,815	210	0.21
<i>Na</i> -ASP-2	Negative	3,350	<0.24	>13,958	1,056	0.02
<i>Na</i> -ASP-2	Negative	3,030	6.00	513	2,779	0.07
<i>Na</i> -ASP-2	Negative	2,320	6.00	418	1,411	0.43
Hepatitis B	NA	<2,000	<0.24	NA	1,774	0.21
Hepatitis B	NA	<2,000	1.49	<1344	2,081	0.80

NA, Not applicable.

TABLE E2. Epitopes recognized by circulating anti-*Na*-ASP-2 IgG and IgE in sera collected from the 9 participants of the phase I trial of the *Na*-ASP-2 vaccine conducted in Brazil

Participant	Vaccine	Urticaria	IgE		IgG	
			Peptides	OD ₄₀₅	Peptides	OD ₄₀₅
A01	<i>Na</i> -ASP-2	No			F123	0.297
					M133	0.283
					W135	1.300
					E137	0.337
A02	<i>Na</i> -ASP-2	Yes	W135	0.305	M45	0.226
			S149	0.398	F123	0.48
					H129	0.344
					W135	0.94
A03	<i>Na</i> -ASP-2	No	F123	0.220	P3	0.187
			W135	0.221	F123	0.368
			S149	0.492	W135	1.020
			M151	0.247		
A04	Hepatitis	No	F123	0.218	M45	0.196
			W135	0.419	F123	0.285
			S149	0.435	W135	0.757
					P175	0.177
A05	<i>Na</i> -ASP-2	No	W135	0.162	W135	0.569
			S149	0.329		
			A161	0.141		
A06	Hepatitis B	No	E97	0.260	A31	0.205
			W135	0.197	M45	0.160
			S149	0.246	N81	0.440
A07	<i>Na</i> -ASP-2	Yes			F123	0.173
			A11	0.177	F123	0.780
			W135	0.312	W135	0.854
			S149	0.398		
A08	<i>Na</i> -ASP-2	—	A161	0.272		
			W135	0.434	F123	0.259
			S149	0.502	W135	0.496
A09	<i>Na</i> -ASP-2	Yes			E137	0.195
			W135	0.257	N5	0.351
			S149	0.432	W135	0.415