REVIEWS ARTICLE

Advances in neglected tropical disease vaccines: Developing relative potency and functional assays for the Na-GST-1/Alhydrogel hookworm vaccine

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Abstract

A new generation of vaccines for the neglected tropical diseases (NTDs) have now advanced into clinical development, with the Na-GST-1/Alhydrogel Hookworm Vaccine already being tested in Phase 1 studies in healthy adults. The current manuscript focuses on the often overlooked critical aspects of NTD vaccine product development, more specifically, vaccine stability testing programs. A key measure of vaccine stability testing is “relative potency” or the immunogenicity of the vaccine during storage. As with most NTD vaccines, the Na-GST-1/Alhydrogel Hookworm Vaccine was not developed by attenuation or inactivation of the pathogen (Necator americanus), so conventional methods for measuring relative potency are not relevant for this investigational product. Herein, we describe a novel relative potency testing program and report for the first time on the clinical lot of this NTD vaccine during its first 60 months of storage at 2–8°C. We also describe the development of a complementary functional assay that measures the ability of IgG from animals or humans immunized with Na-GST-1/Alhydrogel to neutralize this important hookworm enzyme. While 90% inhibition of the catalytic activity of Na-GST-1 was achieved in animals immunized with Na-GST-1/Alhydrogel, lower levels of inhibition were observed in immunized humans. Moreover, anti-Na-GST-1 antibodies from volunteers in non-hookworm endemic areas were better able to inhibit catalytic activity than anti-Na-GST-1 antibodies from volunteers resident in hookworm endemic areas. The results described herein provide the critical tools for the product development of NTD vaccines.
Author summary

As vaccines targeting NTDs advance into clinical trials, product development and vaccine maintenance become critical activities for the success of these vaccines. A key activity during this phase of vaccine development is the “relative potency” of a vaccine or the quality of the immune response that the vaccine elicits in an animal model during storage to ensure its immunogenicity is maintained. As with most NTD vaccines, the Na-GST-1/Alhydrogel Hookworm Vaccine was not developed using traditional methods of attenuating the pathogen (Necator americanus), so traditional measures of relative potency, such as testing the vaccine’s ability to protect against lethal challenge, could not be used. For the first time, we describe the development of a relative potency testing program for an NTD vaccine during five years of storage at 2–8˚C. We also describe the development of a complementary functional assay that measures the ability of IgG from animals or humans immunized with Na-GST-1/Alhydrogel to neutralize this important hookworm enzyme. The results described herein provide, for the first time in an open access format, critical tools for the development of future NTD vaccines.

Introduction

Over the next decade, a new generation of vaccines for the neglected tropical diseases (NTDs), especially those for helminthic parasites such as schistosomiasis and hookworm (Necator americanus) will advance into clinical trials [1]. The Na-GST-1/Alhydrogel Hookworm Vaccine has already entered into the initial stages of clinical development. As with other experimental vaccines for tropical diseases, including recombinant vaccines for Plasmodium falciparum malaria [2, 3], the Na-GST-1/Alhydrogel Human Hookworm Vaccine was not developed using the conventional methods of attenuation or inactivation of the pathogen that induce sterilizing immunity in the host [1]. The aim of the Na-GST-1/Alhydrogel Hookworm Vaccine is to induce neutralizing antibodies that will interfere with the role of glutathione S-transferase-1 (GST-1) in heme detoxification following blood digestion [4–6], inducing parasite death or reducing worm fecundity, thereby reducing protracted morbidity in the host [1, 7–10]. Conventional vaccine potency testing is often characterized by defining the doses of the vaccine that reproducibly protect immunized animals against lethal challenge from a pathogen and is in use for a number of vaccines, including those for tetanus, rabies, diphtheria, pertussis, and clostridia [11–14]. However, conventional vaccine potency testing programs are not feasible for the Na-GST-1/Alhydrogel Hookworm Vaccine due to the following characteristics of hookworms: (i) pathogenesis that results in chronic debilitating morbidity and not lethality, (ii) clinical outcomes that take years (sometimes even decades) to manifest in human hosts, and (iii) vaccine outcomes that are nearly impossible to replicate in laboratory animal models such as iron deficiency anemia from chronic hookworm infection as the animals become “refractory” to long-term infection [1, 15]. The vaccine potency testing program presented here is based on the levels of antibodies detected in the sera of animals immunized with defined doses of Na-GST-1/Alhydrogel [15]. Herein, we describe assessing the potency of the Na-GST-1/Alhydrogel Hookworm Vaccine formulation immediately after its manufacture (“potency at lot release”) and then its stability during 60 months of storage at 2–8˚C (“relative potency”). This vaccine potency assay program can serve as a model for the expanding NTD vaccine community and aid in the product and clinical development of the next generation of recombinant NTD vaccines.
Material and methods

**Na-GST-1/Alhydrogel hookworm vaccine**

*Necator americanus* glutathione-S-transferase-1 (Na-GST-1) is a 24-kDa recombinant protein from *N. americanus* expressed in *Pichia pastoris* and purified by three chromatographic steps [16]. The Na-GST-1 Clinical Drug Product (Aeras Lot# 09-69F-001) was formulated at a concentration of 0.1 mg/mL Na-GST-1 with 0.8 mg/mL of Alhydrogel in a glucose/imidazole buffer (10% dextrose, 10mM imidazole, pH 7.4). The drug product (vaccine) was produced according to current Good Manufacturing Practices (cGMP) on November 17th, 2010 (Aeras, Rockville, MD) and thereafter stored in temperature monitored refrigerators at 2–8˚C.

**Bioassay**

*Vivarium:* Seven to eight-week-old BALB/c mice were obtained from Taconic (Gaithersburg, MD). Mice were housed in a Bio-safety level-2 animal facility with a room temperature of 72 ± 4˚F under negative air pressure and with the air changed every 15–18 hours. A 12-hour day and dark light cycle was maintained in the animal facilities and the mice fed with Teklad Rodent Diet #2018. Mice were identified by ear puncture, and housed five animals per cage. Mice were acclimated to housing conditions one week prior to the start of the study. Mice were observed following immunizations for any signs of ill health (e.g., hunched, scruffy appearance, etc.). The animal studies reported herein were conducted at The George Washington University (Washington, DC), with the approval of its Institutional Animal Care and Use Committee (Protocol A042) and at the Baylor College of Medicine (Houston, TX) in compliance with its Institutional Animal Care and Use Committee (Protocol AN-5765).

*Study Design:* Fig 1 shows the bioassay used for the determination of potency immediately after manufacturing (lot release) and relative potency over 60 months. The pilot dose-ranging study (Fig 1A) utilized research grade recombinant Na-GST-1, and was performed prior to release of the clinical lot. The pilot dose-ranging study and subsequent release study consisted of two immunization cohorts (Fig 1A,B): Cohort 1 was immunized using a prime-only regimen with a terminal bleed on Study Day 28; Cohort 2, was immunized with a prime-boost regimen (Day 0 and Day 28) followed by a terminal bleed on Study Day 42. The study designs used for determination of relative potency over the 60-month stability period are shown in Fig 1B and 1C.

*Mice Immunizations:* BALB/c mice were divided into ten dose groups of ten mice each. BALB/c mice in Group 1 were immunized with Alhydrogel alone; BALB/c mice in Group 2 were immunized with Na-GST-1 alone; and BALB mice in Groups 3 to 9 were immunized in 1.75-fold increasing fractional doses of the vaccine starting from 1 μg to 30 μg of Na-GST-1, with the ratio of the dose of Na-GST-1 to Alhydrogel constant at 0.125 (Table 1). Study Group 10 (6 μg of formulated reference standard Na-GST-1) was included as an in-house reference control (lot# 082709APM) (Table 1). All immunizations were administered by the intraperitoneal route. Fractional dosing generated the doses: i.e., a volume containing the exact amount of Na-GST-1/Alhydrogel drug product was withdrawn into the syringe as opposed to diluting the vaccine to achieve these doses.

**Murine IgG against Na-GST-1 measured by a qualified indirect ELISA**

A qualified indirect ELISA described by Jariwala et al [15] and Bethony et al [17, 18] was used to measure levels of IgG against Na-GST-1 in murine serum samples. Ninety-six-well Polysorp microtiter plates (NUNC) were coated with 1 μg/mL of recombinant Na-GST-1 onto which experimental mouse sera were also added. Colorimetric reactions were read at a wavelength of
492nm on a SpectraMax 340PC (Molecular Devices) using SOFTmax Pro 5.4 for Windows for data capture and analysis.

Four-parameter logistic log modeling of the Standard Calibration Curve (SCC) using a Standard Reference Serum (SRS) of murine IgG against Na-GST-1

A standard reference serum (SRS) of murine IgG against Na-GST-1 was generated in 50 BALB/c mice with immunizations on Day 0 (prime) and then Day 21 (boost), with 0.05 μg

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**Fig 1. Vaccination and bleed schedules of the animal studies performed as part of the potency assay for Na-GST-1/Alhydrogel.** The symbols are as follows: bleed (B), vaccination (V), and sacrifice (S). (A) The schedule of the pilot potency study with two cohorts, each consisting of ten dose groups of ten mice. (B) The schedule for potency at release of cGMP lot #09-69F-001 (T = 0 months). Two cohorts were used for this study, each consisting of ten groups of ten mice. Note: The IgG ELISA results of the pilot potency study (Panel A) showed that the levels of antibodies on Study Day 14 were low so this bleed time point was not performed in subsequent studies. (C) The schedule of the relative potency studies at 3, 6, 9, 12, 18, 24, 36, 48, and 60 months post manufacture. All mice were vaccinated intraperitoneally on Study Day 0 and bled on Study Days -1 and 28 in this final study design.

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Na-GST-1/80μg Alhydrogel co-administered with 5μg CpG 10104 delivered intramuscularly (IM). A negative control (NC) pool was similarly prepared using ten BALB/c mice immunized IM with 80μg Alhydrogel only. The SRS was serially diluted in duplicate along 11 columns of the first two rows of each ELISA plate to generate a dilution–response curve modeled into a SCC by a four-parameter logistic-log function as described by Jariwala et al [15]. The linearity and parallelism of the SCCs were assessed as part of the qualification of the SRS, with the SCCs linearized through a transformation and an Analysis of Variance (ANOVA) test [15].

The Arbitrary Units (AU) of anti-Na-GST-1 IgG were obtained as described by us in Jariwala et al [15]. Indirect ELISAs for these studies were performed at eight different time points post release of the new vaccine lot, with a global SCC (GSCC) established at each time point (Fig 2). The GSCC is assembled from the individual SCCs from each plate in the experimental run (as described in Jariwala et al [15] and Quinn et al [19]). Briefly, all the individual SCC in an experimental run are fit to a single sigmoidal shaped 4-PL function that estimates the combined parameters into a GSCC. The GSCC is then used to interpolate the levels of IgG against Na-GST-1 and to determine the numbers of seroconverted animals to elucidate the ED\textsubscript{50} of the drug product at each time point.

**Determination of a Reactivity Threshold (RT), the median Effective Dose (ED\textsubscript{50}), potency at release, and Relative Potency (RP)**

The value of the Reactivity Threshold (RT) was obtained from the GSCC as discussed by us in detail in Jariwala et al [15] (Table 1). The probit function of SAS 9.2 was used to estimate the ED\textsubscript{50} with its 95% Fiducial Limits (95% FLs) as also described in [15] from the number of seropositive animals in each vaccination group. Based upon this model, the specification (as required by the Investigation New Drug [IND] application to the United States Food and Drug Administration (US-FDA)) for potency testing at release of the newly manufactured lot of Na-GST-1/Alhydrogel clinical drug product was set as the elicitation of an ED\textsubscript{50} with 95% FLs from Na-GST-1 absorbed to Alhydrogel immunized into nine groups of ten BALB/c mice with doses ranging from 1 to 30 μg of Na-GST-1.

### Table 1. The design and results of the Na-GST-1 relative potency study.

| Group | Na-GST-1 (μg) | Alhydrogel (μg) | Injection Volume (mL) | BALB/c Responders by Month\(^a\) |
|-------|---------------|-----------------|----------------------|----------------------------------|
|       |               |                 |                      | N 0 12 18 24 36 48 60            |
| 1     | –             | 240             | 0.30                 | 10 0 0 0 0 0 0 0                  |
| 2     | 30            | N/A             | 0.02                 | 10 0 0 0 0 0 0 0                  |
| 3     | 30            | 240             | 0.30                 | 10 8\(^b\) 9 10 10 9 8          |
| 4     | 17            | 136             | 0.17                 | 10 5 9 10 10 10 7               |
| 5     | 10            | 80              | 0.10                 | 10 2 8 9 6 8 9 5                |
| 6     | 6             | 48              | 0.06                 | 10 1 7 2 7 4 5 4                |
| 7     | 3             | 24              | 0.03                 | 10 0 0 1 1 1 2 1                |
| 8     | 2             | 16              | 0.02                 | 10 0 0 0 0 0 0 0                |
| 9     | 1             | 8               | 0.01                 | 10 0 0 0 0 0 0 0                |
| 10\(^c\)| 6             | 48              | 0.06                 | 10 3 9 9 5 3 7 4               |

\(^{a}\) The number of BALB/c mice seroconverting by dose group, with ten mice per group. Groups 1 & 2 were vaccinated with Alhydrogel 0.8 mg/mL or Na-GST-1 2 mg/mL of clinical drug substance alone, respectively. Groups 3–9 were vaccinated with decreasing fractional doses of the Na-GST-1/Alhydrogel clinical lot.

\(^{b}\) Lower due to death of one BALB/c mouse.

\(^{c}\) Immunizability group using a research standard reference lot of Na-GST-1/Alhydrogel.

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The relative potency (RP) of the clinical vaccine lot was estimated at eight post-release time points by the ED$_{50}$ and 95% FLs, which made it possible to estimate an RP at each of these time points post release to determine the stability of the vaccine [20]. Subsequent to this evaluation, the RP and its 95% FLs are estimated by regressing the dose-response curves and then dividing by their common slope. The RP is calculated by comparing the ED$_{50}$ at each post manufacture stability testing time point to the ED$_{50}$ at release (T = 0 months) by the following expression:

$$M_T = \frac{(a_T - a_s)}{b}$$
\[ M_T = \log(\text{potency ratio}) \]

\[ a_T = \text{intersection of linear regression of responses on log(dose) from the time point being tested} \]

\[ a_S = \text{intersection of linear regression of responses on log(dose) from the original release time point} \]

\[ b = \text{common slope} \]

The antilog of \( M_T \) is the ratio between the release potency time point \( (a_S) \) and then a time point subsequent to release \( (a_T) \) of the clinical drug product. All data are deposited in the Dryad Digital Data Repository (doi:10.5061/dryad.72v34) from the Dryad data repository. [21].

**Desorption of Na-GST-1 from Alhydrogel**

Na-GST-1/Alhydrogel was treated with a sodium citrate buffer to desorb the recombinant protein from Alhydrogel. In brief, a solution of 100 mM sodium citrate, 92 mM dibasic sodium phosphate, pH 8.9 (unadjusted) was prepared and sterile filtered. The desorption buffer was then mixed with the re-suspended formulation at a ratio of 2:1 (buffer: vaccine), inverted ten times, and incubated for 60 minutes at room temperature (mixing by inversion every 20 minutes). The mixture was then centrifuged at 2,000 g for two minutes and supernatant (desorbed protein solution) or pellet (Alhydrogel) mixed with 2X sample buffer and analyzed via SDS-PAGE. Methodology for determining vaccine identity and integrity by SDS-PAGE including densitometry are reported in Plieskatt et al [22]. Pre-cast 4–20% Tris-glycine gels (Invitrogen) were utilized for electrophoresis under reduced (DTT) and non-reduced conditions, followed by Coomassie staining utilizing Phastgel Blue R (GE Healthcare) or silver staining [22]. Densitometry was completed using a GS-800 calibrated densitometer (Bio-Rad) with Quantity One (Bio-Rad) Software for analysis.

**Fractionation of IgG from sera of animals and humans immunized with Na-GST-1/Alhydrogel**

Mouse serum samples obtained from dose-ranging studies of Na-GST-1/Alhydrogel were used to optimize the assay. In brief, IgG were fractionated from Study Day 42 sera of mice immunized with fractional doses of approximately 6, 10, 17, and 30 μg in a prime-boost regimen. Human plasma samples were utilized from two separate Phase 1 studies of the safety and immunogenicity of Na-GST-1/Alhydrogel in non-endemic areas (Washington, DC [NCT01385189] and Belo Horizonte, Brazil [NCT01261130]) and from a N. americanus endemic area in Minas Gerais state, Brazil (NCT01261130). In each trial, subjects were vaccinated on Study Days 0, 56, and 112, with blood collected on Study Day 126 (i.e., two weeks after the third vaccination) for the primary assessment of immunogenicity. Plasma collected prior to prime immunization (Study Day 0) and two weeks after the third immunization (Study Day 126) were IgG fractionated for the purposes of the neutralization assay described below. In the United States Phase 1 trial, 34 of 40 enrolled subjects completed the Study Day 126 visit, whereas in the Brazil trial, 96 of 102 enrolled subjects did.

IgG fractions from human plasma and mouse serum samples were purified by Protein G Spin Plates (Thermo Scientific) according to the manufacturer’s protocol. In brief, Protein G Spin Plates were equilibrated to room temperature and equilibrated with Binding Buffer (0.1M sodium phosphate, 0.15M sodium chloride, pH 7.2). All sera or plasma samples were diluted 1:1 in Binding Buffer, added to the wells of the spin plates, and the spin plates agitated on a plate shaker for 30 minutes at RT. Protein G Spin Plates were centrifuged with collection plates
to remove the flow-through. Four wash steps consisted of the addition of binding buffer to each well followed by centrifugation of the spin plates with collection plates. To elute the total IgG from sera or plasma sample, 0.1M glycine pH 2.0 was added to each well followed by agitation on a plate shaker for one minute. The spin plates were centrifuged to collect the elution into collection plates already containing 1M Tris pH 9.0 to neutralize the pH of the eluate. Purified IgG was stored at 2–8˚C until analysis within seven days.

The Na-GST-1 neutralization assay

A schematic of the neutralization assay is shown in Supplementary S1 Fig. In brief, neutralization of Na-GST-1 activity by purified IgG from mice or humans immunized with Na-GST-1/Alhydrogel was measured utilizing the GST Fluorometric Activity Assay Kit (BioVision). To assay the purified human IgG, the manufacturer’s protocol was followed with modifications to sample volumes and as further noted here. In a 96-well black plate (Thermo Scientific), duplicate sample wells were prepared with a total volume of 100 μl, consisting of 90 μl of Na-GST-1 (0.25 μg) diluted in GST Assay Buffer and 10 μl of purified IgG. Blank wells contained 100 μl of GST Assay Buffer alone. Additional control wells (total volume of 100 μl) contained GST alone or IgG and were measured in quadruplicate. Plates were incubated at 37˚C for one hour with agitation. After incubation, 10 μl of Glutathione was added to each well. To initiate the reaction, monochlorobimane solution was diluted 1:50 in GST Assay Buffer and 100 μl was added to each well. The plate was placed in the SpectraMax Paradigm Multimode Plate Reader (Molecular Devices), shaken for one second, and then fluorescence measured at Ex/Em 380 nm/460 nm every five minutes for one hour. The measurement of Relative Fluorescence Units (RFUs) at 15 minutes was used for analysis. Purified mouse IgG was assayed in the same manner except for an experiment where the volume of purified IgG was varied at 1, 5, 10, and 20 μl.

Western blots for Na-GST-1 specific IgG using sera of animals and plasma of humans immunized with Na-GST-1/Alhydrogel

To confirm the presence of Na-GST-1 specific IgG in the purified mouse and human IgG samples, Western Blots were performed. Five micrograms of reduced and non-reduced recombinant Na-GST-1 were diluted in Sample Buffer (Life Technologies) and heated at 95˚C for five minutes and loaded on 4–20% Tris-glycine gels (Life Technologies) with See Blue Plus2 standard (Life Technologies). For reduced Na-GST-1 samples, β-mercaptoethanol was included in the sample preparation. The gels were run in SDS running buffer (Life Technologies) at 135V for 90 minutes using the PowerEase 500 Power Supply and XCell SureLock Mini-Cell system (Invitrogen). Gels were transferred to nitrocellulose membranes (Life Technologies) in Transfer Buffer (Life Technologies) at 30V for 60 minutes using the PowerEase 500 Power Supply and XCell II Blot Module (Life Technologies) and stained with Ponceau S (Sigma-Aldrich) to confirm transfer of the protein occurred.

Anti-Na-GST-1 IgG (mouse). Blocking, primary antibody incubation, washes, secondary antibody incubation, and developing were performed with the WesternBreeze Chromogenic Immunodetection System (Life Technologies) according to the manufacturer’s protocol. The purified mouse IgG (primary antibody) was diluted 1:200 in supplied buffer.

Anti-Na-GST-1 IgG (human). Following Ponceau S staining, membranes were blocked in PBS with 0.05% Tween 20 (PBS-T) and 3% skim milk powder for 1 hour (room temperature) or overnight (2–8˚C). Membranes were then incubated with purified human IgG diluted 1:200 in blocking buffer for 45 minutes with gentle shaking at room temperature. Following three washes with PBS-T, the membranes were then incubated with the secondary antibody anti-
human total IgG (KPL) diluted at 1:10,000. Membranes were again washed three times with PBS-T, and developed with BCIP/NBT Phosphatase Substrate System (KPL).

Results

Modeling the standard calibration curve and determining its linearity and a reactivity threshold

Following pilot potency assay development as described in by us in [15] and further outlined in the bioassay study design (Fig 1), the reactivity threshold (RT) was determined for each time point during the 60 months of stability testing. Fig 2A shows the sigmoidal 4-parameter logistic log (4-PL) function of the SCC for the potency studies of Na-GST-1/Alhydrogel as described previously by us [15]. The parallelism of the linearized SCCs (N = 9), along with the p-values from the ANOVA test are shown in Fig 2B and Fig 3, with the ANOVA test showing no significant deviation from parallelism for the nine SCCs except at the six-month time point.

| Months Post-Manufacture | RP (95% FL)       | Parallelism |
|-------------------------|-------------------|-------------|
| 60                      | 1.64 (1.01, 2.79) | 0.213       |
| 48                      | 2.71 (1.78, 4.28) | 0.699       |
| 36                      | 2.42 (1.67, 3.62) | 0.457       |
| 24                      | 2.56 (1.73, 3.89) | 0.814       |
| 18                      | 2.15 (1.44, 3.32) | 0.996       |
| 12                      | 2.49 (1.70, 3.75) | 0.654       |
| 9                       | 4.22 (2.76, 6.83) | 0.591       |
| 6                       | 2.77 (1.50, 5.90) | 0.031       |
| 3                       | 3.19 (2.11, 5.06) | 0.733       |

**Fig 3. Relative Potency of Na-GST-1/Alhydrogel.** Relative Potency (RP) and the 95% Fiducial Limits of Na-GST-1/Alhydrogel lot #09-69F-001 at the time of release (0 months) and at post-manufacture testing time points of three months through 60 months of storage. The specification for acceptance was that the upper 95% Fiducial Limit of the RP should not be less than 0.50.

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Determination of the ED$_{50}$ using a probit model

The number of responders by dose group is shown in Table 1, which includes results from potency testing at lot release and at nine subsequent time points post-release (60 months). The ED$_{50}$ at lot release was 15.74 μg (95% FL = 11.74, 23.36) from which the RP specification for the manufactured lot of Na-GST-1/Alhydrogel was established for subsequent stability testing time points. The specification for potency in the Investigational New Drug (IND) application to the US Food and Drug Administration for Na-GST-1/Alhydrogel requires that the upper 95% FL of the RP value remains above 0.50 (Fig 3): i.e., a point that describes a vaccine potency that is half of the potency at the time of release. In other words, if the value of the upper 95% FL falls below the value of 0.50, the vaccine lot of Na-GST-1/Alhydrogel is considered to have “lost” its potency, which means that this lot of vaccine could no longer be used in clinical trials with human volunteers. However, increased potency was observed after three months of storage post-manufacture, with the RP 3.19-times higher than the value obtained at lot release (Fig 3). At subsequent testing time points, the RP remained stable at this value over the remaining 60 months of potency testing (Table 2, Fig 3).

Decrease in desorption efficiency of Na-GST-1 from Alhydrogel post manufacture coincides with a gain in relative potency

The change in relative potency during the first three months post manufacture of Na-GST-1 to Alhydrogel could be attributed to the increased affinity of Na-GST-1 to Alhydrogel during the first three months of the storage as shown represented in Fig 4, which demonstrates the relationship between the RP and the percentage of protein remaining bound to Alhydrogel after desorption conducted at the same time points.

The Na-GST-1 vaccine was formulated as a suspension of 0.1 mg/mL Na-GST-1 and 0.8 mg/mL Alhydrogel and was optimized in a glucose/imidazole buffer to permit 100% absorption of the recombinant protein to Alhydrogel as well as to enhance stability over time [22]. The clinical lot of Na-GST-1/Alhydrogel met other biochemical specifications at release, including 100% absorption to Alhydrogel, identity as Na-GST-1, and purity by SDS-PAGE [16]. Na-GST-1/Alhydrogel demonstrated biochemical stability as indicated by this series of

### Table 2. The median Effective Dose 50 (ED$_{50}$) for Potency at Lot Release and Relative Potency over 60 months for the Na-GST-1/Alhydrogel Hookworm Vaccine.

| Potency measure | Months Post Manufacture | 0$^a$ | 12 | 18 | 24 | 36 | 48 | 60 |
|-----------------|-------------------------|------|----|----|----|----|----|----|
| ED$_{50} \pm$    |                         | 15.74| 6.26| 7.31| 6.12| 6.39| 5.88| 10.44|
| (95% FL)        |                         | (11.74, 23.36) (4.74, 8.28) (2.97, 16.10) (4.56, 8.10) (4.87, 8.33) (2.56, 12.17) (7.21, 16.23) |
| RP$^d$          |                         | 2.49 | 2.15 | 2.56 | 2.42 | 2.71 | 1.64 |
| (95% FL)        |                         | (1.70, 3.75) (1.44, 3.32) (1.73, 3.89) (1.67, 3.62) (1.78, 4.28) (1.01, 2.79) |
| Parallelism$^e$ |                         | 0.654| 0.996| 0.814| 0.457| 0.699| 0.213 |

$^a$ Potency at “lot release” as it is the first time point after current Good Manufacturing Practice (cGMP) manufacture.

$^b$ Effective Dose 50 or the lowest concentration of the vaccine that seroconverts 50% of the animals in a defined dose group of ten animals.

$^c$ FL refers to the 95% Fiducial Limits.

$^d$ RP refers to Relative Potency.

$^e$ Chi-square p-value. Parallelism for Relative Potency degrees freedom (df) = 10.

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stability assays conducted over time, including identity and purity, and remained consistently absorbed to Alhydrogel [16]. In addition to this routine stability testing, the cGMP lot of Na-GST-1/Alhydrogel vaccine was subjected to testing including isoelectric focusing (deamidation) and antigen desorption, with no changes observed in the isoelectric point (which would indicate deamidation) with these data shown in [22]. A sodium citrate buffer (pH 8.9) was utilized to desorb the recombinant protein Na-GST-1 from its adjuvant Alhydrogel. The efficiency of this desorption (i.e., the ability to desorb Na-GST-1 from Alhydrogel) as measured by SDS-PAGE and densitometry, decreased over the first nine months post manufacture but stabilized thereafter (Fig 4). When plotting the RP of Na-GST-1/Alhydrogel clinical drug product as measured by the potency assay over time through eighteen months post manufacture. The X-axis refers to the time points post manufacture.

Western blotting for IgG against Na-GST-1 from mice and humans immunized with Na-GST-1/Alhydrogel

Antibodies (IgG) purified from both mouse sera and human plasma were confirmed to be reactive to the recombinant antigen Na-GST-1. Western blots (Fig 5) performed on sera from a selection of BALB/c mice immunized with Na-GST-1/Alhydrogel and from a selection of
human plasma from a Phase 1 clinical trial of Na-GST-1/Alhydrogel in Brazilian adults confirmed the presence of IgG against Na-GST-1 in these purified samples (Fig 5). Specifically, these purified IgG samples recognized both non-reduced and reduced Na-GST-1 at approximately 24 kilo Daltons (kDa). It should be noted that Na-GST-1 under non-reduced conditions appears as a doublet as previously reported [16, 22].

Inhibition of catalytic activity of Na-GST-1 by IgG from mice immunized with Na-GST-1/Alhydrogel

Having demonstrated the ability to purify IgG from mouse sera and the positive reactivity of such sera with recombinant Na-GST-1, we employed analysis with a fluorescence-based assay to measure possible inhibition of such antibodies to Na-GST-1 catalytic activity. It should be noted that this assay measured Glutathione-S-Transferase activity, and therefore is only representative of Na-GST-1 activity; inhibition through this assay is not necessarily indicative of the mechanism of native Na-GST-1 in *N. americanus*, which is thought to be detoxification of free heme that is the end product of the worm’s hemoglobin digestion pathway [4]. This shows that immunization with Na-GST-1/Alhydrogel can elicit antibodies that block the recombinant protein’s catalytic activity.

First, the non-specific activity present in IgG purified from mouse sera was measured. A commercial mouse IgG (Jackson ImmunoResearch), which is presumably non-reactive to GST, did not inhibit Na-GST-1 glutathione-S-transferase activity (Fig 6, Panel A). To determine if IgG purified from mice immunized with Na-GST-1/Alhydrogel showed a dose-dependent inhibition of Na-GST-1 glutathione-S-transferase activity, sera from mice immunized with increasing doses of Na-GST-1/Alhydrogel (Fig 6 Panel B) were assayed. With increasing doses of Na-GST-1/Alhydrogel, a concomitant increase in the percent of Na-GST-1 inhibited
was observed (Fig 6 Panel B); specifically, sera pooled from mice immunized with doses of approximately 6, 10, 17, and 30 μg of Na-GST-1/Alhydrogel yielded purified IgG that inhibited
Na-GST-1 glutathione-S-transferase activity by 0, 1, 9, and 22%, respectively, utilizing a consistent volume of IgG (10 μL). To determine the impact of various volumes of IgG and if a “dose response” (volume) could be observed, a purified IgG pool of sera from mice immunized with 30 μg of Na-GST-1/Alhydrogel was added to the reaction. In this case, inhibition of Na-GST-1 was 0, 13, 22, and 56% when 1, 5, 10, and 20 μl of this pool were added (Fig 6, Panel C). Finally, Fig 6 Panel D shows the results of a reference pool of purified IgG from mice immunized with 30 μg of Na-GST-1 run in three technical replicates in the neutralization assay to determine the reproducibility of the assay and the effect of different volumes of this reference pool. 10 μl of the purified IgG pool inhibited Na-GST-1 activity by 24 ± 4% and 20 μl of the purified IgG pool inhibited activity by 52 ± 3%.

Inhibition of the catalytic activity of Na-GST-1 by purified IgG from humans immunized with Na-GST-1/Alhydrogel hookworm vaccine

After demonstrating successful inhibition of Na-GST-1 in our enzymatic assay with sera derived from immunized mice, we sought to demonstrate the same inhibitory capability with human plasma samples obtained from Phase 1 trials of Na-GST-1/Alhydrogel conducted in the US and Brazil. As human plasma contains components capable of non-specific inhibition of Na-GST-1 and interferes in the fluorescence-based enzymatic assay. IgG was fractionated from human plasma and used alongside a commercially available purified human IgG as a control, as neither induced non-specific inhibition of Na-GST-1.

The percent inhibition of Na-GST-1 glutathione-S-transferase activity was significantly greater when incubated with IgG purified from the plasma of humans immunized with Na-GST-1/Alhydrogel (any dose) at Study Day 126 (2 weeks after the third immunization) than when incubated with IgG purified from the plasma of humans at Study Day 0 (prior to immunization) (p-value = 0.0002) (Fig 7, Panel A) or with study controls (p-value = 0.0002). Of the 130 individuals with plasma samples at Study Day 126, only 6 samples from the United States trial and 8 samples from Brazilian yielded purified IgG capable of 20% or greater inhibition of Na-GST-1. The relationship between percent inhibition of Na-GST-1 glutathione-S-transferase activity and the dose of Na-GST-1/Alhydrogel administered to individuals is shown in Fig 7, Panel B. In most cases, individuals immunized with Na-GST-1/Alhydrogel had higher inhibition of Na-GST-1 glutathione-S-transferase activity than individuals who received the hepatitis B comparator vaccine (ButaNG, Instituto Butantan, São Paulo, Brazil) (Fig 7, Panel B). However, the dose of Na-GST-1/Alhydrogel or the co-administration of Na-GST-1/Alhydrogel with an aqueous formulation of the immunostimulant Glucopyranosyl-Lipid A (GLA-AF) were not found to be factors in the inhibition of the catalytic activity of Na-GST-1 by purified IgG from human plasma. On the other hand, purified IgG from plasma of individuals resident in hookworm non-endemic areas (USA and Belo Horizonte, Brazil) was significantly higher than the inhibition of Na-GST-1 glutathione-S-transferase activity compared to individuals from a hookworm endemic area in Brazil (Fig 7, Panels C and D) (p = 0.0096) and had significantly greater increases in inhibition from Day 0 to Day 126 (p < 0.0001).

Discussion

The current manuscript focuses on a novel vaccine potency program for the Na-GST-1/Alhydrogel Hookworm Vaccine, with ramifications for the development of other NTD vaccines. In contrast to conventional vaccine potency testing programs, our program is unique in that if the vaccine antigen is presented to an animal immune system in a consistent form and quantity, it will induce a reproducible level of antibody responses to indicate potency at lot release (immediately cGMP post-manufacture) and then relative potency at pre-determined time.
points post-manufacture [11–14]. We also describe the development of a functional assay that measures the ability of IgG antibodies elicited by immunization with Na-GST-1/Alhydrogel to impair the catalytic activity of Na-GST-1 as part of the potency testing program, with the potential for it to be developed into a correlate of protection (CoP) as the vaccine advances through clinical development [23].

The first cGMP-manufactured lot of the Na-GST-1/Alhydrogel Hookworm Vaccine was determined to be “potent” immediately after manufacture (i.e., lot release) and remained potent over 60 months of storage at 2–8°C. A notable increase in relative potency was observed
over the first nine months post release. A factor often implicated in increased vaccine potency immediately after manufacture is the vaccine’s formulation: i.e., a change in the interaction between the protein and the adjuvant. This change may be due to a number of factors, including the auto-extraction of impurities or enhanced binding of the recombinant protein to the adjuvant, resulting in conformational changes to the antigen such as aggregation or deamidation. While no concomitant aggregation or deamidation of the protein was observed three-months post-manufacture, we did observe increased binding of Na-GST-1 to Alhydrogel, as evidenced by the inability to remove the antigen from Alhydrogel through standard de-absorption procedures proximal to this potency time point. The increased binding may have resulted in the exposure of more immunogenic epitopes on the surface of the formulation, making the vaccine more potent in the animals. This coincidence of immunological and biochemical measures illustrates the importance of combining such assays when measuring the stability of recombinant proteins absorbed to Alhydrogel.

Our immunogenicity-based potency assay program was complemented with an assay that attempted to parallel one potential mechanism for the Na-GST-1 Human Hookworm Vaccine in humans [23–25]. Na-GST-1 has peroxidase activity as it catalyzes the conjugation of reduced glutathione to a variety of electrophiles [4–6]. As such, we designed a functional assay (S1 Fig) to measure the percent inhibition of Na-GST-1 glutathione-S-transferase activity when the enzyme is incubated with purified IgG from immunized animals or humans. In pools of sera from mice immunized with increasing doses of Na-GST-1/Alhydrogel, we observed a dose-dependent impairment of Na-GST-1 glutathione-S-transferase activity. Moreover, an increase in the inhibition of glutathione-S-transferase activity was also observed when the volume of purified IgG from mice was increased. A limitation of this catalytic inhibition assay is that it does not directly measure the effect of neutralizing antibodies on the putative heme detoxification role of Na-GST-1 in N. americanus worms. Nor does it take into account the potential effects of GSTs as immunomodulators, as shown for example by the extracellular GST from Onchocerca volvulus (OvGST1), which is thought to participate in the modulation of host immune responses by production of parasite-derived prostanoids that downregulate the effector response [26]. In general, plasma from study participants two weeks after the third immunization with the Na-GST-1/Alhydrogel Hookworm Vaccine showed a significantly higher inhibition of Na-GST-1 catalytic activity compared to baseline regardless of the vaccine dose or co-administration with the immunostimulant GLA-AF. However, few human samples (n = 20) produced greater than 20% inhibition of Na-GST-1 glutathione-S-transferase activity. This low response rate in humans indicates the need for further advances in vaccine formulation, immunization regimen, and possibly the co-administration with another immunostimulant.

Several components of the current potency assay are especially applicable to NTD vaccine production, principal among them being the choice of an animal model and the statistical methods used to measure relative potency [11, 14, 15]. Conventional potency assays use animal models based upon “their permissiveness to lethal infection with the target pathogen” [13]. However, the Na-GST-1/Alhydrogel potency assay measured an antibody response by dose group; hence, the choice of an animal model was not limited to models permissive to hookworm infection such as hamsters or canines [1, 27], neither of which maintain the infection long enough to measure the clinical endpoints of human hookworm disease (e.g., iron deficiency anemia) for which the vaccine will be indicated. Instead, we chose an animal model (i.e., BALB/c mice) based on the ability to induce a reproducible antibody response [15, 28]. Another critical choice was the immunization schedule (0 and 28 days), which depended on several factors, including the animal model, the route of immunization (IP), and the volume of the immunization [15].
A final novel component to this assay was the statistical approach to determine potency and relative potency. The Na-GST-1/Alhydrogel potency testing program varied traditional potency assays by utilizing a ‘quantal’ assay design [2, 14, 29], which contrasts with conventional “quantitative” immunogenicity methods that use ‘measures of central tendency’ to characterize potency such as the mean, median, and standard deviation of an antibody response [11]. As noted by Giersing et al. [2] for potency testing of recombinant malaria vaccines, quantitative potency approaches have the advantage of producing readily analyzable data, but their utility is offset by the poor precision of measures of central tendency due to the a high degree of variation in ELISA assays.

Finally, by modifying a commercially available enzymatic assay into a functional assay for this vaccine, we were able to demonstrate that antibodies raised to Na-GST-1 may also have a functional application. However, further work is needed to optimize such this functional assay before it can become either a part of the current potency testing program or as a correlates of protection in the future.

Supporting information

S1 Fig. Schematic of the process for the Na-GST-1 catalytic inhibition assay. Outset box shows the chemical reaction of monochlorobimane (MCB) with glutathione resulting in fluorescence. (TIF)

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References

1. Bethony JM, Cole RN, Guo X, Kamhawi S, Lightowler MW, Loukas A, et al. Vaccines to combat the neglected tropical diseases. Immunol Rev. 2011; 239(1):237–70. PubMed Central PMCID: PMCPMC3439653. doi: 10.1111/j.1600-065X.2010.00976.x PMID: 21198676

2. Giersing BK, Dubovsky F, Saul A, Denamur F, Minor P, Meade B. Potency assay design for adjuvanted recombinant proteins as malaria vaccines. Vaccine. 2006; 24(20):4264–70. PMID: 16767804
3. Zhu D, McClellan H, Dai W, Gebregziorgis E, Kidwell MA, Aebig J, et al. Long term stability of a recombinant *Plasmodium falciparum* AMA1 malaria vaccine adjuvanted with Montanide(R) ISA 720 and stabilized with glycine. Vaccine. 2011; 29(20):3640–5. PubMed Central PMCID: PMCPMC3089892. doi: 10.1016/j.vaccine.2011.03.015 PMID: 21440641

4. Asojo OA, Ceccarelli C. Structure of glutathione S-transferase 1 from the major human hookworm parasite *Necator americanus* (Na-GST-1) in complex with glutathione. Acta Crystallogr F Struct Biol Commun. 2014; 70(Pt 9):1162–6. doi: 10.1107/S2053230X1401646X PMID: 25195885

5. Zhan B, Liu S, Perally S, Xue J, Fujiwara R, Brophy P, et al. Biochemical characterization and vaccine potential of a heme-binding glutathione transferase from the adult hookworm *Ankylostoma caninum*. Infect Immun. 2005; 73(10):6903–11. PubMed Central PMCID: PMCPMC1230892. doi: 10.1128/IAI.73.10.6903-6911.2005 PMID: 16177370

6. Zhan B, Perally S, Brophy PM, Xue J, Goud G, Liu S, et al. Molecular cloning, biochemical characterization, and partial protective immunity of the heme-binding glutathione S-transferases from the human hookworm *Necator americanus*. Infect Immun. 2010; 78(4):1552–63. PubMed Central PMCID: PMCPMC2849424. doi: 10.1128/IAI.00848-09 PMID: 20145100

7. Loukas A, Gaze S, Mulvenna JP, Gasser RB, Brindley PJ, Doolan DL, et al. Vaccinomics for the major blood feeding helminths of humans. OMICS. 2011; 15(9):567–77. doi: 10.1089/omi.2010.0150 PMID: 21679087

8. Hotez PJ, Bethony JM, Diemert DJ, Pearson M, Loukas A. Developing vaccines to combat hookworm infection and intestinal schistosomiasis. Nat Rev Microbiol. 2010; 8(11):814–26. doi: 10.1038/nrmicro2438 PMID: 20948553

9. Hotez PJ, Bethony JM, Oliveira SC, Brindley PJ, Loukas A. Multivalent anthelmintic vaccine to prevent hookworm and schistosomiasis. Expert Rev Vaccines. 2008; 7(6):745–52. doi: 10.1586/14760584.7.6.745 PMID: 18665774

10. Diemert DJ, Bethony JM, Hotez PJ. Hookworm vaccines. Clin Infect Dis. 2008; 46(2):282–8. doi: 10.1086/524070 PMID: 18171264

11. Arciniega J, Sirota LA. Potential application of the consistency approach for vaccine potency testing. Dev Biol (Basel). 2012; 134:135–9.

12. Hendriksen CF. The consistency approach for the quality control of vaccines. Biologicals. 2008; 36(1):73–7. doi: 10.1016/j.biologicals.2007.05.002 PMID: 17892948

13. Hendriksen CF. Refinement, reduction, and replacement of animal use for regulatory testing: current best scientific practices for the evaluation of safety and potency of biologicals. ILAR J. 2002; 43 Suppl: S43–8.

14. Hendriksen CF. Replacement, reduction and refinement alternatives to animal use in vaccine potency measurement. Expert Rev Vaccines. 2009; 8(3):313–22. doi: 10.1586/14760584.8.3.313 PMID: 19249973

15. Jariwala AR, Oliveira LM, Diemert DJ, Keegan B, Plieskatt JL, Periago MV, et al. Potency testing for the experimental *Na-GST-1* hookworm vaccine. Expert Rev Vaccines. 2010; 9(10):1219–30. doi: 10.1586/erv.10.107 PMID: 20923271

16. Goud GN, Deumic V, Gupta R, Brelsford J, Zhan B, Gillespie P, et al. Expression, purification, and molecular analysis of the *Necator americanus* glutathione S-transferase 1 (*Na-GST-1*): a production process developed for a lead candidate recombinant hookworm vaccine antigen. Protein Expr Purif. 2012; 83(2):145–51. doi: 10.1016/j.pep.2012.03.013 PMID: 22503665

17. Bethony JM, Simon G, Diemert DJ, Parenti D, Desrosiers A, Descomps J, et al. The consistency approach for the quality control of vaccines. Biologicals. 2008; 36(1):73–7. doi: 10.1016/j.biologicals.2007.05.002 PMID: 17892948

18. Goud GN, Deumic V, Gupta R, Brelsford J, Zhan B, Gillespie P, et al. Expression, purification, and molecular analysis of the *Necator americanus* glutathione S-transferase 1 (*Na-GST-1*): a production process developed for a lead candidate recombinant hookworm vaccine antigen. Protein Expr Purif. 2012; 83(2):145–51. doi: 10.1016/j.pep.2012.03.013 PMID: 22503665

19. Jariwala AR, Oliveira LM, Diemert DJ, Keegan B, Plieskatt JL, Periago MV, et al. Potency testing for the experimental *Na-GST-1* hookworm vaccine. Expert Rev Vaccines. 2010; 9(10):1219–30. doi: 10.1586/erv.10.107 PMID: 20923271

20. Goud GN, Deumic V, Gupta R, Brelsford J, Zhan B, Gillespie P, et al. Expression, purification, and molecular analysis of the *Necator americanus* glutathione S-transferase 1 (*Na-GST-1*): a production process developed for a lead candidate recombinant hookworm vaccine antigen. Protein Expr Purif. 2012; 83(2):145–51. doi: 10.1016/j.pep.2012.03.013 PMID: 22503665

21. Brelsford JB, Plieskatt JL, Yakovleva A, Jariwala A, Keegan BP, Peng J, Xia P, Li G, Campbell D, Periago MV, Correa-Oliveira R, Bottazzi ME, Hotez PJ, Diemert D, Bethony JM. Advances in Neglected
Tropical Disease Vaccines: Developing Relative Potency and Functional Assays for the Na-GST-1/Alhydrogel Hookworm Vaccine. Dryad Data Repository.

22. Plieskatt JL, Rezende WC, Olsen CM, Trefethen JM, Joshi SB, Middaugh CR, et al. Advances in vaccines against neglected tropical diseases: enhancing physical stability of a recombinant hookworm vaccine through biophysical and formulation studies. Hum Vaccin Immunother. 2012; 8(6):765–76. doi: 10.4161/hv.19726 PMID: 22495115

23. Plotkin SA. Immunologic correlates of protection induced by vaccination. Pediatr Infect Dis J. 2001; 20(1):63–75. PMID: 11176570

24. Plotkin SA. Correlates of protection induced by vaccination. Clin Vaccine Immunol. 2010; 17(7):1055–65. PubMed Central PMCID: PMCPMC2897268. doi: 10.1128/CVI.00131-10 PMID: 20463105

25. Plotkin SA. Complex correlates of protection after vaccination. Clin Infect Dis. 2013; 56(10):1458–65. doi: 10.1093/cid/cit048 PMID: 23386629

26. Perbandt M, Hoppner J, Burmeister C, Luersen K, Betzel C, Liebau E. Structure of the extracellular glutathione S-transferase OvGST1 from the human pathogenic parasite Onchocerca volvulus. J Mol Biol. 2008; 377(2):501–11. doi: 10.1016/j.jmb.2008.01.029 PMID: 18258257

27. Fujiwara RT, Geiger SM, Bethony J, Mendez S. Comparative immunology of human and animal models of hookworm infection. Parasite Immunol. 2006; 28(7):285–93. doi: 10.1111/j.1365-3024.2006.00821.x PMID: 16842265

28. Schneider B, Jariwala AR, Periago MV, Gazzinelli MF, Bose SN, Hotez PJ, et al. A history of hookworm vaccine development. Hum Vaccin. 2011; 7(11):1234–44. PubMed Central PMCID: PMCPMC3323499. doi: 10.4161/hv.7.11.18443 PMID: 22064562

29. Finney DJ. Probit Analysis. Cambridge, UK: Cambridge University Press; 1971.