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MAJOR ARTICLE

Controlled Human Hookworm Infection: Accelerating Human Hookworm Vaccine Development

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Background. Controlled human hookworm infection (CHHI) is a central component of a proposed hookworm vaccination-challenge model (HVCM) to test the efficacy of candidate vaccines. Critical to CHHI is the manufacture of *Necator americanus* infective larvae (*Na*L3) according to current Good Manufacturing Practice (cGMP) and the determination of an inoculum of *Na*L3 that is safe and reliably induces patent infection.

Methods. cGMP-grade *Na*L3 were produced for a phase 1 trial in 20 healthy, hookworm-naïve adults in the United States, who received either 25 or 50 *Na*L3. Participants were monitored for 12–18 weeks postinfection for safety, tolerability, and patency of *N. americanus* infection.

Results. Both *Na*L3 doses were well tolerated. Early manifestations of infection included pruritus, pain, and papulovesicular rash at the application site. Gastrointestinal symptoms and eosinophilia appeared after week 4 postinfection. The 50 *Na*L3 inoculum induced patent *N. americanus* infection in 90% of this dose group.

Conclusions. The inoculum of 50 *Na*L3 was well tolerated and consistently induced patent *N. americanus* infection suitable for future HVCM trials.

Clinical Trials Registration. NCT01940757.

Keywords. challenge model; controlled human hookworm infection; current good manufacturing practice; hookworm vaccine; *Necator americanus*.

More than 400 million individuals are infected with hookworms, hematophagic nematodes that parasitize the host gastrointestinal tract [1]. Chronic hookworm infection can induce iron deficiency anemia, with children and women of childbearing age being most at risk [2]. Current control efforts rely on mass drug administration (MDA) with a benzimidazole (BZ) that cures established infections but does not prevent re-infection [3]. Furthermore, development of resistance to BZs, as seen in livestock [4, 5], is a significant concern. Given the limitations of MDA, a preventative human hookworm vaccine (HHV) is being developed. The current HHV candidate antigens, *Na*-GST-1 and *Na*-APR-1, have been evaluated in clinical trials in the United States, Brazil, and Gabon and have been found to be safe and immunogenic [6, 7].

The next stage of clinical development for the HHV is to assess vaccine efficacy, which conventionally requires costly field trials in *Necator*-endemic areas, with large sample sizes (~1200 volunteers) and lengthy duration (1–2 years) [8]. To mitigate the expense and risk of such trials, we propose a hookworm vaccination-challenge model (HVCM) to provide early proof of concept of vaccine efficacy that would entail immunizing hookworm-naïve adults from nonendemic areas with candidate vaccines and then challenging them with infective *Necator americanus* larvae (*Na*L3). Efficacy would be determined by the absence of patent infection (no detectable eggs in feces) or a reduction in infection intensity (expressed by eggs per gram [EPG] of feces) compared with unvaccinated controls [7, 9, 10].

Although hookworm-naïve volunteers have been infected with *N. americanus* in prior studies [11–20], we report the first trial in which *Na*L3 produced according to current Good Manufacturing Practice (cGMP) were administered to healthy volunteers to determine the tolerability of different doses and to establish an effective inoculum for future HVCM trials.

METHODS

cGMP Manufacture of *NaL3*

Necator americanus larvae were manufactured by the Immune Modulation Research Group (IMRG) at the

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University of Nottingham, United Kingdom, under an Investigational Medicinal Product Dossier from the UK Medicines and Healthcare products Regulatory Agency (UK MIA [IMP] 3057). Briefly, feces were collected from a chronically infected volunteer who was screened for communicable agents considered relevant for topically applied investigational medicinal products, per 21CFR§1271.3(r)(1) (Supplementary Methods). Fecal material was mixed with amphotericin B and gentamicin for 6 hours, to which activated charcoal (Fisher Scientific, Loughborough, UK) was added, and incubated for 10 days at 28°C according to a modified Harada Mori method to recover *Na*L3 [21, 22].

Recovered *Na*L3 were isolated and washed repeatedly in sterile water. *Na*L3 batches were tested for viability (motility), identity (lectin-binding assay), and microbial contamination (USP <61> and <62>, and then released by IMRG (Supplementary Methods) and transported to George Washington University (GWU) at 19°C–25°C in a temperature-monitored container. Microbial contamination was retested according to USP <61> and <62>) for total aerobic microbial and combined yeast and mold counts, as well as the absence of *Staphylococcus aureus* and *Pseudomonas aeruginosa,* after arrival at GWU and prior to application in the clinical trial under an US Food and Drug Administration (FDA) Investigational New Drug (IND) application (#015752).

Study Design

The phase 1 trial (NCT01940757) was an open-label dose escalation study of 20 healthy, hookworm-naïve adults aged 18–45 years at the George Washington University (Washington, DC). In Cohort 1, 10 volunteers received an inoculum of 25 *Na*L3; in Cohort 2, they received 50 *Na*L3. *Na*L3 were pipetted onto a sterile gauze pad, which was applied to the volar aspect of the forearm and covered with an adhesive bandage for 1 hour. After removal, the gauze was placed into sterile water to count residual *Na*L3.

Cohorts were enrolled in a staggered fashion, with at least 8 weeks of safety data assessed by a Safety Monitoring Committee prior to dose escalation. Participants were evaluated 3, 7, 14, and 28 days postinfection (p.i.) and then weekly until treatment with 3 daily doses of 400 mg albendazole on day 84 for the first 14 participants, and on day 126 for the last 6. At each visit, application sites were examined for erythema, swelling, and tenderness. Solicited symptoms included fever, abdominal pain or bloating, nausea, vomiting, diarrhea, cough, and sore throat. Participants completed daily symptom diaries for the duration of their infections.

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 *Na*L3. Complete blood counts were performed prior to *Na*L3 application and at regular time points starting at week 5 (Supplementary Methods). The study was approved by the GWU Institutional Review Board (IRB), and written informed consent was obtained from all volunteers. Inclusion and exclusion criteria are listed in the Supplementary Methods.

Detection of Patency

Beginning 5 weeks p.i., fecal samples were examined weekly by both saline flotation and quantitative polymerase chain reaction (qPCR) methods to detect *N. americanus* eggs [10]. Samples were further analyzed by the McMaster (MM) technique and qPCR to assess intensity of infection, expressed as EPG of feces [9].

Statistical Analysis

Time to patent infection was displayed using Kaplan-Meier estimates, with log-rank tests used to compare the distribution of time to patency between dose groups and censoring of early withdrawals or those failing to develop patent infection. Proportions with patent infection were compared between dose groups by the Fisher exact test. Locally weighted regression was used to display trends in eosinophil counts and fecal EPGs by study day. Mann-Whitney *U* tests were used to evaluate differences in EPG and eosinophil counts between dose groups and study day. Sign tests were used to determine if median eosinophil counts were above the upper limit of the normal range. A Cox proportional hazards model was used to evaluate the effects of covariates (eg, *Na*L3 dose or eosinophil count) on time to patency. A generalized linear mixed-effects model was constructed using the PROC GLIMMIX procedure to determine the effects of eosinophil count and *Na*L3 dose on EPG levels quantified by MM or qPCR. To compare the accuracy of infection intensity, as measured by MM and qPCR, signed rank tests were used to evaluate differences between EPG determined by the 2 methods. Receiver operating characteristic (ROC) curves were used to compare the accuracy of MM and qPCR in discriminating between patency and no patency, with the saline flotation method used as the gold standard. Analyses were conducted using SAS, version 9.3 (Cary, NC), and figures were constructed using R software (version 3.3.2).

Power Calculations for HVCM

Data from the current study were used to calculate the sample sizes required for the HVCM. Primary sample size analysis was based on comparing the proportion of individuals with patent infection in the 3 intervention groups (ie, 3 different vaccine formulations) with the proportion with patent infection in a control (placebo) group using a 1-sided Fisher exact test, for a total of 3 comparisons. The Holm-Bonferroni procedure was used to control the familywise error rate at .05.

RESULTS

Of 31 adults screened, 6 declined or were lost to follow-up before enrollment, 5 were ineligible, and 20 (5 males and 5

Table 1. Incidence and Severity of Application Site, Respiratory and Gastrointestinal Solicited Adverse Reactions Following Application of 25 or 50 *Na*L3 by CHHI

	25 *Na*L3 (n = 10)			50 *Na*L3 (n = 10)		
	Mild	Moderate	Severe	Mild	Moderate	Severe
	No.	No.	No.	No.	No.	No.
Application site reactions						
Rash	8	1	–	10	–	–
Pruritus	10	–	–	8	2	–
Pain	1	–	–	4	–	–
Tenderness	4	–	–	8	–	–
Swelling	2	–	1	4	1	–
Bruising	3	–	–	1	–	–
Respiratory reactions						
Sore throat	2	2	–	7	–	–
Cough	2	2	–	7	–	–
Gastrointestinal reactions						
Abdominal bloating	4	3	–	4	2	–
Abdominal pain	5	3	–	3	2	–
Nausea	3	2	–	3	2	–
Vomiting	1	2	–	–	–	–
Diarrhea	3	2	–	5	–	–
Flatulence	6	–	–	1	1	–

Data are number of participants experiencing an event. Participants with more than 1 occurrence of the same adverse reaction are recorded only once, at the maximum severity experienced.
Abbreviation: CHHI, controlled human hookworm infection.

females in each of the 2 cohorts) met eligibility criteria and were enrolled (Supplementary Figure 1). Reasons for exclusion were concomitant medical diagnoses such as irritable bowel syndrome, Hashimoto's thyroiditis, and iron deficiency anemia. The median age of enrolled participants (range) was 25 (19–45) years. Two participants in the 50 *Na*L3 cohort agreed to participate in an IRB-approved substudy and were not treated with albendazole but have remained infected to serve as donors of fecal samples, from which *N. americanus* eggs will be produced for future HVCM studies. Mean percentages of residual larvae were 5.60% and 5.40% for the 25 and 50 *Na*L3 cohorts, respectively ($P = .56$, Wilcoxon rank-sum test).

Safety of CHHI

Application of *Na*L3 to healthy, hookworm-naïve adults was well tolerated. No participants were withdrawn or required early treatment due to adverse events or discomfort (1 participant in Cohort 1 withdrew early and was treated with albendazole on day 63 due to moving unexpectedly from the area). Common reactions consisted of early skin reactions at *Na*L3 application sites and later gastrointestinal symptoms. *Na*L3 application site reactions were mostly mild to moderate rash and pruritus (Table 1). Rash occurred in 9/10 and 10/10 participants in the 25 and 50 *Na*L3 cohorts, respectively, and ranged from maculopapular to vesicular (Figure 1). Rashes were mild in severity, except for 1 moderate case in the 25 *Na*L3 cohort; topical corticosteroids were used in 6/20 participants for symptom relief. Onset of rash was typically noted immediately upon removal of the gauze pad following *Na*L3 application and lasted a median (range) of 26 (4–69) days. Residual skin hyperpigmentation occurred in 3 participants in the 50 *Na*L3 cohort and lasted a median of 107 days after resolution of the rash; in 2 cases, the hyperpigmentation was still present, albeit fading, at the final visit.

Gastrointestinal complaints were common (Table 1) and occurred primarily after week 4 p.i. (Figure 2). The incidence and timing of these symptoms were not significantly different between the 2 dose cohorts (Supplementary Table 1). Increased flatulence (7/10 in the 25 *Na*L3 cohort and 3/10 in the 50 *Na*L3 cohort), abdominal bloating (7/10 in both cohorts), and abdominal pain (8/10 in the 25 *Na*L3 cohort and 5/10 in the 50 *Na*L3 cohort) were the most common gastrointestinal complaints, all of which were mild or moderate in severity. Four episodes of vomiting occurred in 3 participants in the 25 *Na*L3 cohort, although

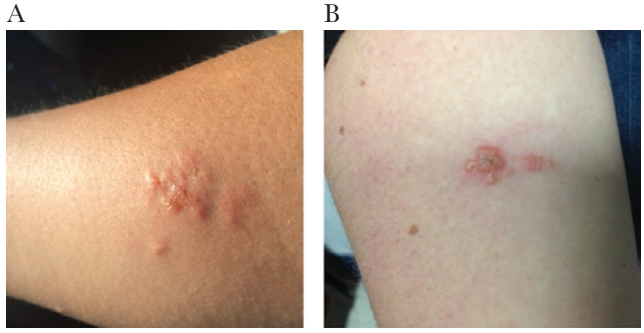

Figure 1. Representative images of rash induced by application of *Na*L3 to the forearm. A, Day 7 postapplication of 25 *Na*L3. B, Day 35 postapplication of 50 *Na*L3.

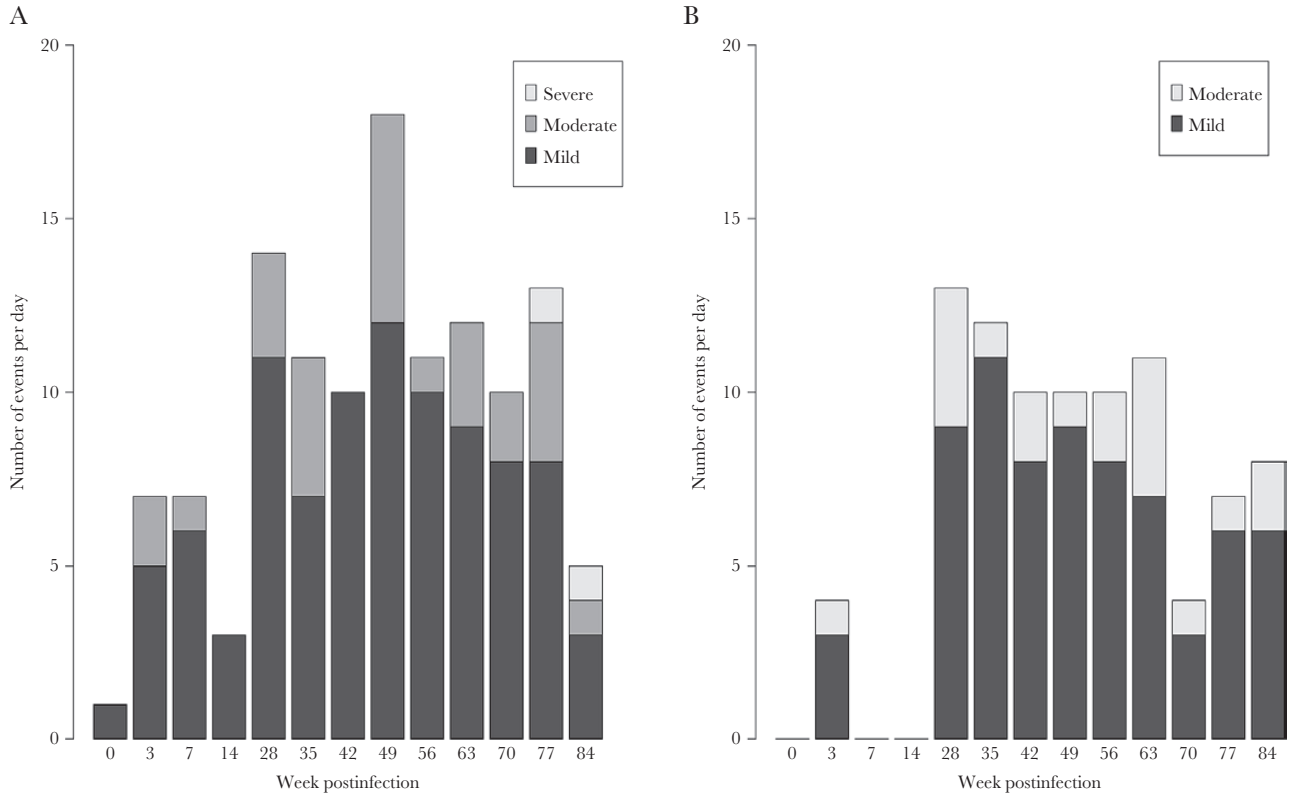

Figure 2. Prevalence of gastrointestinal symptoms by study week. Bar graph represents sum of participants in each dose cohort who experienced abdominal pain, abdominal bloating, nausea, vomiting, or diarrhea that was assessed as being related to controlled human hookworm infection. A, 25 *Na*L3 cohort. B, 50 *Na*L3 cohort.

none were considered related to CHHI. Other solicited adverse events possibly related to infection with *N. americanus* included respiratory symptoms due to larvae transiting through the lungs and upper airways. Cough was reported by 6 and 4 participants in the 25 and 50 *Na*L3 cohorts, respectively, while 4 and 7 participants complained of transient sore throat. The median onset of respiratory symptoms (range) was 2 (8–43) days p.i.

Patent Infection and Intensity of Infection

Fifty *Na*L3 resulted in significantly more (9/10 vs 3/10) participants developing patent *N. americanus* infection, as determined by saline flotation, than 25 *Na*L3 ($P = .02$), whereas 40% (n = 4) of the 25 *Na*L3 group had patent infection, as determined by qPCR, compared with 90% (n = 9) with 50 *Na*L3 (Figure 3). The difference in the proportion with patent infection when measured by qPCR between the 2 groups was just above the level of significance ($P = .06$). A significant difference was found between the 25 and 50 *Na*L3 groups in the distribution of time to patency based on Kaplan-Meier curves ($P = .04$) when assessed by qPCR, although not by saline flotation ($P = .11$).

Two participants in the 25 *Na*L3 cohort had eggs in feces quantifiable by MM, ranging from 16.67 to 33.33 EPG (Figure 4A). Four of the 25 *Na*L3 cohort with patent infections, as determined by qPCR, had quantifiable eggs in feces, also by qPCR (range, 1.5–123.6 EPG). Nine in the 50 *Na*L3 cohort had quantifiable egg counts by both MM and qPCR, ranging from 16.33 to 166.66 EPG by MM and from 1.2 to 373.2 EPG by qPCR (Figure 4). When compared by study day, there were no statistically significant differences in distribution of EPG or "visit-to-visit" changes in EPG between dose groups (Supplementary Table 2).

There were no statistically significant differences in the distribution of time to patency or intensity of infection by either MM or qPCR. MM was slightly more accurate than qPCR by ROC curve analysis. Using saline flotation as the gold standard, the value of the area under the curve (AUC) for detecting fecal eggs using the MM (AUC_{MM}, 0.97) was greater than that for qPCR (AUC_{qPCR}, 0.88), although the difference was not significant ($P = .16$) (Figure 5).

Eosinophil Counts

Those who received 50 *Na*L3 had a more rapid and greater increase in eosinophil count that declined faster than in those who received 25 *Na*L3 (Figure 6). Starting at 49 and 42 p.i., median eosinophil levels in volunteers in the 25 and 50 *Na*L3 dose groups increased significantly (both $P < .02$) above the upper limit of the normal range and remained high until treatment. Eosinophilia peaked 56 and 42 days p.i. for the 25 and 50 *Na*L3 groups, respectively. When compared on each study day, there were no statistically significant differences in distribution of eosinophil counts between dose groups (Supplementary

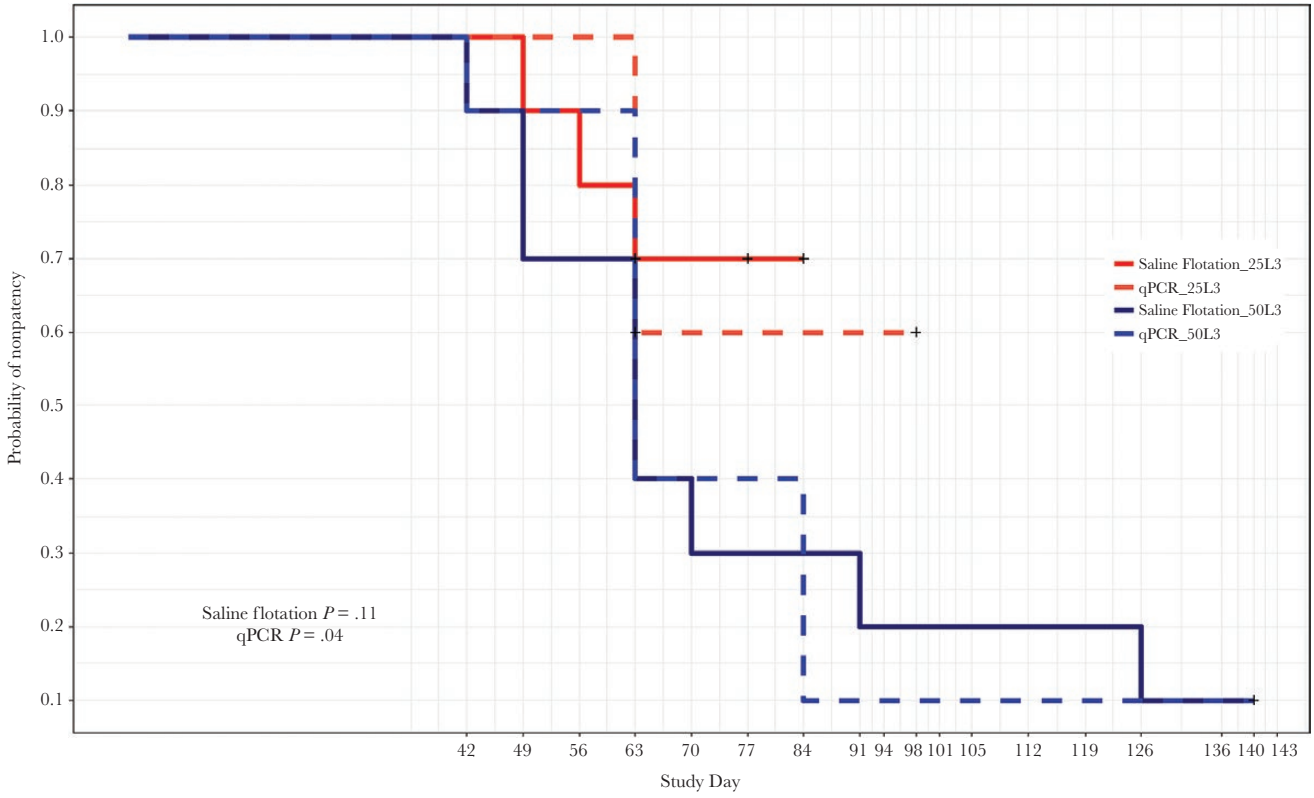

Figure 3. Time to patency for controlled *Necator americanus* infection as shown by Kaplan-Meier curves for volunteers exposed to either 25 *N. americanus* third-stage larvae (*Na*L3), as shown by the red line, or 50 *Na*L3, as shown by the blue line. Solid lines represent the proportion of individuals in the dose group with patent *N. americanus* infection, as detected by saline flotation, while dotted lines represent the proportion with patent infection, as determined by quantitative polymerase chain reaction. Crosses indicate censored data. Abbreviation: qPCR, quantitative polymerase chain reaction.

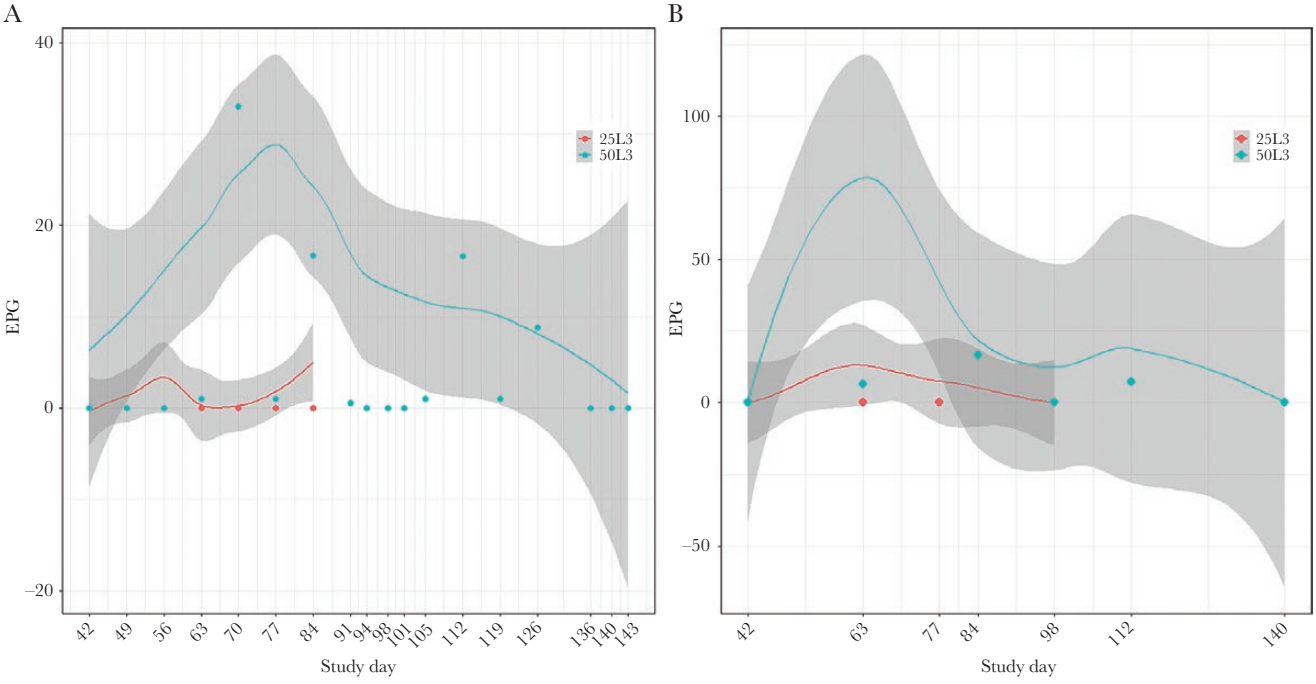

Figure 4. Eggs per gram (EPG) of feces, as determined by the McMaster (A) and quantitative polymerase chain reaction (B) methods for controlled human hookworm infection with an inoculum of 25 *Necator americanus* third-stage larvae (*Na*L3), as shown by the red line, or 50 *Na*L3, as shown by the blue line. Each dot represents the median EPG of the inoculum group by study day, and the solid lines show the trend in EPG for each inoculum group using locally weighted regression. The gray shading shows the upper and lower boundaries of the 95% confidence interval around the trend line.

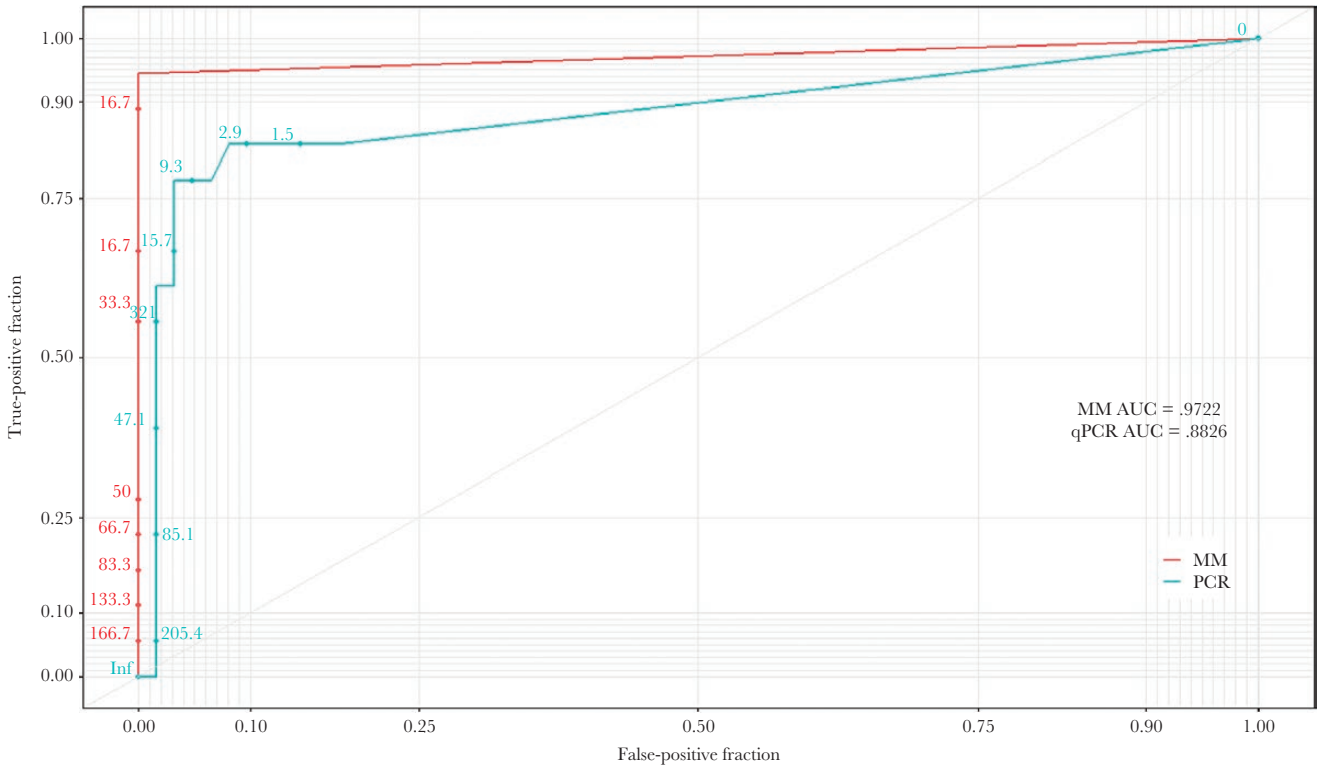

Figure 5. Receiver operator characteristic curves comparing the accuracy (area under the curve) of quantitative polymerase chain reaction with the conventional McMaster method to indicate patency (eggs in stool) from controlled human hookworm infection with 50 *Necator americanus* third-stage larvae (*Na*L3) using the saline flotation technique as the gold standard. Abbreviations: AUC, area under the curve; MM, McMaster; PCR, polymerase chain reaction.

Table 3). However, "visit-to-visit" changes in eosinophil count declined significantly faster (P = .03) in the 50 *Na*L3 cohort compared with the 25 *Na*L3 cohort from days 70 to 77.

Interaction Between Eosinophil Counts and Na*L3 Dose on Time to Patency*

In a Cox regression model, neither *Na*L3 dose nor eosinophil count significantly predicted the probability of patent *N. americanus* infection (P > .05). In addition, 50 *Na*L3 resulted in a 2.72 times (P = .15) or 2.55 times (P = .12) greater probability of patency than 25 *Na*L3, as determined by saline flotation or qPCR, respectively.

Eosinophil Count and Infection Intensity

Longitudinal fecal egg counts were analyzed using a generalized linear mixed-effects model with random intercept and an unstructured covariance matrix. The response variable EPG was assumed to have a Poisson distribution, and residual pseudo-likelihood was used as the estimation procedure. The dose of *Na*L3 and the eosinophil count induced by each dose predicted intensity of infection (EPG) when quantified either by MM or qPCR (P < .001). Parameters associated with EPG are reported in Supplementary Table 4.

Power Calculations for HVCM

Assuming a rate of 90% patent hookworm infection, as observed in the 50 *Na*L3 dose cohort in the current study, and a greater than 80% rate of protection in at least 1 of 3 vaccination groups (eg, different doses or adjuvant formulations) compared with controls who did not receive the hookworm vaccine, a sample size of 48 (12 per group) would be sufficient to demonstrate the efficacy of a prophylactic hookworm vaccine such as *Na*-GST-1 or *Na*-APR-1, at an alpha of 0.05/3 and a power of 87.7%.

DISCUSSION

This manuscript describes the first administration of *Na*L3 obtained from a human donor and manufactured under cGMP conditions to infect hookworm-naïve volunteers in a clinical trial in the United States. Moreover, this is the first CHHI trial to be conducted under an IND with the FDA. As such, the results enable us to use CHHI to test the efficacy of experimental hookworm vaccines in an HVCM under the existing US regulatory framework.

Both the 25 and 50 *Na*L3 inocula were safe and well tolerated, with application site and gastrointestinal reactions being the most common adverse events in both groups. Of note, skin reactions (mild to moderate pruritus, pain, erythema, and rash) occurred in most participants, lasted several weeks in a minority, and in some participants resulted in residual hyperpigmentation. Interestingly, the dermatologic findings were more significant and prolonged than reported in previous CHHI studies [18, 19, 23]. Gastrointestinal complaints were mild to

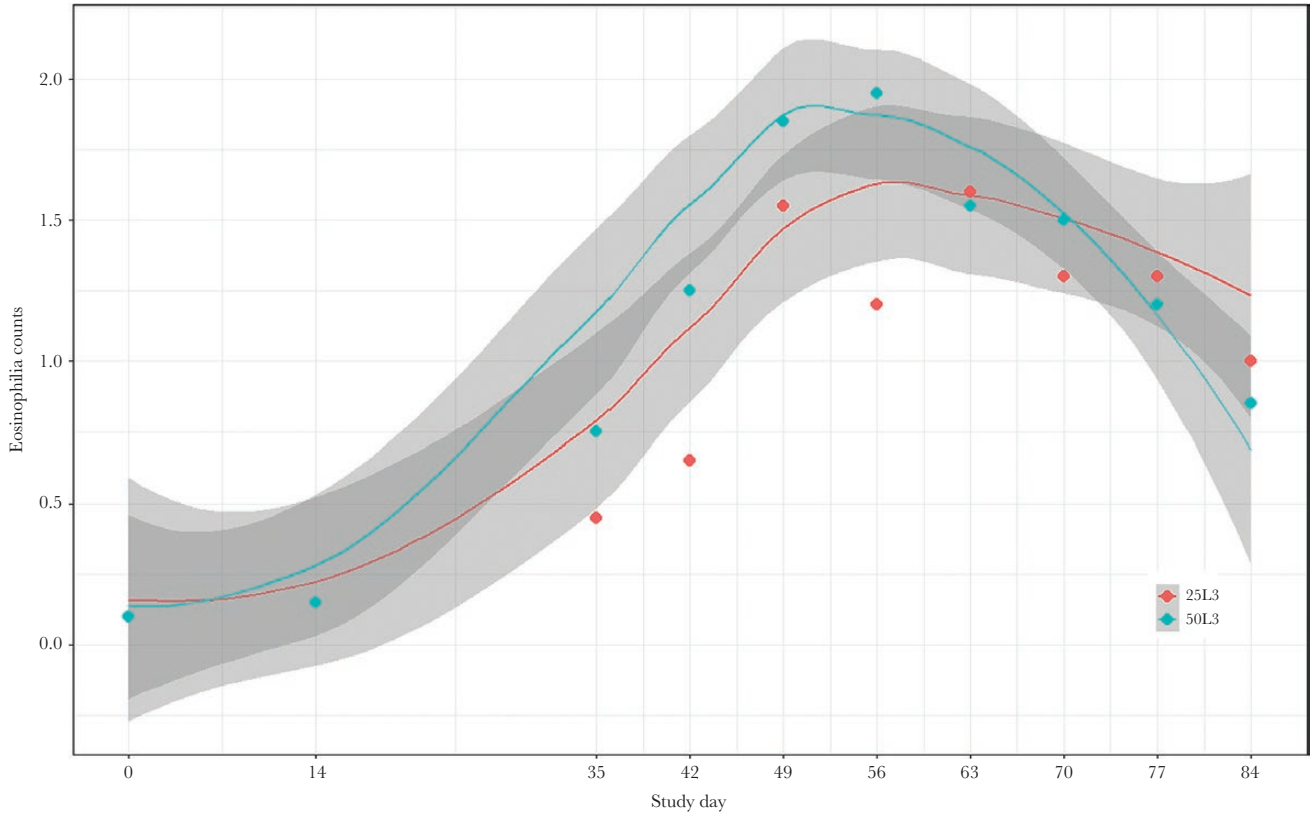

Figure 6. Peripheral eosinophil counts of volunteers infected with 25 *Necator americanus* third-stage larvae (*Na*L3) in red or 50 *Na*L3 in blue. Dots represent median eosinophil counts by study day, and the lines show the trend of eosinophil counts for each cohort using locally weighted regression. The upper and lower boundaries of the 95% confidence interval around the trend line are depicted by the gray shading.

moderate in severity and coincided with onset of patency. All participants stated that they would participate in the study again if given the opportunity.

Fifty *Na*L3 induced a significantly higher proportion of patent *N. americanus* infections than 25 *Na*L3 and also resulted in quantifiable fecal egg counts by conventional MM or qPCR. One possible explanation for the lower rate of patency in the 25 *Na*L3 group is that fewer *Na*L3 applied to the forearm traversed the dermis and lungs and succeeded in migrating to the intestine, or, if they did reach the intestine, they were too few in number to have resulted in effective mating. Based on these data, we estimate for an HVCM trial that 50 *Na*L3 would be sufficient to demonstrate the efficacy of an experimental vaccine using only 48 individuals divided into 3 groups (eg, different doses or formulations) compared with an unvaccinated control group, with 12 volunteers per group (Supplementary Figure 2).

The current CHHI study enabled an unprecedented observation of the host response to *N. americanus* infection. As skin-penetrating nematodes, *Na*L3 have been thought to traverse the dermis within 48–72 hours p.i., with 98% traversing the lungs within the first 120 hours p.i., after which they undergo tracheo-esophageal migration and enter the lumen of the gastrointestinal tract for the fourth molt [24]. As we have shown, marked eosinophilia was observed in both dose groups, although 50 *Na*L3 resulted in a significantly earlier onset, higher peak, and more rapid decline than 25 *Na*L3. Eosinophilia during experimental hookworm infection has long been thought to coincide with larvae traversing lung parenchyma [13, 25]. However, as described in animal models [26], *Na*L3 may have already reached the gastrointestinal tract by 14 days p.i., in which case development of eosinophilia would be concomitant with initiation of blood feeding and recruitment of inflammatory cells to feeding sites [25].

The current study allowed comparisons between conventional coprological methods such as the saline flotation and McMaster techniques with novel molecular methods (qPCR) for the detection of *N. americanus* eggs. Both conventional methods detected the same time to patency, while qPCR detected more patent infections in the 25 *Na*L3 group than conventional techniques. No difference was observed in measurements of infection intensity by MM or qPCR. However, the MM method was slightly more accurate than qPCR, as determined by ROC analysis (Figure 5). The main advantage of qPCR is that frozen fecal samples can be used, whereas conventional coprological methods require fresh specimens.

The incorporation of CHHI into hookworm vaccine clinical development would dramatically improve the efficiency of vaccine efficacy testing. Currently, efficacy studies for a preventative vaccine propose treating hookworm-infected volunteers in

endemic areas prior to vaccination and determining efficacy by comparing incidence of reinfection or mean intensity of reinfection. Such a trial would require a sample size of approximately 1200 volunteers and last for several years [8]. However, an HVCM trial would last only 3 months following completion of vaccinations and require far fewer volunteers (eg, 12 adults per group, with 1 group serving as unvaccinated infectivity controls), such that multiple vaccine candidates or adjuvant formulations could be assessed simultaneously to facilitate rapid downselection of the most promising product for phase 3 efficacy testing.

Our findings support the use of CHHI as a tool to assess hookworm vaccine efficacy and provide the framework for ensuring reproducible and accurate results that can be used to justify regulatory applications for efficacy testing. Moreover, for the concept of using controlled hookworm infection as a potential therapeutic for autoimmune and allergic diseases [12, 15, 23, 27], production of high-quality *Na*L3 in compliance with cGMP and administration to study participants according to current Good Clinical Practice will be required.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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