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A history of hookworm vaccine development

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The human hookworms *Necator americanus* and *Ancylostoma duodenale* remain among the most common infections of humans in areas of rural poverty in the developing regions of the world, with an estimated 1 billion people infected with one or more of these parasites. Herein, we review the nearly 100 years of research, development, animal testing and fieldwork that have led to our current progress in recombinant hookworm vaccines. We begin with the identification of hookworm at the start of the 20th century in Southern US, then discuss the progress in developed countries to eliminate human hookworm infection, and then the industrial development and field use in the 1970s a canine hookworm vaccine (*Ancylostoma caninum*), and finally our progress to date in the development and clinical testing of an array of recombinant antigens to prevent human hookworm disease from *N. americanus* infection. Special attention is given to the challenges faced in the development of a vaccine against a blood-feeding nematode, including the epidemiology of infection (high prevalence of infection), pathogenesis (chronic infection that increases with the age of the host), and a robust immune response that fails to confer the protection in the host and a concomitant absence of correlates of protection by a successful vaccine could be developed and tested. Finally, we provide the optimal and acceptable profiles of a human hookworm vaccine, including the proposed indication, target population and route of administration, as developed by the Human Hookworm Vaccine Initiative, the only group currently working on vaccines targeting this parasite.

Understanding the Impact of Hookworm Infection

Human hookworms are some of the world's most common parasitic helminths and are prevalent in South and Central America, sub-Saharan Africa and East Asia. Until half a century ago, hookworms were also prevalent in the US, Southern Europe and Australia.[1,2] As a result of considerable economic growth and development, especially in the years following World War II, which included programs of mass chemotherapy with anthelminthic medications, improved sanitation and health education, the soil-transmitted helminths (STHs), which also include *Ascaris lumbricoides* and *Trichuris trichiura*, as well as the focus of the current review the hookworms *Necator americanus* and *Ancylostoma duodenale* were eliminated from most of these regions. However, the STHs, especially the hookworms, remain the most common infections of humans in areas of rural poverty in the developing regions of the world, with an estimated 1 billion people infected with one or more of these parasites. The epidemiology of hookworm infection (either *Necator americanus* or *Ancylostoma duodenale*) as it relates to the rationale for a hookworm vaccine has been extensively reviewed by us elsewhere.[3-5] In brief, current control efforts focus on the administration of anti-nematode drugs ("anthelminthics") from the benzimidazole (BMZ) class of drugs, either albendazole or mebendazole.[3-5] While BMZ class anthelminthics are highly effective at removing established ("patent") infections, they do not confer lasting protection against new infection—hence, individuals resident in hookworm endemic areas are rapidly re-infected even after successful treatment. For this reason, the extensive use of chemotherapy has failed to interrupt and, in some cases, even limit transmission of these blood-feeding nematode parasites—with individuals becoming re-infected to their previous levels of infection within 12 months of treatment.[3-5] Current control efforts center on mass drug administration (MDA), which refers to the administration of BMZ class anthelminthics to entire populations resident in endemic areas irrespective of their individual infection status, has also raised concerns about the rates of drug failure (mebendazole) when used in a single dose and the possible emergence of drug resistance, as seen in veterinary uses of the BMZ class of drugs.[6] These concerns have stimulated efforts to develop a hookworm vaccine that would provide long-term protection against disease due to *N. americanus*, the most prevalent hookworm worldwide.[3-5]

History of Hookworm Infection

Evidence of hookworm infections stems back to Pharaonic times in the Old World and to pre-Columbian times in the New World.[7] While *A. duodenale* was identified over 1.5 centuries ago, *N. americanus* was more recently discovered only a century ago in the Southern US.[7] Shortly after the turn of the 20th century, Stiles and his colleagues conducted several scientific experiments and issued public health reports concerning

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hookworm infection in the US, and its impact on the population, especially school-aged children.[8-10] Prompted by these reports, the Rockefeller hookworm eradication campaign was soon underway in the Southern US and quickly expanded globally.[11] Despite some successes, the overall impact of the campaign on reducing the prevalence and intensity of hookworm infection remains controversial.[12,13]

In 1947, Norman Stoll[14] made the first global estimate of the number of individuals infected with human hookworm when he claimed that nearly one third of the world's population (over 600 million cases at that time) was infected with hookworm, which would make hookworm one of the most common human infections.[14,15] Over the next half of a century, efforts have been undertaken to reduce the prevalence of hookworm in regions as diverse as North America, Asia, sub-Saharan Africa and Latin America through a combination of improvements in infrastructure and widespread anthelminthic drug treatment.[16] Today, endemic hookworm has been essentially eliminated from the US.[17,18] Economic development in countries such as Taiwan, Japan and South Korea during the 1960–1970s also resulted in significant control of hookworm transmission, with national prevalence levels currently below 1%.[14] Additionally, over the past 20 y, the People Republic of China has successfully decreased the national hookworm infection prevalence levels, especially in eastern China.[19] Latin America and the Caribbean have experienced noticeable decreases in the prevalence of hookworm infections over time with the implementation of various national treatment programs coupled with economic growth.[20] Today, hookworm remains highly prevalent in regions (especially rural) of the low- and middle-income countries of Africa, Asia and the Americas where economic development has not yet taken place, and is not expected to anytime soon.[15] Despite the successful control of hookworm in several parts of the world, an estimated 576–740 million cases still exist worldwide, a figure very close to Stoll's original estimate more than 60 years ago.[21]

Natural History of Hookworm

The life cycles of *N. americanus* and *A. duodenale* as they relate to vaccine development have been reviewed extensively by us in reference 3–5, and are summarized in **Figure 1**. Briefly, humans become infected with hookworms when third-stage infective larvae (L3) penetrate the skin and then migrate into subcutaneous venules and lymphatics before traveling via the afferent circulation to the pulmonary capillary bed. From there, L3 enter the respiratory tree through the alveolae and ascend the bronchioles and the bronchi, pass over the epiglottis, and enter the gastrointestinal tract. In the small intestine, L3 molt twice to become adult male and female hookworms where they can live for five years or more. The adult worms, approximately one centimeter in length, attach to the mucosa and submucosa, rupture capillaries and arterioles, and then feed on host blood and mucosal tissues. The hookworms mate and female worms then produce thousands of eggs that exit the body in the feces. The eggs hatch in soil with adequate moisture and high temperature and then molt twice into L3 that seek higher ground to come into contact with human skin.

Intestinal blood loss is the main clinical manifestation of human hookworm infection.[3-5] Heavily and even moderately infected patients with underlying iron or protein nutritional deficiencies can develop hookworm disease—the clinical entity that specifically refers to the resulting iron deficiency anemia [IDA], which is a microcytic and hypochromic, anemia, that occurs when hookworms feeding on the blood host with poor nutritional background.[3-5] Hookworm-induced blood loss is estimated to be as high as 9·0 mL per day in heavy infections, with hookworm burdens of 40–160 worms sufficient to cause anemia.[22] In school-aged children and adults in resource-poor countries, where host iron stores are often lower than those in developed countries, there is a well-established relationship between the intensity of hookworm infection, intestinal blood loss and anemia.[3-5]

The Human Immune Response to Hookworm Infection

As with other helminths that infect humans, the robust immune response is induced by hookworm infection fails to confer protection against new or established infection. This phenomenon has been extensively reviewed elsewhere for hookworm[23] and for helminths in general, as well as the consequences it has on the discovery and development of vaccines against these parasites. The major impediment to the development of vaccines for these infections is that there is no evidence of protection in humans.

An important reason for this "major impediment" is the natural history of hookworm infections: individuals can be infected for years, starting as young as 12 months of age, with hookworms sometimes infecting up to 70% of individuals at risk in endemic areas (i.e., 70% an entire village may be infected). As such, it is difficult to find individuals in endemic areas who have not been continuously infected for months, years or even decades. More specifically, it is nearly impossible to find hookworm-näive individuals (i.e., individuals who have never been infected), follow them longitudinally and observe them for incident or "new" infection in order to elucidate the manner in which the human immune response evolves over time to this pathogen. Hence, most of our knowledge of the human immune responses to hookworm infection comes from individuals who have failed to mount a protective immune response against the pathogen, leaving us without a model for a "successful" immune response that could be used to predict vaccine-induced protection or at least determine a "correlate of protection" (CoP).[24,25] The lack of a CoP profoundly effects the pre-clinical and clinical development of hookworm vaccines in variety of ways; for example, the lack of a CoP makes difficult to test the variation in "potency" of different manufactured lots of a hookworm vaccine as we do not know the biologically effective level of antibody needed to induce protection. or if one even exists Additionally, the lack of CoP or a model of protection necessitates the inclusion of much higher sample sizes in phase 2 and 3 trials of candidate hookworm vaccines, since the endpoints must be clinically-based rather than an easier-to-measure CoP.

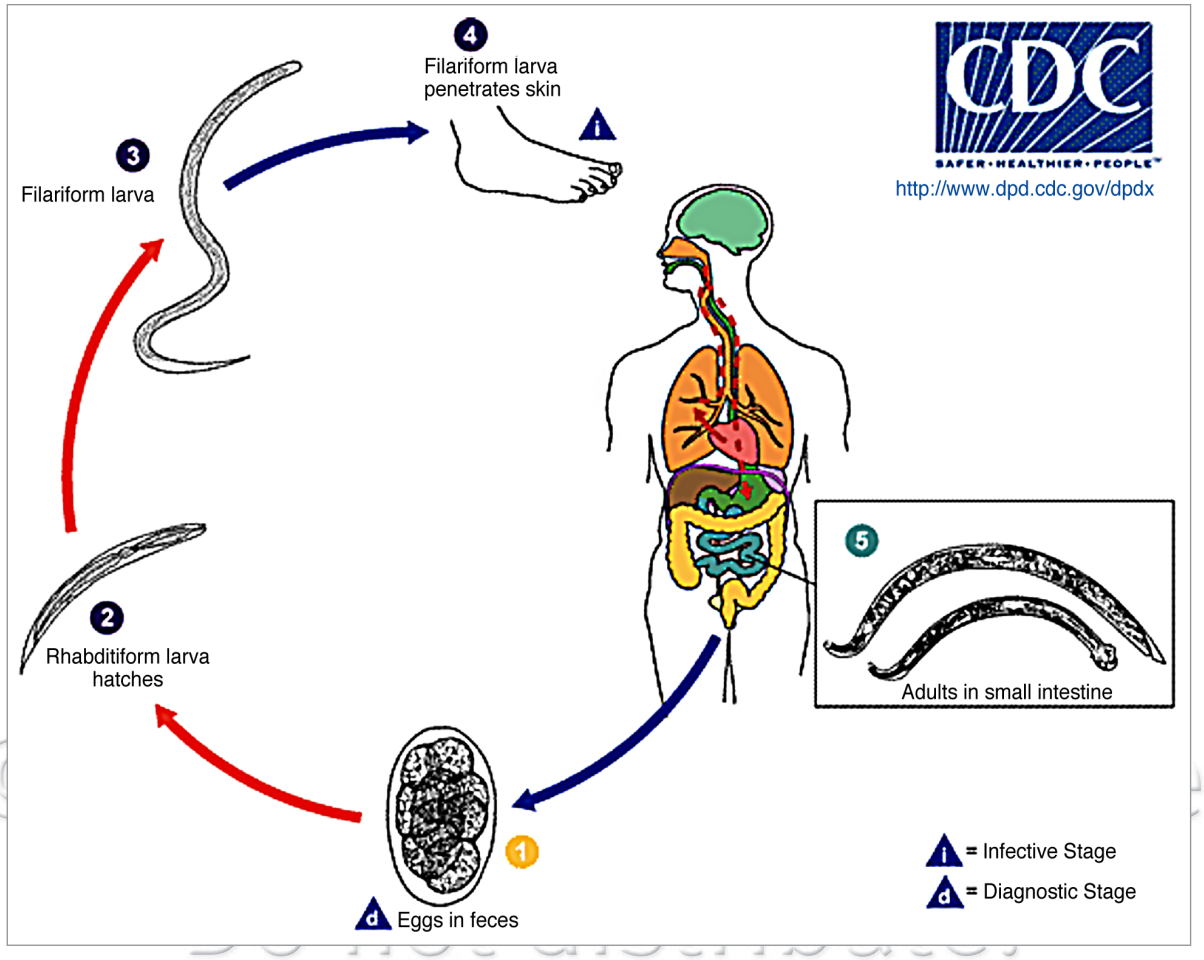

Figure 1. The life cycle of the human hookworms as presented by www.cdc.gov/parasites/hookworm/biology.html Hookworm eggs are passed in the stool and larvae hatch in 1 to 2 days (1). The released larvae grow in the feces or soil and after 5 to 10 days become infective third-stage larvae as those used by Miller and other to form an irradiated larval vaccine (irL3) (2-3). On contact with the human host, L3 penetrate the skin and are carried through the blood vessels to the heart and then to the lungs (4) where they penetrate pulmonary alveoli, ascend the bronchial tree to the pharynx and are swallowed. The larvae reach the he lumen of the small intestine and mature into blood-feeding adults (5), by attaching to the intestinal wall. Several of the vaccine candidate antigens mentioned in this review target this stage, including Na-GST-1 and Na-APR-1.

While host immune response to hookworm infection is robust and comprehensive in scope, activating both strong humoral and cellular immune responses, it fails to elicit protection. Naturally-infected humans mount strong antibody responses against hookworms, involving all isotypes and most IgG subclasses, with IgG4 and IgE exhibiting the greatest increases. These antibody responses can be detected to all stages of the pathogen in the host (L3, L4 and L5).[26,27] It is well accepted that a T-helper type 2 (Th2) cellular immune responses is elicited during hookworm infection (as with other helminth infections such as schistosomiasis, onchocerciasis and filariasis[28-32]). As part of this Th2 response, individuals develop elevated levels of total IgE and parasite-specific IgE, increased levels of interleukin [IL]-4, IL-5, IL-10 and IL-13, and an increase in eosinophils and mast cells. There has been a long-standing hypothesis that parasite-specific IgE can have a partially protective effect against helminth infections such as Schistosoma spp.[33,34] Our own studies have shown that elevated levels of IgE to the recombinant *Ac*-ASP-2 (*Ancylostoma caninum* Ancylostoma Secreted Protein-2), are associated with a decreased risk of heavy hookworm infection.[35]

The Th2 response during helminth infection is induced against a background of potent, parasite-induced immunoregulation, referred to as a "modified" Th2 response.[28-32] This response consists of alternatively activated macrophages, Foxp3$^+$ CD4 regulatory T [Treg] cells, and CD4$^+$ Tr1-IL-10 producing T cells.[28-32] The effect of this response is to create an immune environment so extensively downregulated that it may protect the host not only from the strong inflammatory effects of helminth infections, but also against the effects of other IgE-mediated disorders such as atopy, asthma and anaphylaxis.[32] Reduced allergic responses have been shown in studies of infection of mice with various helminth infections (see Erb[36] for review). Moreover, epidemiological evidence suggests that hookworm infection is associated with reduced skin reactivity to common allergens and a lowered risk of extrinsic asthma.[37] As seen with other helminth infections, hookworm is associated with a systemic down-modulation of immune responsive, with measurable attenuation of

responses to bystander antigens to immunization with routinely administered vaccines.

As mentioned above, there is no CoP (as defined either by Qin[38] or by Plotkin[24,25]) that has been identified for human hookworm infection. The fact that the immune system reacts vigorously to hookworm infection and fails to harm the parasite not only hampers identification of target vaccine molecules for antigen discovery (about which much has been written[3-5,28,39]), but also provides challenges for development of potential vaccine antigens.

Hookworm in the Laboratory

The life cycle of many viral and bacterial pathogens are easy to maintain in vitro and permissive animal models exist for them as well, making the testing of vaccines targeting these organisms for efficacy, immunogenicity and potency relatively straightforward. However, like many other Neglected Tropical Diseases (NTDs), the life cycle of hookworms is difficult to maintain in vitro or as laboratory strains within animal models. In some cases, only a single stage of the life cycle can be consistently maintained in the laboratory. Moreover, like many NTDs, the laboratory animals that permit hookworm testing are large, expensive and require extra care to maintain (e.g., canines). There also remains considerable scientific debate as to whether these animal models reproduce the infection as it occurs in the human host (see Fujiwara et al.[40] for review).

The current models used in the testing of experimental hookworm vaccines are canines and hamsters. Canines can be experimentally infected with *A. caninum*, which closely resembles human *N.* hookworm infection.[40] The hamster *Mesocricetus auratus* can be infected with either *A. ceylanicum* or *N. americanus*. These animal models are of limited benefit for the following reasons:

(1) While initially permissive to infection by third-stage infective larvae (L3), both models become refractory to the infection over time.[41,42] For example, canines develop natural resistance to hookworm infection after 20 weeks of infection, after which time the parasites are expelled from the host.[41,42] This makes the immunization and challenge model difficult to mount and a long-term pathological endpoint such as anemia difficult to induce given such a short time span of infection.

(2) Neither canines nor hamsters easily reproduce the clinical endpoints seen with human hookworm disease—specifically, development of IDA is uncommon. The relationship between worm burden and intestinal blood loss is not straightforward in either model, probably due to the method of infection (a single bolus challenge of infective larvae), which has to be given over a short time frame to permissively infect a large group of animals.

(3) Finally, the hamster model, especially for *N. americanus*, has the further limitation that even under optimal conditions, less than 20% of infective larvae develop into adult hookworms in the gastrointestinal tract during challenge infections.[43]

Over the last few decades there have been several attempts to create alternative animal models for hookworm infection.[40] The need for alternative experimental models has been driven by the need to develop new anthelminthic therapies as well as a vaccine for this parasite. Hookworm infections have been attempted in species such as rabbits, chickens and chimpanzees.[40] Mice have also been challenged with Ancylostoma spp and *N. americanus*. However, none of these are permissive hosts for the human hookworms; in many cases, their infective larvae fail to mature into blood-feeding adult hookworms. Consequently, mice challenged with hookworm larvae do not go on to experience intestinal blood loss.

The First Hookworm Vaccine

A detailed history of the advances leading to canine and human vaccines can be found in **Figure 2**. Briefly, in 1964, Miller[44] showed that *Ancylostoma caninum* larvae could be attenuated using 40,000 röntgens of X-ray. As the amount of radiation increased, the larval infectivity decreased and the pathogenicity was also reduced. He also observed that female larvae irradiated at 40,000 röntgens or more were consistently sterile. In a single subcutaneous vaccination in dogs 3–4 months old using 1,000 *A. caninum* larvae exposed to 40,000 röntgens of X-ray, a single subcutaneous vaccination resulted in a high degree of resistance to the challenge burden of normal worms in terms of hematological, clinical and coprological changes. Miller then conducted a series of experiments to investigate the effect of double vaccination with irradiated *A. caninum* larvae (irL3) and if the determine the route of vaccine administration [subcutaneous (s.c.) or oral] had any effect on the immunogenic efficiency.[44,45] He concluded that double oral vaccination was not as effective as double s.c. vaccination in dogs that were 3–4 months of age, by measuring the establishment of adult hookworms after challenge with infective larvae. Both routes of vaccination seemed equally effective in terms of resistance to the pathogenic effects of hookworm infection, involving hematologic, coprologic (eggs per gram of feces), and clinical observations after challenge with infection with L3. The arrest of irL3 in somatic locations along their migratory route (primarily the lungs) after subcutaneous inoculation generated a more intense immunogenic stimulation of the canine host.[44,45] Consequently, Miller favored subcutaneous vaccination for the commercialized version of the irL3 vaccine.[42,46]

As mentioned above, Miller believed that the presence or absence of somatic migration (L3 migrating to the lungs) was a primary moderator in the increased efficacy observed when irradiated larvae were administered subcutaneously rather than orally.[45,46] This hypothesis was established on the observed basis that nearly 75% of irL3 became "arrested" before or died prior to reaching the intestines, the location where the hookworm cause substantial host blood loss. Miller believed that the larvae's journey to the intestines concluded in the lungs, which was the case in a hypothesis put forth for an experimental irradiated schistosome vaccine[47] and for irradiated *Nippostrongylus brasiliensis* larvae.[48] Moreover, Miller showed that subsequent challenge infections of vaccinated dogs was due to the administration of irradiation to third stage infective larvae (L3), which attenuated the L3. As stated by Miller,[44] three characteristics of attenuation were observed when irL3 were used to immunize canines:

(1) a reduction in larval infectivity;[44]

(2) a reduction in the pathogenicity of the worms that eventually reached the small intestine;[44] and

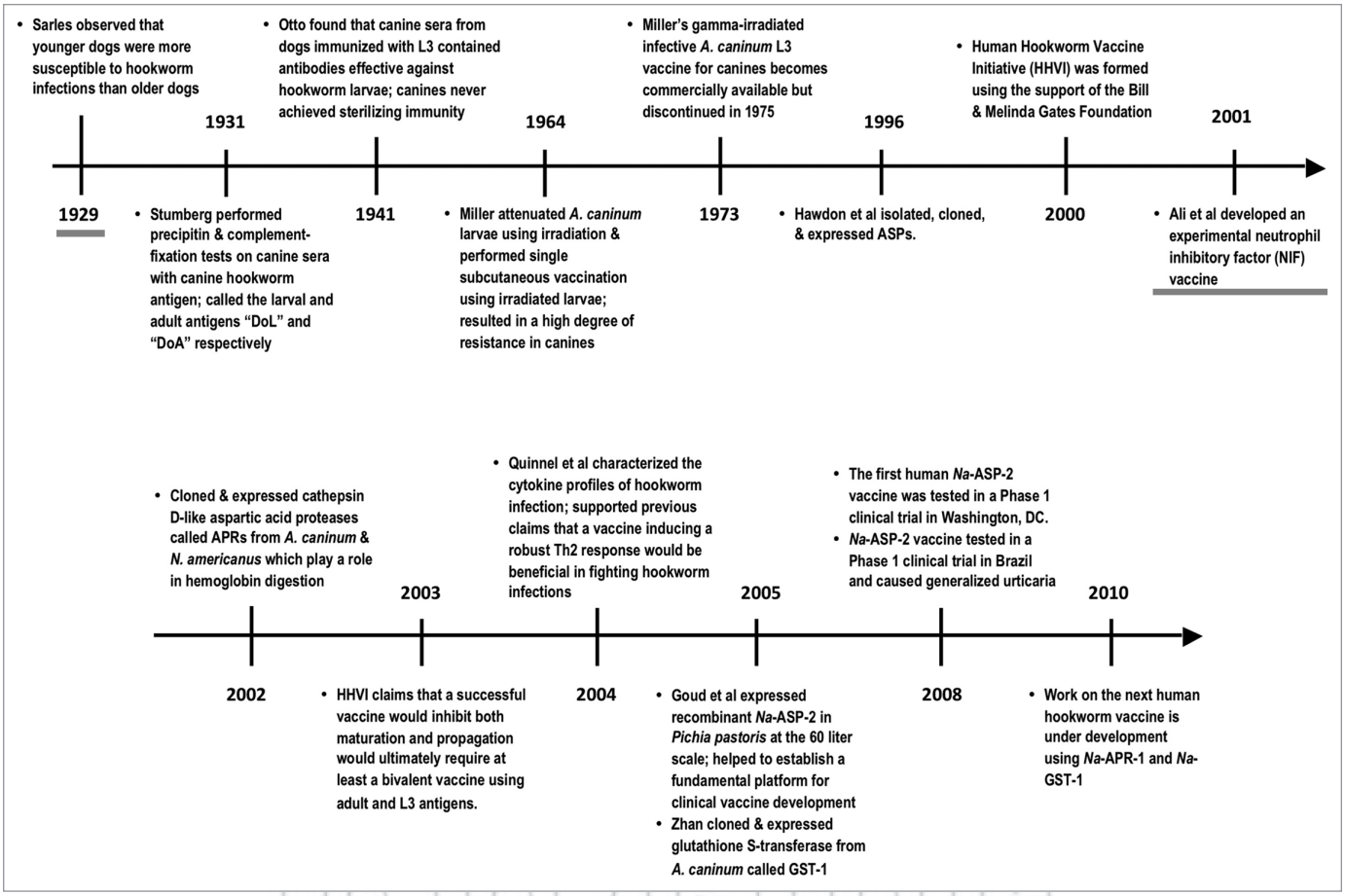

Figure 2. A century of parasitology that has led to the successful industrial-scale development of a canine hookworm vaccine and the experimental testing of a human hookworm vaccine.

(3) a sterilizing effect on female worm's fecundity as measured by eggs per gram of feces (epg).[47]

Until this point in time, successful passive immunization for helminth parasites exhibited variable and contradictory results primarily because of experimental techniques. A few years after establishing his "characteristics of attenuation," Miller conducted a series of experiments revealing that the immunity of double vaccinated dogs with X-irradiated *A. caninum* larvae could be passively and adoptively transferred to susceptible pups using serum and/or lymphoid cells.[49] Despite a lack of immunological reagents at this time (e.g., monoclonal antibodies, cell surface markers), these findings on passive immunization enabled Miller to conclude that the mechanism of resistance induced to irL3 was the result of an immunological phenomenon rather than a mechanical event (the attenuated L3 being trapped in the lung).

Miller used these "characteristics of attenuation" for the, product development, industrial manufacture and USDA licensing of the 1st hookworm vaccine, which consisted of gamma-irradiated infective *A. caninum* L3 vaccine for canine.[42] The vaccine was field tested by approximately 1,500 practicing veterinarians across the US.[41,42] While the vaccine proved to be safe for the dogs and even effective (90%) for canine, it had a considerably short shelf-life (6 mo) and a unique set of storage condition. The vaccine was discontinued in 1975 due to commercial failure; most veterinarians were unwilling to incorporate it into their vaccination programs because of difficulties in storing the vaccine, the short shelf life, the lack of sterilizing immunity, and economics—it prevented routine de-worming of canines, a routine and rather lucrative source of income for veterinarians.[42]

A Chance for Learning

Although the irL3 vaccine failed commercially, it provide compelling evidence that the possible existence of human hookworm vaccine and the expectations (see Target Product Profile in **Table 1**) of such a vaccine.[39] First, dog owners complained about the appearance of eggs in feces following vaccination. Miller countered that it was an "unrealistic expectation for successful banishing of all hookworm infection: and that sterilizing immunity to such macro-parasite was probably not option; a reducing in the number of worms and not complete elimination of the worms was sufficient to have an important clinical effect".[42] Indeed, such expectation on the part of the dog-owners was not unreasonable. The majority of human vaccines are indeed sterilizing in nature, eliciting a rapid and specific immune response that kills the pathogen—a characteristic vital to combating

Table 1. The optimal and acceptable profiles of a human hookworm vaccine given

Item	Optimal Scenario	Acceptable Scenario
Vaccine Indication	Prevention of moderate to heavy hookworm infections due to Necator americanus, as determined by quantitative fecal egg counts using the thresholds recommended by the World Health Organization	Prevention of heavy hookworm infections due to Necator americanus, as determined by quantitative fecal egg counts using the thresholds recommended by the World Health Organization
Target population	Children less than 10 years of age	Children less than 5 years of age
Route of Administration	Intramuscular Injection	Intramuscular Injection
Product Presentation	Multi-dose vials containing 5–10 ml liquid formulation consisting of 1–2 recombinant antigens plus adjuvant(s) and preservative(s). Volume of delivery, 0.5 ml	Single-dose vial containing 1.2 ml liquid formulation consisting of 1 recombinant antigen plus adjuvant. 1.0 ml volume of delivery
Dosage Schedule	Maximum of 2 immunizations per child, regardless of age, with the second injection given at 1–2 months after the first	Three (3) immunizations per child, regardless of age, delivered according to a 1, 1–2 and 4–12 month schedule
Expected Efficacy	80% efficacy against moderate/heavy infections for at least 5 years	50% efficacy against heavy infections for at least 2 years
Shelf life	5 years	2 years
Storage	Refrigeration between 2 and 8 degrees Celsius. Cannot be frozen. Can be out of refrigeration (at temperatures up to 25 degrees Celsius) for up to 72 hours	Refrigeration between 2 and 8 degrees Celsius. Cannot be frozen. Temperature monitoring required

viral and bacterial pathogens that reproduce asexually, such as varicella and measles or the effect of toxins such as from tetanus. Additionally, the success of human vaccines against viral and bacterial pathogens are measured in terms of the decreasing the incidence of disease in populations receiving the vaccination and also generate "herd immunity." However, given the size and the life cycle of this blood-feeding parasitic nematodes, a human hookworm (or any other helminth vaccine for that matter) is unlikely have a sterilizing effect (see Maizels[28,50] for classic and still timely reviews of the challenges facing helminth vaccinologists)(**Box 1**). Hence, an important lesson learned from Miller is to product profile of a human hookworm vaccine (see **Table 1**), the indication of which would be to reduce the total worm burden, resulting in an associated decrease in the morbidity from hookworm diseases (**Table 1**).[39] For example, Miller claimed that the irradiated canine hookworm vaccine was discontinued due to "the failure of veterinarians to differentially diagnose hookworm infection from hookworm disease."[42] In other words, it failed to induce a sterilizing immunity in canines. However, as Miller goes on to argue, the degree of pathology generated from "hookworm is directly related to the number of worms residing within the host"[42] and not simply infection alone; i.e., there is a critical difference between "hookworm infection" and "hookworm disease", and it is based on the intensity of infection. The more hookworms infect the human or canine host the greater the chance for hookworm disease; hence, a human hookworm vaccine should target "hookworm disease" and not "target hookworm infection". More specifically, a human hookworm vaccine should diminish the risk for moderate and heavy worm burdens and the possible clinical sequelae.[3,42,54,55]

Box 1. The Major Hurdle. The major hurdle in the development of a hookworm vaccine, as well as vaccines against other intestinal parasites, is that protection against these helminths has never been demonstrated in humans—a crucial impendent to the making of a prophylactic vaccine (for a classic review of these challenges see ref. 28).

A critical characteristic of the irradiated canine hookworm vaccine developed by Miller was that the efficacy of the vaccine depended on the viability of the larvae.[39] More specifically, the attenuated larvae in the canine vaccine were able to live and generate antigens that would be secreted upon entry in the host.[39,42] This viability factor has proven to be extremely informative for the development of a recombinant human hookworm vaccine. While immunizing canines with live attenuated hookworm larvae appears to have a low risk-benefit ratio, immunizing children with live attenuated hookworm larvae is both unpractical for hookworm control. Consequently, the observation dealing with larval viability coupled with findings that larval-secreted antigens induce protective immunity[39,42] inspired era of successful antigen discovery 30 years later (**Box 2**).

A Model of Human Hookworm Infection

During the early 1980s, Carroll and Grove started doing work with the hookworm *Ancylostoma ceylanicum*.[53-58] This species of hookworm could be a model of human hookworm due to its ability to infect humans. In 1984, they began a series of experiments to observe the parasitological, hematologic and immunologic responses to these hookworms in dogs.[53-58] They successfully stimulated transitory lymphocytes using adult *A. ceylanicum* antigens. Carroll and Grove further observed that IgG antibodies to the antigens persisted for an indefinite period of time while specific IgM antibodies only lasted temporarily. Carroll and Grove later discovered that two immunizing doses of 1 mg of protein from *A. ceylanicum* suspended in Freund's complete adjuvant

Box 2. Limitations of field epidemiological of the immune response to hookworm and the impact on vaccine development.

- Only a snapshot of a failed immunological situation can be taken, in which generations of the parasite have already successfully colonized its human host for months, years or possibly, even decades. While hookworms are though to live for 5 to 7 y in their human host 21, individuals are constantly becoming re-infected, substituting "old" infections with "new" infection and in many cases accumulating infections. In fact, the prevalence and intensity of hookworm infection increases with age, such that the elderly often have the greatest number of parasites in endemic areas.[86,87]
- It is difficult to classify humans into the different phases of hookwrom infection: (1) exposure, (2) pre-patency (larvae), patency (estbalsihed blood feeding infection) and post-patency (cured) as most individuals resident in endemic areas have already been infected for several years. Indeed, given the chronicity (5 to 7 y per worm) and the prevalence (up to 70% in some communities), it is often difficult to find hookworm näive individuals to follow longitudinally in order to elucidate the evolution of an immune response to pathogen from initial exposure to acute infection to chronic infection.
- Moreover, it is not ethically acceptable to follow hookworm-infected individuals for long periods of time without offering treatment, thus hampering longitudinal studies, on the evolution of chronic infection.
- The most common study design of the immune response to hookworm infection comes from the rather "unnatural" situation of the treatment-reinfection study design conducted in high-transmission areas. In this study design, patients are treated for their established hookworm infections with one of the usually very effective BMZ class of anthelminthic drugs and their immune response monitored until they become re-infected. Usually within 12 mo. A comparison is then amde betwen individuals who become reinfected and those who do not. See Quinnell et al. for an elegant example of this method.

significantly reduced fecal egg output and total intestinal adult hookworm numbers.[53-58] They concluded that injection of soluble adult hookworm antigens preceding an infection would establish specific protective immunity. Simultaneously, Hotez and Cerami proposed developing vaccines comprised of recombinant soluble enzymes.[59,60] These observations further improved the outlook for human hookworm vaccine development using soluble molecules rather than entire parasites that have previously been irradiated. Using such soluble molecules completely eliminated the chance of accidental infection that persisted with vaccines constructed from whole hookworm larvae. These findings proved to revolutionize the search for a successful human hookworm vaccine during the two decades to follow.[39]

The Human Hookworm Vaccine

Table 1 shows the optimal and acceptable profiles of a human hookworm vaccine, including the proposed indication, target population and route of administration, as developed by the Human Hookworm Vaccine Initiative, the only group currently working on vaccines targeting this parasite. *Ancylostoma Secreted Protein-2* of *N. americanus* (*Na*-ASP-2) is a 21 kDa protein that is secreted by infective hookworm larvae upon entry into the host.[61-64] Vaccination of hamsters and dogs with recombinant ASP-2 results in reduced worm burdens and fecundity following challenge infection.[35] Furthermore, sera from vaccinated animals inhibit the migration of infective hookworm larvae through tissue in vitro. Studies of populations living in hookworm endemic areas have also shown that antibodies to ASP-2 are associated with a reduced risk of developing heavy hookworm infections.[35] Based on these results, *Na*-ASP-2 was chosen as a lead hookworm vaccine candidate.[39] In a phase 1 study in hookworm-naïve adults living in the US, *Na*-ASP-2 adjuvanted with Alhydrogel® was well-tolerated and immunogenic.[65] However, a phase 1 safety and immunogenicity trial of this vaccine in healthy adults from a hookworm endemic area in rural Brazil has to be halted when 3 participants developed immediate, generalized urticarial reactions following a single injection of 10 ug of recombinant *Na*-ASP-2. Subsequent analysis showed that the urticarial reactions were associated with elevated levels of IgE antibodies specific for *Na*-ASP-2, which were present before immunization, most likely as a result of previous infection with hookworm.[66] A survey of adults and children from the same hookworm-endemic area revealed that a significant proportion had elevated levels of IgE to *Na*-ASP-2, therefore halting further development of the vaccine. These findings had a major implication for the development of vaccines against not only hookworm, but all helminths given their common propensity to induce strong T helper cell type 2 (Th2) immune responses and elevated levels of IgE to both parasite and bystander antigens.

Systemic allergic reactions are rare for licensed vaccines, averaging 0.65 cases per million doses.[67] Few of these reactions implicate the immunizing agent itself, as was the case with *Na*-ASP-2. More common are reports of allergic reactions to incipient components such as animal protein (gelatin), antibiotics (neomycin) or latex (vial stoppers).[68] However, a comparable situation was reported for the clinical testing of the circumsporozoite Multiple Antigen Meptide (MAP) vaccine, an experimental vaccine against *Plasmodium falciparum*.[69] During phase 1 trials in malaria-unexposed individuals, vaccinees developed Iimediate-type hypersensitivity (ITH) reactions associated with elevated levels of IgE to MAP.[69] However, in contrast to the data presented here, vaccinees developed MAP-specific IgE only after repeated vaccinations and were not sensitized by a natural parasite infection such as was the case with *Na*-ASP-2. The mechanism behind these ITH reactions was hypothesized to be the presence of recombinant malarial proteins that were unbound to adjuvant, which cross-linked IgE attached to mast cells to stimulate release of histamine and other mast cell mediators involved in the pathogenesis of acute urticaria.[69] We propose a similar hypothesis whereby *Na*-ASP-2 cross-linked the IgE on mast cells armed during years of chronic infection, thus triggering a substantial histamine release.

It is possible that intrinsic structural or biological properties of the *Na*-ASP-2 molecule were responsible for the ITH reactions in previously infected individuals.[70] Few molecules have the necessary properties to bind and crosslink IgE[71] (Traidl-Hoffmann 2009). An intriguing possibility is that humans have targeted a restricted range of antigens from metazoan parasites, such as helminths, to enable host defense without inducing self-harm or tolerance.[72] In this context, *Na*-ASP-2 would be both an attractive target for antibody-mediated defense against hookworm infection and a target for IgE. During penetration of host tissue, infective L3 of *N. americanus* encounter host-specific signals

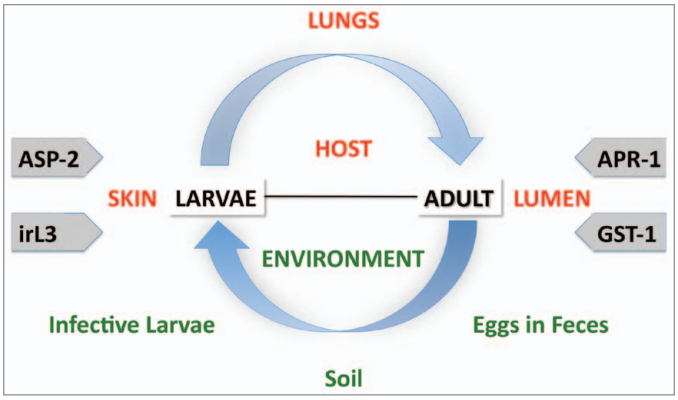

Figure 3. The strategy for a human hookworm vaccine. Our previous strategy focused on excretory/secretory factors from the infective larval stage (L3). A newer strategy focuses on inhibit the consumption and detoxification of heme by the adult hookworm.

that induce a programmed chain of developmental events that result in the successful 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,[65] both of which belong to the Pathogensis Related Protein (PRP) superfamily.[61-64] Recombinant *Na*-ASP-2, expressed in *Pichia pastoris*, has shown significant protection in laboratory animal models, with sera from animals immunized with ASP-2 inhibiting the migration of infective larvae in vitro through host tissue.[35,73] The latter observation suggests that antibodies against *Na*-ASP-2 might attenuate larvae during tissue migration and prevent them from reaching the host intestine and therefore developing into healthy adult hookworms.[35,39]

New Generation Hookworm Vaccines

A new generation of hookworm vaccines have been described in detail in reference 3–5, and are shown in **Figure 3** in regards to their place in the hookworm life cycle. In brief, from more than 20 potential target proteins involved in the hookworm blood feeding process,[51,74,75] two lead proteins have emerged as promising candidate antigens for further clinical development, based on preclinical efficacy testing and reduced concern of hypersensitivity reactions as supported by data from human immunoepidemiology studies: *Na*-GST-1 and *Na*-APR-1. Each vaccine antigen is an enzyme (or modified enzyme) required for parasite blood feeding. When used as a vaccine, we hypothesize that they will induce neutralizing antibodies that will interfere with parasite blood-feeding and cause parasite death and reduced worm fecundity (**Fig. 3**). *N. americanus* hookworms depend on host hemoglobin for survival.[75] Following hemolysis, adult *N. americanus* use an ordered cascade of hemoglobinases beginning with the cleavage of intact hemoglobin by the aspartic protease *Na*-APR-1.[74-77] The freed heme generates toxic oxygen radicals that can be bound and detoxified by homodimeric glutathione S-transferases (*Na*-GSTs).[78]

Na-glutathione S-transferase 1 (*Na*-GST-1) is a 24-kDa protein comprised of 205 amino acids and one N-glycosylation site.[79] Recombinant *Na*-GST-1 has been expressed in *P. pastoris* at high yield (>0.2 g/L) and purified by three chromatographic steps. The X-ray crystal structure of this protein has been solved.[79] In dogs challenged with infective larvae, vaccination with *Ac*-GST-1 (the canine hookworm ortholog) induced high levels of antibody and resulted in substantially lower worm burdens (40%) and fecal egg counts compared with controls.[77] Using the hamster model of hookworm infection, approximately 57% protection (as measured by worm burden) was achieved by immunization with either *Ac*-GST-1 or *Na*-GST-1.[79,80] Additionally, recent immunoepidemiological studies indicate that individuals living in hookworm endemic areas of Brazil who acquire anti-*Na*-GST-1 IgG1 antibodies have a significantly reduced risk of having heavy hookworm infections (unpublished data).

N. americanus-Aspartic Protease-1 (*Na*-APR-1) is a 45-kDa recombinant protein consisting of 407 amino acids with a C-terminal histidine tag.[77,81] For stability and safety reasons, this aspartic protease has been inactivated by the mutation of the two canonical aspartic acid residues to alanines.[76] The recombinant protein has been expressed in multiple systems, with tobacco plants (*Nicotania benthamiana*) producing the highest yields with the greatest stability and solubility being achieved in the presence of zwitterionic detergents (unpublished data). In animals models, immunization with recombinant *Na*-APR-1 or its canine ortholog *Ac*-APR-1 substantially diminished blood loss, adult hookworm burden and fecal egg counts, as well as reduced gut pathology compared with controls animals immunized with adjuvant alone.[74,76]

Both *Na*-GST-1 and *Na*-APR-1 have been formulated with the aluminum salt adjuvant Alhydrogel®. However, in addition to Alhydrogel®, it may be advantageous to co-administer these formulated antigens with one of the new generation of immunostimulants such as the Toll-like receptor agonist GLA-AF (an aqueous formulation of synthetic glucopyranosyl lipid A) as described in[82-85] to improve not only the quantity of the antibody response (more antibody), but also the quality of this response (better affinity and the develop or greater memory B cells production) (**Box 3**).

Box 3. Technical challenges for the development of a human hookworm vaccine.
1. The difficulty of maintaining human hookworms in animal models and the cost of maintaining the hookworm in laboratory-canine model.
2. The absence of an laboratory animal that is permissive to human hookworms and can accurately reproduce human disease (anemia).
3. Paucity of in vitro functional tests to determine the effectiveness of the immune response induced by an experimental hookworm vaccine.
4. The lack of a protective immune response in humans and the consequent absence of Correlates of Protection (CoP) that can guide the discovery of vaccine antigens and be used to assess their effectiveness in preclinical and clinical trials.
5. No model of an effective immune response in humans to determine the biological effect of the vaccine in humans.

Current Plans

By simultaneously targeting two enzymes in the blood digestion and detoxification pathway, we hypothesize that the bivalent human hookworm vaccine will elicit neutralizing antibodies that will have a substantial impact on host worm burden (as measured by quantitative fecal egg counts) and blood loss. It is anticipated that vaccination with both recombinant proteins will be needed to disrupt this complex system of hemoglobin digestion, and thereby reduce parasite fecundity and survival at a more rapid rate. Based on previous animal trials conducted during preclinical efficacy testing of each antigen individually, it is anticipated that the impact of the HHV on human populations will prevent the establishment of adult hookworms in the range of 40–50%. By preventing the establishment (or re-establishment with reinfection) of adult hookworms, most (≥80%) moderate and heavy infections would be prevented along with the associated IDA.

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