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INVITED REVIEW

Progress in the development of a recombinant vaccine for human hookworm disease: The Human Hookworm Vaccine Initiative

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Abstract
Hookworm infection is one of the most important parasitic infections of humans, possibly outranked only by malaria as a cause of misery and suffering. An estimated 1.2 billion people are infected with hookworm in areas of rural poverty in the tropics and subtropics. Epidemiological data collected in China, Southeast Asia, and Brazil indicate that, unlike other soil-transmitted helminth infections, the highest hookworm burdens typically occur in adult populations, including the elderly. Emerging data on the host cellular immune responses of chronically infected populations suggest that hookworms induce a state of host anergy and immune hyporesponsiveness. These features account for the high rates of hookworm reinfection following treatment with anthelminthic drugs and therefore, the failure of anthelminthics to control hookworm. Despite the inability of the human host to develop naturally acquired immune responses to hookworm, there is evidence for the feasibility of developing a vaccine based on the successes of immunizing laboratory animals with either attenuated larval vaccines or antigens extracted from the alimentary canal of adult blood-feeding stages. The major antigens associated with each of these larval and adult hookworm vaccines have been cloned and expressed in prokaryotic and eukaryotic systems. However, only eukaryotic expression systems (e.g., yeast, baculovirus, and insect cells) produce recombinant proteins that immunologically resemble the corresponding native antigens. A challenge for vaccinologists is to formulate selected eukaryotic antigens with appropriate adjuvants in order to elicit high antibody titers. In some cases, antigen-specific IgE responses are required to mediate protection. Another challenge will be to produce anti-hookworm vaccine antigens at high yield low cost suitable for immunizing large impoverished populations living in the developing nations of the tropics.

Keywords: Hookworm; *Necator*, *Ancylostoma,* vaccines, recombinant proteins, expression systems

1. "The Great Infection of Mankind"

The World Health Organization (WHO) estimates that approximately two billion people worldwide are infected with the soil-transmitted nematode helminths *Ascaris lumbricoides*, *Trichuris trichirua*, and the hookworms *Necator americanus* and *Ancylostoma duodenale*. Approximately 400 million of these infected individuals are children of school age and 300 million suffer from heavy worm burdens sufficient to result in severe disease. Overall, it is believed that, together with schistosomiasis, the soil-transmitted helminth infections account for 40% of the global morbidity caused by all infectious diseases, exclusive of malaria (WHO, 2000).

Because they injure their human hosts directly by causing intestinal blood loss leading to iron deficiency and protein malnutrition, some investigators implicate hookworms as the most important helminthic cause of global disease burden. Professor Norman Stoll of the Rockefeller Institute and Foundation considered hookworms "The Great Infection of Mankind" (Stoll, 1962), second only to malaria as the major cause of human misery in the tropics.

Despite its global importance hookworm has, until recently, been overlooked as a public health problem. There are several reasons for this situation. Among them, hookworm disease and anemia, which result from parasite-induced blood loss are frequently insidious and seldom dramatic in their clinical presentation; they are not usually associated directly with high mortality. Therefore, clinical experts often fail to link human hookworm infection to a significant cause of morbidity worldwide. Moreover, because of the misconception that hookworm could be prevented by increased use of shoes in tropical countries, hookworm was often not deemed worthy of scientific study. Possibly for these reasons, hookworm was not listed as one of the six major tropical infections of the Tropical Disease Research (TDR) program established by the WHO during the 1980s (Hotez *et al.*, in press).

Our ignorance regarding the public health importance of hookworm began to change during the early 1990s when new quantitative estimates of disease burden based on the concept of disability adjusted life years (DALYs) revealed hookworm's global impact (Murray and Lopez, 1996). DALY estimates determined that the disease burden from hookworms exceeds three tropical infectious diseases under investigation in the WHO-TDR program: African trypanosomiasis, Chagas disease, and leprosy. Hookworm was also found to outrank dengue fever (Murray and Lopez, 1996).

More recent DALY estimates for the global burden of disease in 2001 indicate that hookworms cause even greater disease burden than previously believed (WHO, 2002). Hookworm exceeds conditions such as schistosomiasis and both hepatitis B and C. New data on the epidemiology of iron deficiency anemia in East Africa indicate that hookworm accounts for a large percentage of the huge DALY estimates resulting from this condition (Stoltzfus *et al.*, 1997a; Stoltzfus *et al.*, 1997b; Dreyfuss *et al.*, 2000). Iron deficiency anemia currently accounts for a higher percentage of DALYs than HIV-AIDS and is almost as important as malaria! Hookworm-associated iron deficiency has been noted to be particularly common in coastal communities in the tropics (Lwambo *et al.*, 1991). The observation that hookworm anemia occurs in an estimated 44 million women in pregnancy with resultant health consequences for the fetus and newborn (Bundy *et al.*, 1995), will likely add to these disease burden estimates.

Newer findings that hookworms may induce a state of host immunological hyporesponsiveness (see below) and could promote susceptibility to intercurrent viral, bacterial or protozoan infections such as measles, HIV-AIDS (Borkow *et al.*, 2000; Wolday *et al.*, 2002) and tuberculosis (Borkow and Bentwich, 2000; Borkow *et al.*, 2001) have tremendous importance in areas where these diseases overlap. High rates of hookworm are known to occur in Subsaharan Africa (Albonico *et al.*, 1995; Behnke *et al.*, 2000; Bradley *et al.*, 1993; Brooker *et al.*, 2000a; Brooker *et al.*, 2000b; Lwambo *et al.*, 1999; Palmer and Bundy, 1995; Partnership for Child Development, 1998; Stoltzfus *et al.*, 2001; Stephenson *et al.*, 1989), South China (Hotez *et al.*, 1997; Liu *et al.*, 1999; Zhan *et al.*, 2000; Gandhi *et al.*, 2001; Bethony *et al.*, 2002; Hotez, 2002), Southeast Asia (Humphries *et al.*, 1997), India and Nepal (Haswell-Elkins *et al.*, 1988; Dreyfuss *et al.*, 2000), and in the Americas (Bloch and Rivera, 1977; Labiano-Abello *et al.*, 1999). In each of these regions HIV-AIDS is spreading rapidly. Therefore, a firm link between hookworm and HIV-AIDS would forever alter our concept of the importance of hookworm as a cause of global disease burden.

2. The emerging epidemiology of hookworm

Traditionally, the epidemiology of hookworm has been considered to be much like other soil-transmitted helminth infections, such as ascariasis and trichuriasis, i.e., an infection in which the heaviest worm burdens occur in children of school age (Bundy, 1990). Indeed, the clinical effects of pediatric hookworm anemia are well described in the literature (Hotez, 1989), including physical, mental and cognitive growth retardation effects (Smilie and Augustine, 1926; Hotez, 1989; Sakti *et al.*, 1999; Beasley *et al.*, 2002; Lwambo *et al.*, 2000; Hotez, 2000; Stoltzfus *et al.*, 2001).

While heavy hookworm burdens still occur among children in some tropical areas (Stephenson *et al.*, 1989; Labiano-Abello *et al.*, 1999), studies conducted worldwide within the last decade indicate that the peak prevalence and infection intensities for hookworm often occur in individuals in middle age, or even the elderly (Gandhi *et al.*, 2001; Bethony *et al.*, 2002). Shown in Figure 1 are the relationships between age and prevalence or age and intensity of hookworm in two different helminth-endemic regions of China (Hainan Province) and Brazil (Minas Gerias State). These represent areas of high hookworm transmission and endemicity (Gandhi *et al.*, 2001; Bethony *et al.*, 2002), and demonstrate that both prevalence and intensity increase as a function of age. Especially striking is the strong correlation ($r = 0.69$; $P < 0.001$) between age and egg counts shown in Hainan. In the Hainan study, a variance components analysis revealed that age and gender made the most important contributions to infection intensity (28–30%), with age alone responsible for 27% of this variation (Bethony *et al.*, 2002). Figure 2 compares the age-related intensities of hookworm infections in both Hainan and Minas Gerias with other endemic helminth

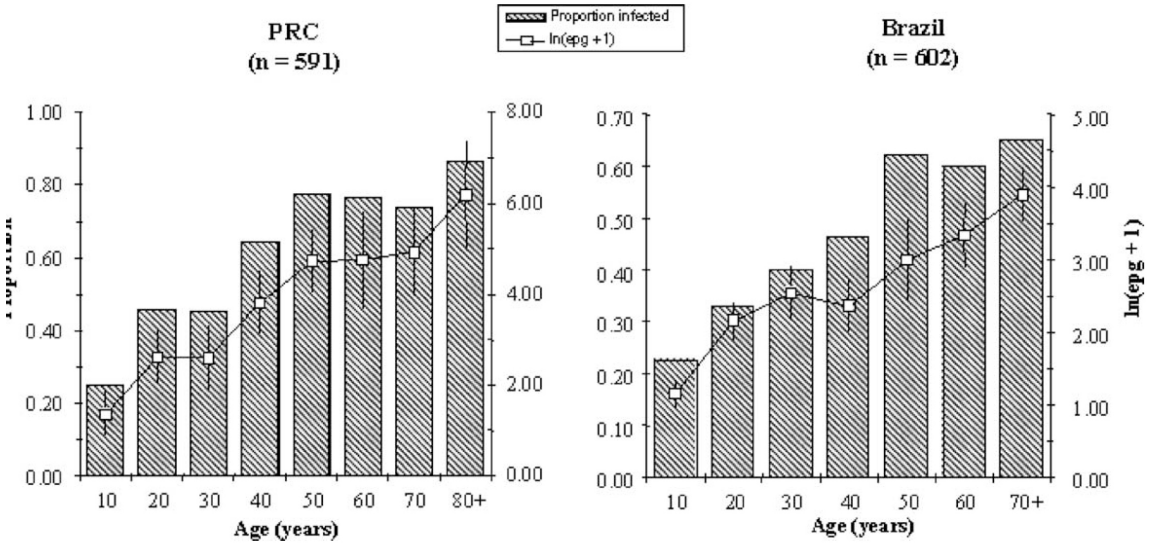

Figure 1. The prevalence and intensity of infection with *Necator americanus* increases with age in two endemic areas: Hainan Province, People's Republic of China (1999) and Minas Gerias, Brazil (2000). Data are from cross-sectional studies. Analysis of variance showed that egg counts were significantly different ($P < 0.001$) among the age intervals, and that the eldest four age intervals were significantly different ($P < 0.05$) from the younger age intervals, but not different from each other.

infections. In Hainan, *Ascaris* and *Trichuris* infections decrease after the age of 20, while in Minas Gerias the intensity of schistosomiasis diminishes after the age of 10. This is in distinct contrast to hookworm epidemiologic patterns. Similar patterns for infection have been observed in other *N. americanus* endemic areas (Liu *et al.*, 1999; Wang *et al.*, 1999; Zhan *et al.*, 2000; Humphries *et al.*, 1997).

The association between increasing age and increasing prevalence and hookworm burden reveals an important public health problem for developing countries, since the elderly are seldom mentioned as a group either at high-risk for infection or the consequent morbidity associated with high worm burdens. The influence of aging on the prevalence and intensity of *Necator* infection has important public health consequences. In some parts of the developing world, such as China, the elderly are one of the most rapidly expanding age groups (Hotez, 2002).

The mechanisms by which hookworms establish chronic infections among the middle-aged and elderly are under active investigation. Increasing evidence suggests that this phenomenon may have an immunologic basis. In Papua New Guinea, Quinnell *et al.* (1993, 1995) observed

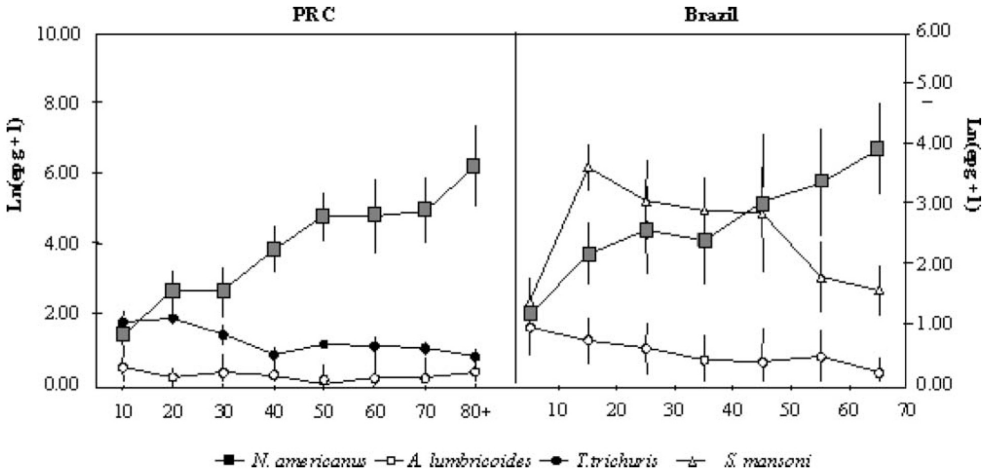

Figure 2. The age distribution of *Necator americanus* differs from other soil-transmitted helminths such as *Ascaris lumbricoides*, *Trichuris trichiura,* as well as *Schistosoma mansoni*. Panel A shows the relationship between age and infection intensity for three STHs in a cross-sectional study from Hainan, People's Republic of China. *Ascaris lumbricoides* and *Trichuris trichiura* epgs peak among children and then decline into adulthood. The intensity of infection with *Necator americanus* increases with age, peaking in eldest age category (80+). Panel B shows the distribution for *Necator americanus* in relation to other helminth infections, from Minas Gerais, Brazil. The distribution of *Schistosoma mansoni* infection intensity shows a traditional convex curve with age, increasing dramatically in young adults. The intensity of infection with *Necator americanus* again increases steadily with age, peaking in the eldest age categories.

that experience with hookworm infection does not confer resistance, except in a selected few individuals who somehow acquire specific IgE responses against hookworm antigens (Pritchard and Walsh, 1995; Pritchard *et al.*, 1995). Susceptibility to reinfection was confirmed among volunteers who remained susceptible to hookworm reinfection even after infecting themselves with *N. americanus* (Maxwell *et al.*, 1987). In support of the inability of the human host to mount natural immunity to *Necator* infection is the preliminary observation that hookworm antigens may interfere with host lymphoproliferation and IL-4 levels during chronic infections (J. M. Bethony and M. E. Bottazzi, unpublished observations). This might even be an active immunosuppressive process that occurs through hookworm-derived secreted antigens. Several investigators have isolated and characterized hookworm macromolecules that interfere with host cellular immune responses (Moyle *et al.*, 1994; Culley *et al.*, 2000).

3. Rationale for hookworm vaccine development

The steady increase of hookworm burdens as a function of age and the apparent absence of naturally acquired immunity in endemic areas have serious implications for national and international control efforts. The public health control of hookworm and other soil-transmitted helminths currently relies heavily on the use of benzimidazole anthelminthic drugs, especially albendazole (Albonico *et al.*, 1999). However, studies sponsored by the WHO and other organizations have shown that high rates of hookworm reinfection can occur within just a few months following benzimidazole treatments (Albonico *et al.*, 1995). Presumably because of the absence of acquired immunity following natural infection, treated individuals remain susceptible to reinfection following exposure to third-stage infective hookworm larvae (L3) in the soil. This biological feature of the host–parasite relationship greatly limits the sustainability of anthelminthic chemotherapy-based control. In addition, most anthelminthic deworming programs worldwide currently target children in order to improve their physical growth, school performance, and cognitive development. While this approach might effectively, over time, reduce endemic exposure and worm burdens for *Ascaris* and *Trichuris* infections, there is no reason to believe that it would have an impact on endemic hookworm. It is therefore not surprising to discover that more than 1.2 billion individuals are infected with hookworms despite the widespread availability of albendazole.

An additional concern regarding the feasibility of sustainable control with benzimidazoles is the possibility of emerging anthelminthic drug resistance among human hookworm populations. In some nematode species benzimidazole resistance occurs secondary to the spread of a single point mutation in nematode tubulin alleles. This phenomenon has already resulted in widespread benzimidazole resistance among soil-transmitted helminths of ruminant livestock in the Southern Hemisphere (Conder and Campbell, 1995), and might partially account for an observed failure of mebendazole chemotherapy for human hookworm in southern Mali (De Clercq *et al.*, 1997).

The unique epidemiology of hookworms resulting from their apparent immunosuppressive properties, together with the potential for emerging anthelminthic resistance, necessitates a search for alternative or complementary approaches to public health control that do not rely exclusively on anthelminthics. One approach for consideration is the development and use of anti-hookworm vaccines.

4. Feasibility of hookworm vaccine development

Is it feasible to develop a vaccine for an infection in which natural experience with the pathogen does not confer immunity? The concept of natural acquisition of immunity was, after all, the cornerstone for the development of first-generation attenuated vaccines against poliomyelitis, measles, and numerous other infections. A central challenge for hookworm vaccine development will be to stimulate an artificial immune response that is unique and results in disease burden reduction. In this sense, an anti-hookworm vaccine must overcome hurdles similar to those faced by vaccinologists who tackle HIV-AIDS and malaria.

Existing evidence that it is feasible to vaccinate against hookworms is based on three independent lines of research (Hotez *et al.*, 1996, 1999, in press):

1. Studies first conducted in the Department of Helminthology at the Johns Hopkins School of Hygiene and Public Health during the 1930s in dogs and mice demonstrated that artificial immunity against challenge infections with *Ancylostoma caninum* L3 could be obtained by administering multiple inocula of living L3 either orally or subcutaneously (McCoy, 1931, Foster, 1935; Kerr, 1936; Otto and Kerr, 1939). The immunity conferred using multiple doses of living L3 was shown to be antibody mediated and could be administered passively (Otto, 1940, 1941). Typically, the immunity elicited by living L3 "vaccination" was not sterilizing but instead operated by reducing hookworm burdens relative to non-vaccinated control animals. The L3-vaccinated dogs were protected against hookworm disease characterized by heavy intestinal blood loss, but they were not protected against hookworm infection. Following very heavy challenge infections the mortality of severe hookworm disease was prevented by vaccination. Subsequent studies on the mechanisms of vaccine immunity demonstrated that host immune responses were directed primarily against the antigens secreted by L3 during host entry (Sarles, 1938; Taliaferro and Sarles, 1939; Sheldon and Groover, 1942).
2. Later, during the 1960s, the principles established by the development of living L3 vaccines were employed to develop an attenuated hookworm vaccine. This was

accomplished by first damaging *A. caninum* L3 with ionizing radiation, including X-rays, gamma-rays and ultraviolet light (Miller, 1971, 1978, 1987). The irradiated L3 needed to remain viable in order for the vaccine to work—presumably viability was essential in order to ensure that the damaged L3 continued to secrete antigens after host entry. Ionizing radiation allowed the vaccinator to increase the dose of L3 used in an inoculum thereby decreasing the number of doses required to achieve effective immunity. Ultimately, an attenuated canine hookworm vaccine was developed and marketed first in Florida and later in the Eastern United States during the early 1970s (Miller, 1978). The product soon failed commercially, however, because of its high production costs, limited shelf-life viability and inability on the part of both pet owner and veterinarian to appreciate how a vaccine that does not elicit sterilizing immunity (i.e., does not prevent against hookworm infection) is still useful for protecting against hookworm disease.

3. Extracts of the esophagus of adult *A. caninum* elicit acquired immunity (Thorson, 1956). This parasite organ is enriched with proteases and much of the antibody in vaccinated dogs has the capacity to neutralize parasite protease activity (Thorson, 1956). These efforts confirmed a prediction first made by Asa Chandler during the 1930s that anti-enzyme antibodies have importance in mediating protective immunity against helminths (Chandler, 1932, 1936). More recently, studies conducted to develop a vaccine against *Haemonchus contortus*, a blood-feeding trichostrongyle nematode of sheep that is phylogenetically related to hookworms, identified the major antigens aligning the brush border membrane of the blood-feeding adult parasite alimentary canal (Knox, 2000). Among these antigens are parasite gut-derived glycoproteins that comprise a complex of proteases and other components (designated "H-gal-GP"), which bind to peanut lectin. Although the H-gal-GP complex is not ordinarily recognized during natural infection, and is considered a hidden antigen, the complex when administered with a saponin-derived adjuvant (Quil A) elicits high levels of vaccine protection with respect to adult worm burdens and fecundity (Knox and Smith, 2001).

To summarize, the feasibility of developing anti-hookworm vaccines is based on more than 70 years of successful protection in laboratory animals, which is elicited either by living L3 and their secreted antigens or by the antigens comprising the adult parasite alimentary canal.

5. Hookworm antigen discovery and selection

Hookworm antigens were selected as lead candidates for further testing on the basis of their link with either attenuated larval vaccines or the H-gal-GP complex.

5.1. L3 secreted antigens

Progress on attenuated hookworm larval vaccines pointed to the importance of secreted L3 antigens as the major immunogenic macromolecules associated with vaccine protection. However, it was found that *A. caninum* L3 constitutively release only trace amounts of protein (Hawdon *et al.*, 1995). A major breakthrough was the observation that L3, stimulated at 37 °C in the presence of a cocktail composed of an ultrafiltrate of canine serum and glutathione (or its derivatives), release comparatively large amounts of parasite-derived proteins (Hawdon and Hotez, 1996; Hawdon *et al.*, 1995, 1996, 1999; Zhan *et al.*, 2002a). This observation made it possible to isolate the major secreted L3 antigens, obtain partial amino acid sequences of the purified proteins, and subsequently clone and sequence their corresponding cDNAs.

5.1.1. Ancylostoma secreted proteins

The two most abundant gene products released by L3 under host-stimulatory conditions are cysteine-rich secretory proteins (CRISPs) belonging to the pathogenesis related protein (PRP) superfamily. The PRP superfamily is composed of a phylogenetically diverse array of animal and plant proteins including insect venom allergens, mammalian testis and epididymal proteins, and plant-pathogenesis related protein (Henriksen *et al.*, 2001). The major L3 *Ancylostoma* secreted proteins (ASPs) are composed of ASP-2 (Hawdon *et al.*, 1999), a 24 kDa protein with a single PRP domain, and ASP-1 a 45 kDa double PRP domain protein corresponding to a heterodimorphic repeat (Hawdon *et al.*, 1996). Isolation of approximately 100 pmoles of each protein required the collection of up to 300,000 *A. caninum* L3, which were obtained by coproculture from the feces of infected donor dogs. This was followed by partial amino acid sequencing of the purified protein and then the synthesis of degenerate oligonucleotides for cDNA library screening (Hawdon *et al.*, 1996, 1999). Orthologues of both *asp-1* and *asp-2* cDNAs were also subsequently isolated and cloned from *A. duodenale*, *Ancylostoma ceylancium* and *N. americanus* (Zhan *et al.*, 1999). The orthologues exhibit high degrees of amino acid sequence similarity to each other. For instance, the *N. americanus* ASP-1 (Na-ASP-1) exhibits 97% identity to *A. caninum* ASP-1 (Ac-ASP-1).

The biological function of the ASPs is not known. One study conducted with an ASP orthologue from *Onchocerca volvulus* suggests that it may exhibit angiogenic properties (Tawe *et al.*, 2000). However, ASP orthologues are also present in the free-living nematode *Caenorhabditis elegans* and the plant pathogen *Meloidogyne incognita*, and therefore also presumably have a function that is unrelated to the host–parasite relationship. Adult hookworms also produce single and double domain ASPs, which immunolocalize to a number of different nematode organs including the cuticle, cephalic glands, and the brush border of the alimentary canal (Zhan *et al.*, 2003).

There are several lines of evidence that point to the ASPs as promising vaccine candidates: (1) Natural product single and double domain ASPs were shown to protect sheep and guinea pigs against challenge infections with *H. contortus* (Kooyman *et al*., 2000; Sharp *et al*., 1992; Sharp and Wagland, 1998; Schallig *et al*., 1997a, 1997b, 1997c). Vaccine protection relies on the presence of high levels of anti-ASP IgE (Kooyman *et al*., 2000). (2) Antibodies obtained from the abomasal mucus and draining lymph nodes from calves immunized with multiple infections of *Ostertagia ostertagi* recognize an ASP orthologue (De Maere *et al*., 2002). In addition fractions of helminth antigens that protect cattle against *O. ostertagi* infection are enriched in single domain ASPs (Geldhof *et al*., 2003). (3) Vaccination of mice with a recombinant *Escherichia coli* fusion protein composed of a polyhistidine tag and amino acids 96–424 of either Ac-ASP-1 or Na-ASP-1 results in host immune responses, which inhibit the extra-intestinal migration of *A. caninum* L3 into the lungs following oral infection (Ghosh *et al*., 1996; Ghosh and Hotez, 1999; Liu *et al*., 2000). Anti-Ac-ASP-1 antibody also passively protects against larval migration (Ghosh and Hotez, 1999). (4) In two cross-sectional epidemiologic studies it was determined that residents of Hainan Province, China and Minas Gerais State, Brazil who harbor less intense hookworm burdens (as measured by quantitative egg counts) exhibit high titers of circulating antibodies against a single domain ASP from L3 (Jeff Bethony, unpublished observation).

5.1.2. Astacin-like metalloprotease (MTP)

Hookworm L3 also releases a 62 kDa zinc metalloprotease upon host-stimulation (Zhan *et al*., 2002a). The MTP-1 enzyme is of the astacin class represented by a metalloprotease from the crayfish *Astacus astacus*. In addition to a catalytic domain composed of zinc in the enzyme active site coordinated by the imidazoles of conserved histidine residues, MTP-1 also contains an epidermal growth factor domain of unknown function. MTP-1 is an immunodominant protein and was first cloned and isolated by screening an *A. caninum* cDNA expression library with pooled sera from hookworm-infected individuals who harbor low worm burdens and are putatively resistant (Zhan *et al*., 2002a). Calves immunized with *O. ostertagi* L3 also recognize an MTP-1 orthologue (De Maere *et al*., 2002). Studies examining the vaccine potential of Ac-MTP-1 from *A. caninum* are under evaluation, as is an orthologue from *Ancylostoma ceylanicum* (see below).

5.1.3. Other L3 antigens

In addition to the ASPs and MTP-1, *Ancylostoma* L3 also releases macromolecules with amino acid similarities to a 60 kDa acetylcholinesterase (ACH) and a 16 kDa (144 amino acids) putative transthyreitin (TTR), which may bind retinol binding proteins (Hawdon *et al*., unpublished). Previously, a secreted acetylcholinesterase has shown promise as a vaccine target against *Dictyocaulus viviparous* (McKeand, 2000) and *Trichostrongylus colubriformis* (Griffiths and Pritchard, 1994). Each of these proteins is being evaluated as vaccine candidates, as is a hookworm L3 surface protein similar to Ov-103 from *O. volvulus*.

5.2. Adult hookworm antigens

5.2.1. Secreted antigens

There is no evidence that the excretory–secretory proteins released by adult hookworms elicit high levels of vaccine protection. The major protein secreted by adult hookworms is TMP, a 16 kDa orthologue of mammalian tissue inhibitor of metalloprotease (Zhan *et al*., 2002b). Ac-TMP comprises almost 10% of the protein secreted in vitro by adult *A. caninum* (Zhan *et al*., 2002b). The function of this molecule for adult hookworms at the site of attachment in the mammalian intestine is unknown. However, of interest is the observation that regions of amino acid sequence similarity between Ac-TMP and mammalian TIMPs are not those predicted to be in contact with their target matrix metalloproteases (Zhan *et al*., 2002b). This suggests the possibility that Ac-TMP has a function other than metalloprotease inhibition. In addition to TMP, adult *A. caninum* hookworms also release serine protease inhibitors including those involved in the coagulation cascade such as factor Xa (Cappello *et al*., 1995, 1996) and VIIa-tissue factor (Stanssens *et al*., 1996), and some adult-specific ASPs (Zhan *et al*., in press).

5.2.2. H-gal-GP orthologues

The H-gal-GP complex from the brush border of the alimentary canal of *H. contortus* is composed of multiple components including a metalloendopeptidase of the neprilysin family, an aspartic protease, a cysteinyl protease, a cystatin, a thrombospondin orthologue, and a galectin (Knox, 2000). As noted above, the complex elicits high levels of protection against *H. contortus* challenge infections in sheep (Knox, 2000; Knox and Smith, 2001), although each component has so far not been examined individually as a natural product. Orthologues of each of the protease components of the complex have been cloned from hookworms including *A. caninum*-metalloendopeptidase-1 (Ac-MEP-1) (Jones and Hotez, 2001), *A. caninum*-cysteinyl proteases (Ac-CP-1 and Ac-CP-2) (Harrop *et al*., 1995; Loukas *et al*., 2000), and *A. caninum*-aspartic proteases (Ac-APR-1 and Ac-APR-2) (Harrop *et al*., 1996; Williamson *et al*., 2002, 2003a, 2003b). Immunolocalization studies confirm that some of these proteases align the brush border membrane of the alimentary canal of adult hookworms (Figure 3). These proteases are believed to operate in a coordinated fashion to facilitate host hemoglobin degradation during blood feeding (Brinkworth *et al*., 2000, 2001).

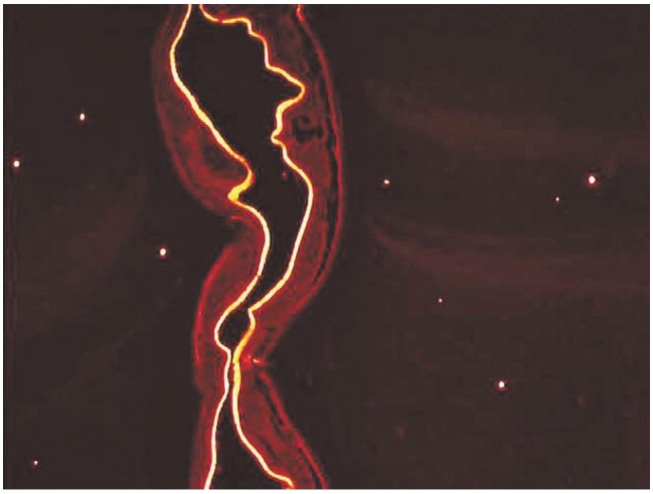

Figure 3. Immunolocalization of Ac-MEP-1 (neprilysin-like adult hookworm metalloprotease) to the brush border membrane of the adult hookworm alimentary canal.

6. The rules of engagement

Work conducted during the decade of the 1990s on *H. contortus* vaccine protection experiments in sheep and *A. caninum* vaccine protection experiments in mice has provided some useful paradigms that might be applicable to preclinical vaccine protection experiments in laboratory animals.

1. Many of the candidate vaccine antigens under evaluation are hydrolytic enzymes, including proteases and acetylcholinesterases from L3 and adult stages. While the natural products isolated from parasites typically maintain their enzymatic activity, the activity is lost when the protease cDNAs are expressed in *E. coli*. Therefore, expression of bioactive molecules usually requires eukaryotic vectors.
2. Similarly, in instances where natural product antigens protect against challenge infections, the corresponding recombinant protein expressed in *E. coli* fails to reproduce the protection. In most cases this is because the conformational epitopes are lost during *E. coli* expression. Incorrect disulphide bond formation probably accounts for most of the lost epitope expression, particularly for cysteine-rich proteins like the ASPs, rather than the absence of correct glycosylation. The failure of *E. coli* to produce correctly folded antigens for vaccination was observed for L3 ASPs from *H. contortus* (Sharp and Wagland, 1998) and *A. caninum* (see below), and for the H-gal-GP complex components (Knox and Smith, 2001).
3. In some cases eukaryotic expression restores the conformational epitopes lost during *E. coli* expression. This has been demonstrated for baculovirus expressed ASP from *H. contortus* (Sharp and Wagland, 1998) and yeast-expressed Ac-ASP-1 (see below).
4. Among the eukaryotic vectors that have been employed to express candidate nematode vaccine antigens are baculovirus, insect cells, and yeast. To date, it has not been possible to predict which vector system will work best. However, baculovirus and insect cells will likely prove to be too expensive for the manufacture of a public sector hookworm vaccine. Leading the yeast expression vectors is the methanol-utilizing organism *Pichia pastoris*, which produces proteins in high yields and is relatively inexpensive compared to other eukaryotic expression systems.
5. In the few studies that have attempted to define the mechanisms associated with vaccine protection, achieving high levels of host antibody is required for reduction in worm burdens (Ghosh and Hotez, 1999). In some cases host IgE may be the principal effector antibody. This was suggested by the finding that low *H. contortus* worm burdens in sheep associated with high levels of IgE directed *H. contortus* ASP (Kooyman *et al.*, 2000). The importance of IgE antibody is also underscored by preliminary epidemiologic data suggesting that host IgE directed against ASP-2 was associated with reduced hookworm intensity, as determined by quantitative egg counts (Jeff Bethony, unpublished data).
6. Achieving high levels of host antibody using baculovirus, insect cell, or yeast-expressed proteins will be challenging, particularly when these proteins are formulated with aluminum-based adjuvants such as alum or alhydrogel. The requirement for high levels of immunogenicity has prompted the search for alternative adjuvants.

7. Hookworm antigen expression

Over the last 2 years, focused efforts have been made to express the lead candidate antigens in both prokaryotic and eukaryotic systems (Table 1).

7.1. L3 hookworm antigens

Expression of *Na-asp-1*, *Ac-asp-2*, and *Ac-mtp-1* cDNAs in *E. coli* has so far produced recombinant fusion proteins containing polyhistidine tags, which are neither soluble nor correctly refold in the absence of harsh denaturants, including ionic detergents. In addition, the *E. coli* recombinant fusion proteins frequently are expressed in inclusion bodies that are heavily contaminated with other bacterial constituents. Frequently it is not possible to separate the recombinant fusion proteins away from these contaminants. In the case of the ASPs, the high number cysteines (10 in ASP-2 and 20 ASP-1) presumably cause recombinant polypeptides to form with aberrant disulphide bond formation. Recombinant *E. coli* Na-ASP-1 does not exhibit conformational epitopes corresponding to the native protein. As shown in Figure 4, polyclonal antibodies prepared against *E. coli* Na-ASP-1 fail to immunoprecipitate the corresponding native protein from soluble L3 extracts. Similarly, recombinant Ac-MTP-1 expressed in *E.*

Table 1. Lead candidate hookworm vaccine antigens

Antigen	Description	MW (kDa)	Expression vector
L3 secreted antigens			
ASP-1	Pathogenesis-related protein	45	*Pichia pastoris*
ASP-2	Pathogenesis-related protein	24	*P. pastoris* and insect cell
MTP-1	Astacin metalloprotease	62	Baculovirus
TTR	Transthyreitin	16	*Escherichia coli* and P. pastoris
ACH	Acetylcholinesterase	60	*P. pastoris*
Adult gut-membrane antigens (H-gal-orthologues)			
MEP-1	Neprilysin metalloprotease	99	Insect cells
APR-2	Aspartic protease	45	Baculovirus
CP-1	Cysteinyl protease	35	Insect cells and *P. pastoris*
CP-2	Cysteinyl protease	35	P. pastoris
ASP-5	Pathogenesis-related protein	24	*P. pastoris* (pending)

coli does not exhibit enzymatic activity. In order to overcome these hurdles and express recombinant proteins either having catalytic activity or appropriate conformational epitopes, their corresponding cDNAs had to be re-engineered in eukaryotic vectors, including baculovirus, insect cells, or yeast. Polyclonal antibodies prepared against *P. pastoris* expressed Na-ASP-1 immunoprecipitate the native protein from soluble L3 extracts (Figure 4). To date, the following L3 recombinant proteins have been successfully expressed in *P. pastoris*: Na-ASP-1, Na-ASP-2, and Ac-TTR, from *N. americanus* and *A. caninum*, respectively, as well as Ay-ASP-1 and Ay-ASP-2, the orthologues from *A. ceylanicum*. One of the L3 acetylcholinesterases has also been expressed (M. Selkirk, personal communication). To date it has not been possible to express soluble Ac-MTP-1 and Ac-ASP-2 in yeast. However, Ac-ASP-2 as well as catalytically active Ac-MTP-1 was recently expressed in baculovirus.

7.2. Adult hookworm antigens

Two of the small protease inhibitors released by adult *A. caninum* hookworms, Ac-TMP and Ac-AP (factor Xa inhibitor anticoagulant peptide) have been expressed as soluble recombinant fusion proteins in *E. coli*. However, none of the H-gal-GP orthologous proteases expressed in *E. coli* are soluble or exhibit enzymatic activity. Ac-MEP-1, Ac-CP-1, a cysteinyl protease, and the aspartic proteases, in contrast have been expressed as bioactive, soluble enzymes in either viral or non-viral insect cell expression systems (Loukas, Williamson, *et al.*, unpublished). Ac-CP-2 has been expressed in *P. pastoris* in biologically active form. Work is in progress to express the adult-stage-specific ASPs in eukaryotic systems (Zhan *et al.*, in press).

8. Hookworm antigen-adjuvant formulation and vaccine testing

Two laboratory animal systems are now in routine use for preclinical vaccine development: hamsters challenged with L3 of *A. ceylanicum* and dogs challenged with L3 of *A. caninum*. An advantage of the hamster model is its relatively low cost compared to dogs. In addition, hamsters infected with *A. ceylanicum* experience weight and blood loss similar to human disease from heavy infections (Bungiro *et al.*, 2001). However, *A. ceylanicum* does not ordinarily cause intestinal blood loss in humans and the hamster is not a natural host for the parasite. In addition, the number of adult hookworms that establish in hamsters is low—this requires the use of large numbers of hamsters in order to achieve adequate sample sizes for comparing experimental and control groups. In contrast, dogs are the natural hosts of *A. caninum*, and this model closely resembles *A. duodenale* hookworm infection. The ability to achieve high adult worm burdens in the laboratory

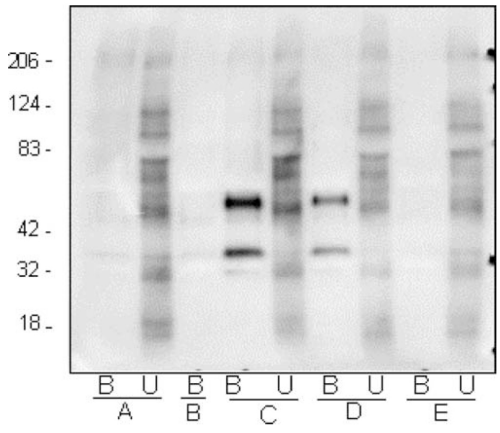

Figure 4. Immunoprecipitation of Ac-ASP-1 from biotinylated extracts of *A. caninum* L3. B, protein bound to strepavidin resin (immunoprecipitated). U, protein unbound to strepavidin resin (non-immunoprecipitated). Group A, canine antiserum to two doses of irradiated *A. caninum* L3. Group B, canine antiserum to alum (adjuvant control). Group C, canine antiserum to Na-ASP-1 expressed in *Pichia pastoris* and administered with alum in four doses separated by 3 weeks. Group D, canine antiserum to Na-ASP-1 expressed in *Pichia pastoris* and administered with alum in four doses on days 1, 4, 60, and 64. Group E, canine antiserum to Na-ASP-1 expressed in *Escherichia coli* and administered with alum in four doses on days 1, 4, 60, and 64.

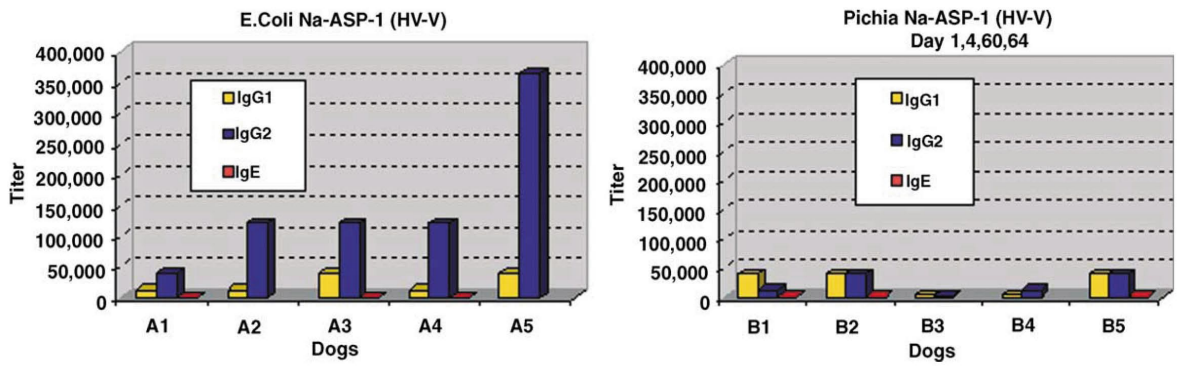

Figure 5. Comparison of antigen-specific IgG1, IgG2, and IgE canine antibody titers in purpose-bred beagle dogs (n = 5 per group) vaccinated with 150 μg of *Escherichia coli* Na-ASP-1 (four doses) or *Pichia pastoris* Na-ASP-1 (four doses using two different schedules). An additional group of dogs (n = 5) was vaccinated with *A. caninum* irradiated L3 (two doses).

reduces the sample size required for comparing experimental and control groups. For vaccine studies, purpose-bred beagles are usually vaccinated with hookworm antigens formulated with adjuvants beginning at 8–10 weeks of age. Up to 2 months are usually required to complete the immunization series, followed by challenge infections (Hotez *et al.*, 2002).

In addition to the two Ancylostoma systems under investigation, some efforts have been made to adapt *N. americanus* to laboratory hamsters. Recently, Xiao Shuhua and his colleagues adapted *N. americanus* by passing it through 100 generations in a Chinese strain of the golden hamster (Xue *et al.*, in press). By doing so, they have obviated the requirement for exogenous steroids and can obtain reasonable levels of adult hookworm burdens by infecting hamsters at 9–10 weeks of age. In contrast, other investigators have had to rely on infecting neonatal hamsters (Behnke and Pritchard, 1987).

To date, studies in dogs and hamsters have revealed that *E. coli* recombinant fusion proteins are more immunogenic than recombinant proteins expressed in baculovirus, insect cells, or yeast. Shown in Figure 5 is a direct comparison of pre-challenge antibody titers in dogs (five per group) vaccinated either with *E. coli* Na-ASP-1 or *P. pastoris* Na-ASP-1 formulated with alhydrogel. The former exhibits far higher IgG2 antibody titers, although the IgG1 and IgE responses are similar. However, it is not clear whether the IgG2 responses might be directed against bacterial lipopolysaccharide or *E. coli* protein contaminants. As noted above (Figure 4), many of the peptide sequences recognized by canine anti-*E. coli*-Na-ASP-1 do not correspond to conformational epitopes on the native protein.

The comparatively low immunogenicity of eukaryotic recombinant hookworm antigens formulated with aluminum-based adjuvants (e.g., alum and alhydrogel) points out a new requirement to explore alternative adjuvants. Among those under investigation, are oil–water emulsions such as montanide ISA-720 (Seppic) and ASO3 (GlaxoSmithKline), as well as adjuvants containing saponin derivatives such as ASO2 (GlaxoSmithKline) and Quil A. ASO2 is an oil–water emulsion that contains QS-21 (derived from saponin) and a lipid A derivative. Both ISA-720 and ASO2 have been used successfully in human clinical trials (Bojang *et al.*, 2001; Doherty *et al.*, 1999).

The importance of eliciting strong immune responses against the candidate recombinant hookworm antigens is highlighted by studies, which examine the relationship between antibody titers and hookworm burdens. As shown in Figure 6, high levels of antigen-specific IgE directed against recombinant *E. coli* Na-ASP-1 and Ac-TTR inversely correlate with hookworm burdens. In the case of *E. coli* Ac-MTP-1, the inverse relationship between antibody titer and worm burden is best correlated with IgG2 (Figure 6). Because many of these antibodies directed against these *E. coli* proteins probably do not recognize conformational epitopes, the levels of vaccine protection should improve significantly by employing recombinant antigens expressed in eukaryotic systems. Also of great promise is the exploitation of new platform technologies for the development of anti-hookworm vaccines, including the use of hepatitis B core particles for the expression of peptide epitopes from candidate vaccine antigens (Birkett *et al.*, 2002). Among them are amino acids 291–303 of Na-ASP-1, which correspond to a predicted hydrophilic loop in the native molecule.

To summarize, each of the lead antigens has been expressed in eukaryotic vectors and is now under evaluation as candidate vaccines in one or more of three different laboratory animal challenge models (*A. caninum* in dogs, *A. ceylanicum* in hamsters and *N. americanus* in hamsters). Results from these studies will provide the basis for up-selection leading to process development and pilot manufacture.

9. Future prospects

Even after process development and pilot manufacture is completed, however, there are still a number of hurdles that must be overcome before an anti-hookworm vaccine is produced for the developing world. Among them are the following.

1. No human clinical trials have ever been conducted with a recombinant nematode vaccine.
2. Preliminary studies conducted in Brazil suggest that patients chronically infected with hookworm are functionally immunosuppressed and do not develop lymphoproliferative responses to hookworm antigens (J. Bethony and M. E. Bottazzi, unpublished observations). However, some patients who are treated for hookworm with anthelminthic drugs acquire a renewed capacity to respond to hookworm antigens. This suggests that human anti-hookworm vaccinations may need to be preceded by an initial round of anthelminthic treatment.
3. Little is known on the role of cross reactivity and regulation of the immune response in individuals carrying more than a single helminthic infection, or its correlation with the development of resistance to infection and reinfection.
4. If the requirement to develop IgE responses against some of the lead candidate hookworm vaccine antigens is confirmed, then it may become necessary to choose adjuvant formulations and doses designed to enhance allergic-type responses. This could contribute to the vaccine's ultimate toxicity.
5. Many of the hookworm antigens selected for further development contain partial amino acid sequences that are homologous with mammalian proteins. The potential for cross reactivity to autoantigens require investigation.
6. Hookworm is a disease of the "poorest of the poor" in the developing nations of the tropics. For this reason, there is practically no commercial market for an anti-hookworm vaccine. The institutions for developing and manufacturing public sector vaccines are still rudimentary. This is particularly unfortunate, given that the next generation of vaccines for diseases in developing countries, e.g., hookworm, HIV-AIDS, malaria, will require high levels of technological sophistication (Hotez, 2001; Broder *et al.*, 2002).
7. Immunity to hookworm vaccines must be long-lasting in order to protect at-risk individuals exposed to hookworm L3 in adulthood.
8. The geographic variation of the candidate hookworm antigen amino acid sequences is largely unknown. However, preliminary studies with Na-ASP-1 indicate that for this particular antigen, geographic variation is not significant (J. Hawdon, unpublished observation).

Despite these obstacles, the potential impact of developing a hookworm vaccine is huge. Because of its contribution to the burden of disease caused by iron deficiency anemia, hookworm is arguably the most significant human helminth pathogen worldwide. Its possible role in promoting increased susceptibility to other intercurrent pathogens, including HIV-AIDS will ensure hookworm's disease burden impact.

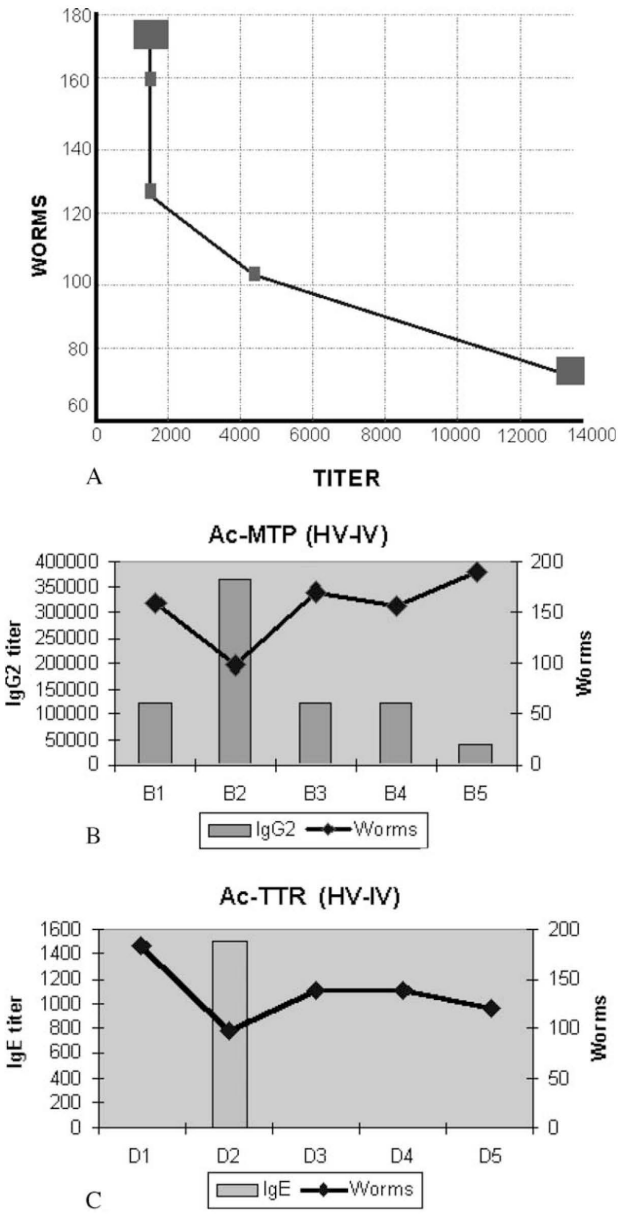

Figure 6. Inverse relationships of antibody titers to *Escherichia coli* expressed recombinant fusion proteins to hookworm burden in vaccinated dogs. Purpose-bred beagles were vaccinated at 8–10 weeks of age and each received three to four doses of the recombinant protein formulated with an aluminum-based adjuvant for ASO2 (GlaxoSmith-Kline). (A) Anti-Na-ASP-1 IgE, (B) anti-Ac-MTP-1 IgG2, (C) anti-TTR IgE. Figure 6B was modified from a figure published in Hotez *et al.* (2002).

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Further Reading

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