



Progress in the Development of a Recombinant Vaccine for Human Hookworm Disease: The Human Hookworm Vaccine Initiative

**DISEASE CONTROL
PRIORITIES PROJECT**

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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 in 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 energy and immune hyporesponsiveness. These features account for the high rates hookworm reinfection following treatment with anthelmintic drugs and therefore, the failure of anthelmintics 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 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.

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1. “The Great Infection of Mankind”

The World Health Organization estimates that approximately 2 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 percent 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 (Hotez, 2003). There are several reasons for this situation. Among them, hookworm disease and anemia, which results from parasite-induced blood loss is frequently insidious and seldom dramatic in its clinical presentation; it is 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 misconcep-

tion 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, 2003).

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; Hotez, 2003).

More recent DALY estimates may determine that hookworms cause even greater disease burden than previously believed. New data on the epidemiology of iron deficiency anemia in East Africa indicates that hookworm accounts for a large percentage of the huge DALY estimates resulting from this condition (Stoltzfus et al, 1997 a and b; 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) has tremendous importance in areas where these diseases overlap. High rates of hookworm are known to occur in Sub-Saharan Africa (Albonico et al, 1995; Behnke et al, 2000; Bradley et al, 1993; Brooker et al, 2000a; 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 treated 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 anaemia 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, 2000; 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 occurs in individuals in middle age, or even the elderly (Gandhi et al, 2001; Bethony et al, 2002). Shown in Fig. 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 Geras State), respectively. 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). Fig. 2 compares the age-related intensities of hookworm infections in both Hainan and Minas Geras with other endemic helminth infec-

tions. In Hainan, *Ascaris* and *Trichuris* infections decrease after the age of 20, while in Minas Gerais 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 suggest that this phenomenon may have an immunologic basis. In Papua New Guinea, Quinnell et al (1993; 1995) observed 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 impli-

cations 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 anthelmintic 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 anthelmintic chemotherapy-based control. In addition, most anthelmintic 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 anthelmintic drug resistance among human hookworm populations. Benzimidazole resistance occurs secondary to the spread of a single point mutation in nematode tubulin alleles. This phenomenon has already resulted in widespread benzimidazole drug 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 anthelmintic resistance, necessitates a search for alternative or complementary approaches to public health control that do not rely exclusively on anthelmintics. 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; 2003):

- 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 *Ancylostoma 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 binds 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.

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 comprised 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, 2002). 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.

Ancylostoma secreted proteins (ASPs). 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 comprised 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 comprised 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 heterodimeric repeat (Hawdon et al, 1996) (Fig. 3). Isolation of approximately 100 pmol 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*, *A. 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, unpublished).

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; Schallig et al, 1997a; 1997b; 1997c; Sharp et al, 1992; Sharp and Wagland, 1998). 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-1 orthologue (De Maere et al, 2002). 3) Vaccination of mice with a recombinant *E. coli* fusion protein comprised of a polyhistidine tag and amino acids 96-424 of either Ac-ASP-1 or Na-ASP-1 result 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).

Astacin-like metalloprotease (MTP). Hookworm L3 also release a 62 kDa zinc metalloprotease upon host-stimulation (Zhan et al, 2002). The MTP-1 enzyme is of the astacin class represented by a metalloprotease from the crayfish *Astacus astacus*. In addition to a catalytic domain comprised of zinc in the enzyme active site coordinated by the imidazoles of conserved histidine residues, MTP-1 also contains epidermal growth factor and CUB domains of unknown function (Fig. 4). 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 *A. ceylanicum* (see below).

Other L3 antigens. In addition to the ASPs and MTP-1, *Ancylostoma* L3 also release macromolecules with amino acid similarities to a 60 kDa acetylcholinesterase (ACH) and a 16 kDa (144 amino acids) putative transthyretin (TTR), which may bind retinol binding proteins (Hawdon et al, unpublished). Previously, a secreted acetylcholinesterase has shown promise as a vaccine target against *Dictyocaulus viviparus* (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 *Onchocerca volvulus*.

Adult Hookworm Antigens

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 percent 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).

H-gal-GP orthologues. The H-gal-GP complex from the brush border of the alimentary canal of *H. contortus* is comprised 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, 2002), *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, 2002a; 2002b; 2002c). Immunolocalization studies confirm that some of these proteases align the brush border membrane of the alimentary canal of adult hookworms (Fig. 5). These proteases are believed to operate in a coordinated fashion to facilitate host hemoglobin degradation during blood feeding (Brinkworth et al, 2000; 2001).

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 have 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 disulfide 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 shown recently for IgE directed against *H. contortus* ASP (Kooyman et al, 2000).

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 two years, focused efforts have been made to express the lead candidate antigens in both prokaryotic and eukaryotic systems (Table 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 disulfide bond formation. Recombinant *E. coli* Na-ASP-1 does not exhibit conformational epitopes corresponding to the native protein. As shown in Fig. 6, 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. 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. As shown in Fig. 6, polyclonal antibodies prepared against *P. pastoris* expressed Na-ASP-1 immunoprecipitate the native protein from soluble L3 extracts. To date, the following L3 recombinant proteins have been successfully expressed in *P. pastoris*: Na-ASP-1 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.

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 and 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.

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 host of *A. caninum*, and this model closely resembles *A. duodenale* hookworm infection. The ability to achieve high adult worm burdens in the laboratory 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 two 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 (unpublished) adapted *N. americanus* by passaging it through 100 generations in a Chinese strain of the golden hamster. By so doing, 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 Fig 7 is a direct comparison of pre-challenge antibody titers in dogs (5 per group) vaccinated either with *E. coli* Na-ASP-1 or *P. pastoris* Na-ASP-1 formulated with alhydrogel. The former exhibit 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 (Fig. 6), 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 Fig. 8, 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 (Fig. 8). 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:

- 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 anthelmintic 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 anthelmintic 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.

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12. Table

Table 1.

Lead Candidate Hookworm Vaccine Antigens

L3 Secreted Antigens

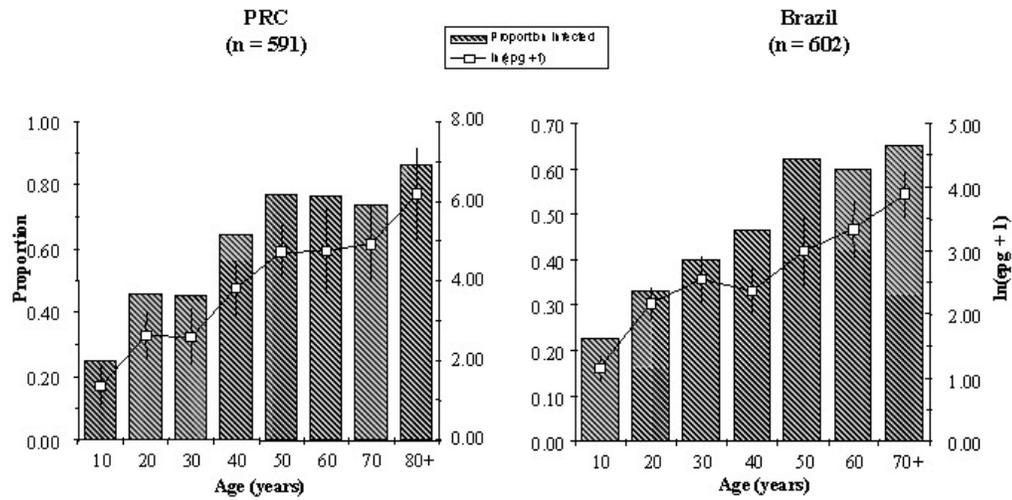
Antigen	Description	MW	Expression Vector
ASP-1	Venom allergen	45 kDa	Pichia pastoris
ASP-2	Venom allergen	24 kDa	P. pastoris & Insect Cell
MTP-1	Astacin metalloprotease	62 kDa	Baculovirus
TTR	Transthyretin	16 Kda	E. coli and P. pastoris
ACH	Acetylcholinesterase	60 kDa	Pichia pastoris

Adult Gut-Membrane Antigens (H-gal-GP Orthologues)

Antigens	Description	MW	Expression Vector
MEP-1	Nepriylsin metalloprotease	99 kDa	Insect Cells
APR-2	Aspartic protease	45 kDa	Baculovirus
CP-1	Cysteinylyl protease	35 kDa	Insect Cells and P. pastoris
CP-2	Cysteinylyl protease	35 kDa	Pichia pastoris
ASP-5	Venom allergen	24 kDa	P. pastoris (pending)

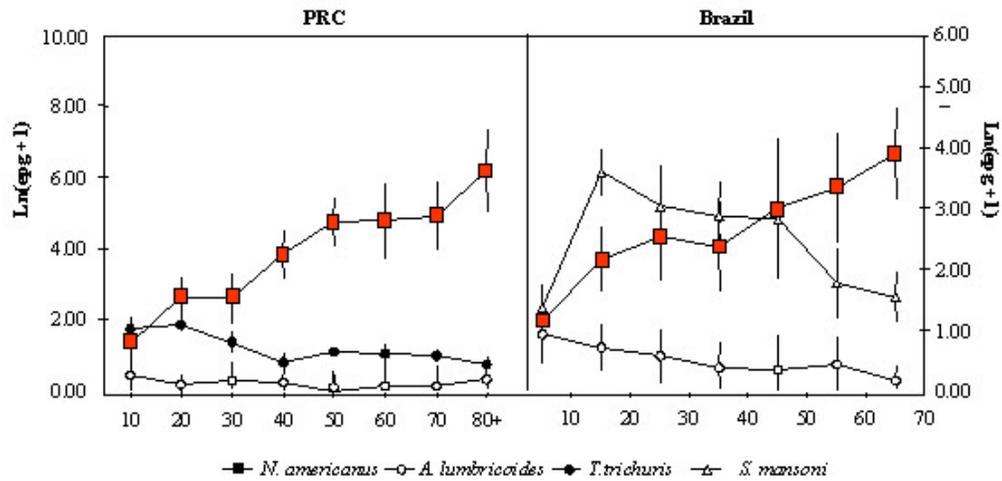
13. Figures

Figure 1



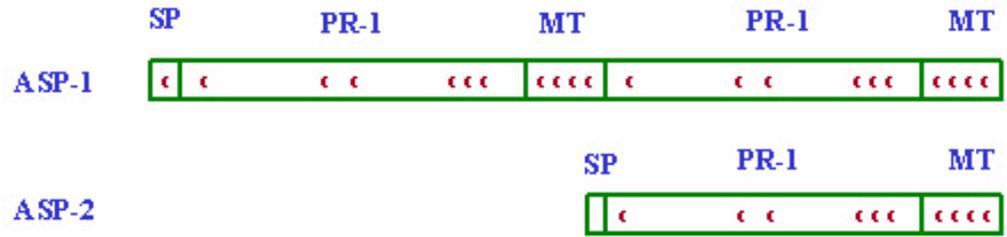
The prevalence and intensity of infection with *Necator americanus* increases with age in two endemic areas: Hainan Province, PRC (1999) and Minas Gerias, Brazil (2000). Data are from cross-sectional studies. Analysis of variance showed that egg counts were significantly different ($P < 0.001$) among the age intervals, and that the eldest 4 age intervals were significantly different ($P < 0.05$) from the younger age intervals, but not different from each other.

Figure 2



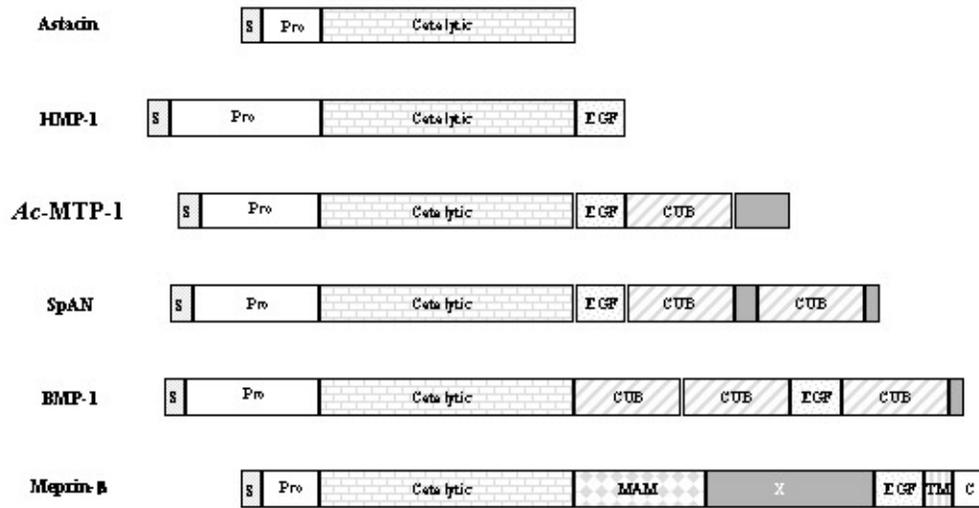
The age distribution of *N. americanus* differs from other STHs such as *A. lumbricoides*, *T. trichiura*, as well as *S. mansoni*. Panel A shows the relationship between age and infection intensity for three STHs in a cross-sectional study from Hainan, PRC. *A. lumbricoides* and *T. trichiura* peak epg among children and then decline into adulthood. The intensity of infection with *N. americanus* increases with age, peaking in eldest age category(80+). Panel B shows the distribution for *N. americanus* in relation to other helminth infections, Minas Gerais, Brazil. The distribution of *S. mansoni* infection intensity shows a traditional convex curve with age, increasing dramatically in young adults. The intensity of infection with *N. americanus* again increases steadily with age, peaking in the eldest age categories.

Figure 3



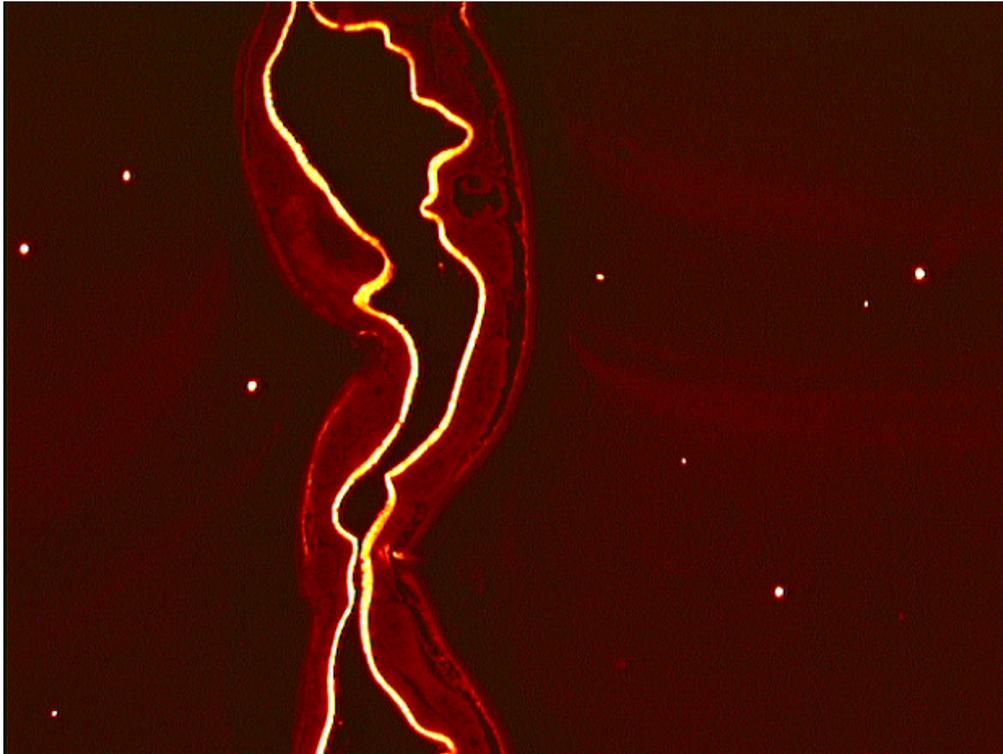
Schematic diagram of the protein domain structures of ASP-1 and ASP-2. ASP-1 corresponds to a heterodimeric repeat of the single domain ASP-2. Modified from Hotez et al (1999).

Figure 4



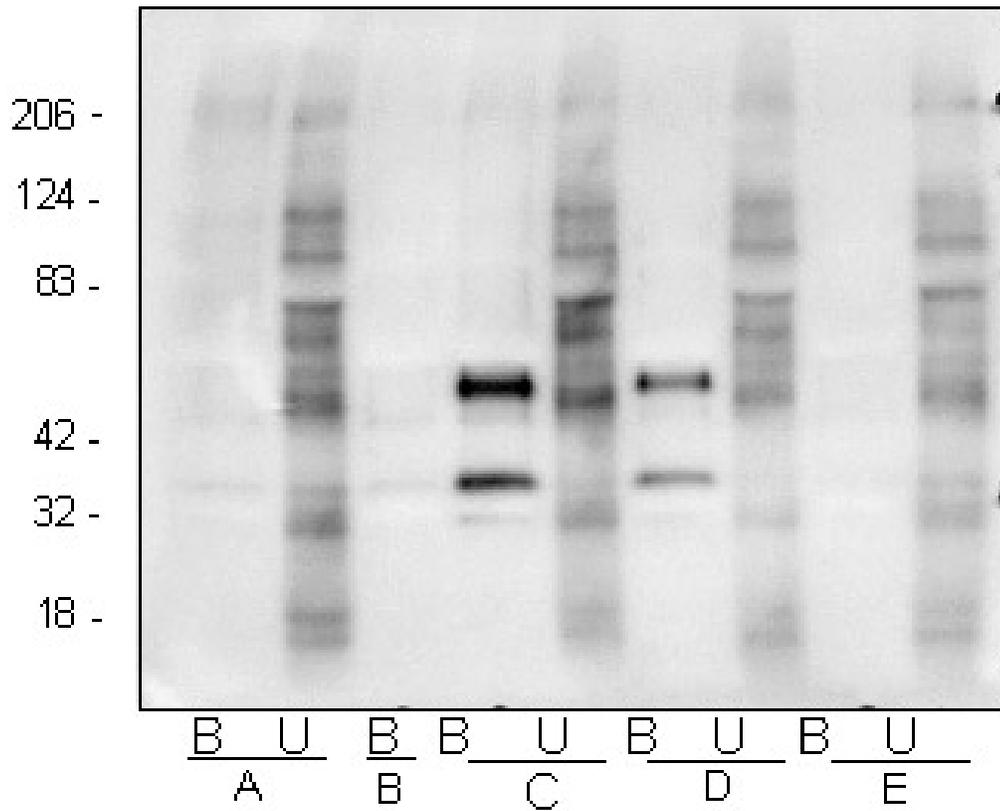
Schematic diagram comparing the domain structures of Ac-MTP-1 from *Ancylostoma caninum* with other members of the astacin family of zinc metalloproteases, including astacin, HMP-1, Hydra metalloprotease 1, SPAN, sea urchin SPAN protein, BMP-1, human bone morphogenic protein 1, and rat meprin-beta. S, signal peptide; Pro, pro-peptide domain; catalytic domain; EGF, epidermal growth factor-like domain; CUB, Clr/s complement-like domain; MAM, adhesion domain; X, unknown domain; TM, transmembrane domain; C, cytoplasmic domain. Reproduced from Zhan et al (2002).

Figure 5



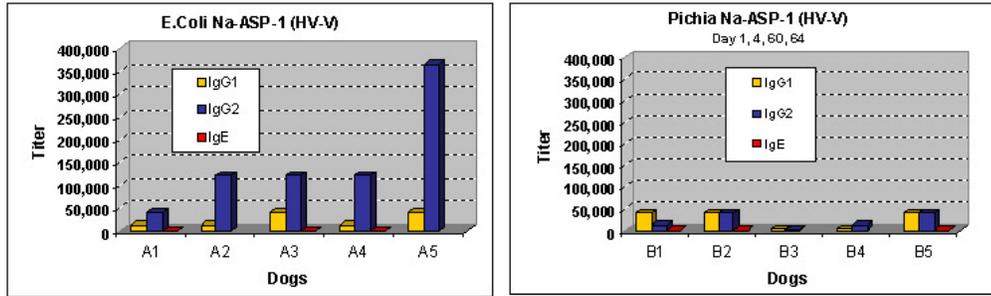
Immunolocalization of Ac-MEP-1 (nepilysin-like adult hookworm metalloprotease) to the brush border membrane of the adult hookworm alimentary canal.

Figure 6



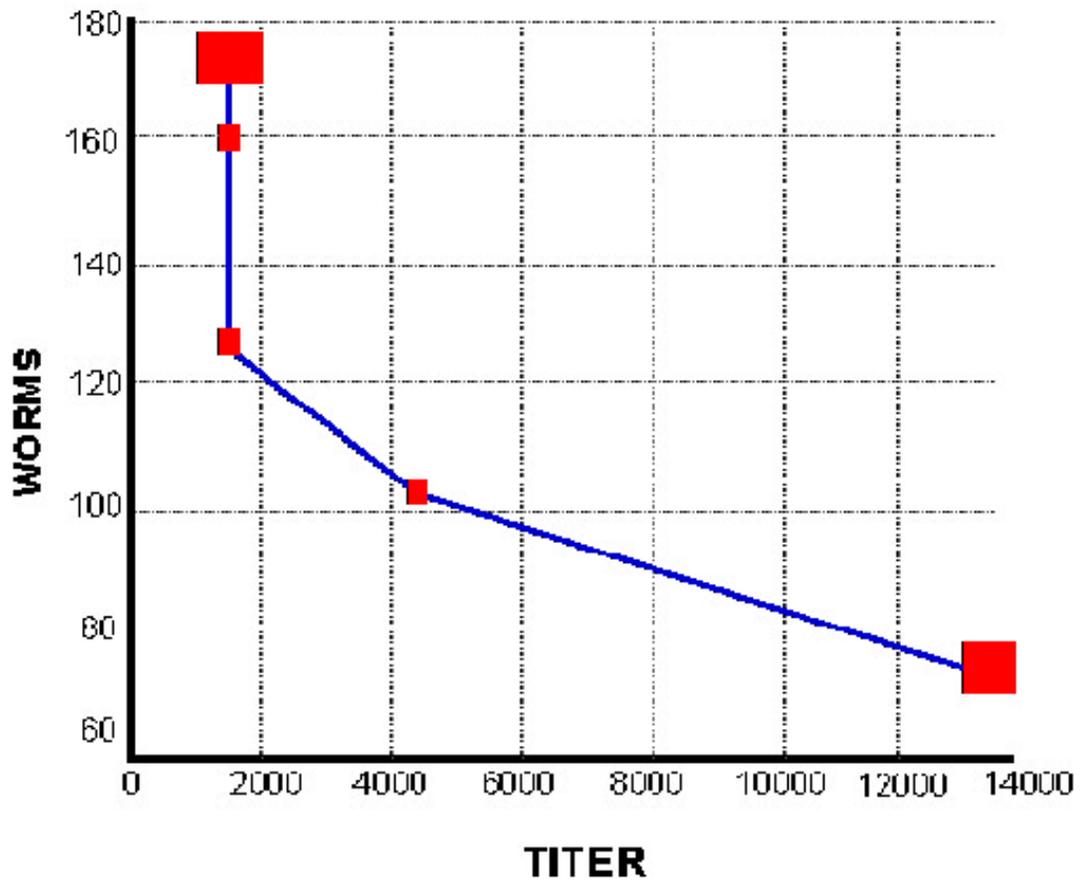
Immunoprecipitation of Ac-ASP-1 from biotinylated extracts of *A. caninum* L3. B, protein bound to streptavidin resin (immunoprecipitated). U, protein unbound to streptavidin resin (non-immunoprecipitated). Group A, Canine antiserum to two doses of irradiated *A. caninum* L3. Group B, Canine antiserum 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 three 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, 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, 64.

Figure 7



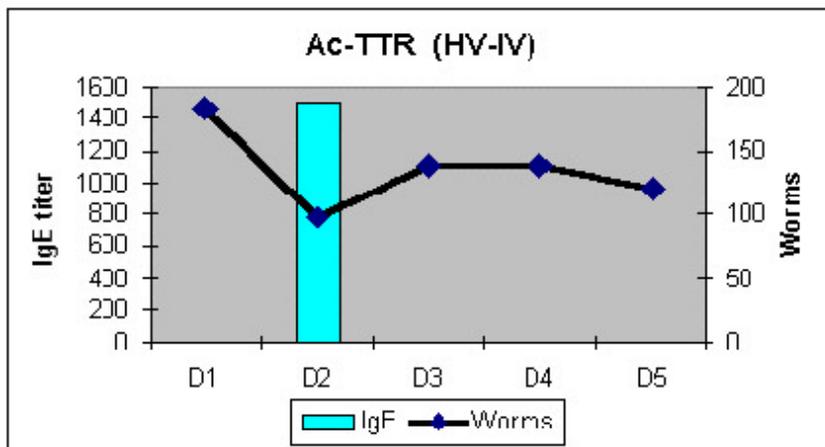
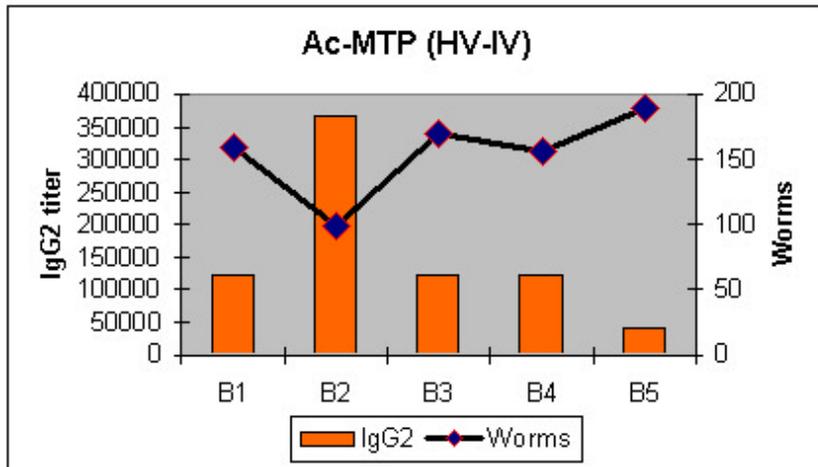
Comparison of antigen specific IgG1, IgG2, and IgE canine antibody titers in purpose bred beagle dogs (n = 5 per group) vaccinated with 150 micrograms of E. coli Na-ASP-1 (4 doses) or P. pastoris Na-ASP-1 (4 doses using two different schedules). An additional group of dogs (n =5) was vaccinated with A. caninum irradiated L3 (2 doses).

Figure 8



Inverse relationships of antibody titers to *E. 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 3-4 doses of the recombinant protein formulated with an aluminum-based adjuvant for ASO2 (GlaxoSmithKline). A) Anti-Na-ASP-1 IgE, B) Anti-Ac-MTP-1 IgG2, C) Anti-TTR IgE. Fig. 8B was modified from a figure published in Hotez P., Ashcom J., Zhan B. et al. (2003).

Figure 9



DCPP Working Papers

1. At Least One-Third Of Poor Countries' Disease Burden Is Due To Malnutrition. By John B. Mason, Philip Musgrove and Jean-Pierre Habicht. March 2003.
2. Progress In The Development Of A Recombinant Vaccine For Human Hookworm Disease: The Human Hookworm Vaccine Initiative. By Peter J. Hotez, Bin Zhan, Jeffrey M. Bethony, Alex Loukas, Angela Williamson, Gaddam Narsa Goud, John M. Hawdon, Azra Dobardzic, Reshad Zook, Yan Wang, Sen Liu, Idong Essiet-Gibson, Sophia Chung-Debose, Shushua Xiao, David Knox, Michael Meagherf, Mehmet Inan, Rodrigo Correa-Oliveira, Paul Vilc, Herman R. Shephard, Walter Brandt, and Philip K. Russell. March 2003.
3. Soil Transmitted Helminth Infections: The Nature, Causes And Burden Of The Condition. By Peter J. Hotez, Nilanthi de Silva, Simon Brooker and Jeffrey Bethony. March 2003.
4. Dicounting. By Dean T. Jamison and Julian S. Jamiseo n. March 2003.

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