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TITLE:
Advancing a multivalent 'Pan-anthelmintic' vaccine against soil-transmitted nematode infections

ABSTRACT:
ASCARIS LUMBRICOIDES: The Sabin Vaccine Institute Product Development Partnership is developing a Pan-anthelmintic vaccine that simultaneously targets the major soil-transmitted nematode infections, in other words, ascariasis, trichuriasis and hookworm infection. The approach builds off the current bivalent Human Hookworm Vaccine now in clinical development and would ultimately add both a larval Ascaris lumbricoides antigen and an adult-stage Trichuris trichiura antigen from the parasite stichosome. Each selected antigen would partially reproduce the protective immunity afforded by UV-attenuated Ascaris eggs and Trichuris stichosome extracts, respectively. Final antigen selection will apply a ranking system that includes the evaluation of expression yields and solubility, feasibility of process development and the absence of circulating antigen-specific IgE among populations living in helminth-endemic regions. Here we describe a five year roadmap for the antigen discovery, feasibility and antigen selection, which will ultimately lead to the scale-up expression, process development, manufacture, good laboratory practices toxicology and preclinical evaluation, ultimately leading to Phase 1 clinical testing.

Rationale for a Pan-anthelmintic vaccine:
The three major soil-transmitted nematode infections, in other words, ascariasis, trichuriasis and hookworm infections, are highly prevalent neglected tropical diseases that rank near the top of the list of most common human afflictions [1]. According to some estimates, approximately 800 million people are infected with the roundworm, Ascaris lumbricoides, and 600 million people with the whipworm, Trichuris trichiura or hookworms, mostly by Necator americanus [1,2]. There is widespread geographical overlap of these three soil-transmitted nematode infections (also referred to as soil-transmitted helminth, intestinal helminth, intestinal nematode or geohelminth infections) in impoverished areas of sub-Saharan Africa, East Asia and South Asia and tropical regions of Central and South America [3]. Coinfections with two or even all three soil-transmitted nematode infections are extremely common in children [2,3]. The WHO currently estimates that 874.5 million children are infected or exposed to A. lumbricoides, T. trichiura and hookworms, and therefore, require regular and periodic anthelmintic treatment ('deworming') (Table 1)
[4]. Such children are often chronically infected and suffer from long-term disabling consequences including growth stunting, reductions in physical fitness, and cognitive and intellectual delays [2]. Moreover, there are millions of pregnant women in developing countries with soil-transmitted nematode infections, especially hookworm infection [5]. Recent estimates from the Global Burden of Disease Study 2010 indicate that soil-transmitted nematode infections are responsible for 5.18 million disability-adjusted life years, which leads all neglected tropical diseases [6]. In addition, ascariasis is responsible for 2,700 deaths annually [7].
Global control of soil-transmitted nematode infections is based on annual (or sometimes twice-annually) mass drug administration with either albendazole or mebendazole for children between the ages of 1 and 14 years who live in areas where the prevalence of these infections exceeds 20% [4]. The WHO estimates that in 2011, 30.6% of the world's children who require mass treatment actually received their medication [4]. Currently, pregnant women do not consistently receive anthelmintic treatments even though they too might benefit from deworming in their second or third trimester [5].
Aside from the low treatment coverage for children, a situation that is being remedied through expanded donations of albendazole and mebendazole and new global policies including a recent World Health Assembly resolution [8], there are concerns that pediatric mass drug administration alone may not be sufficient to effect global control of soil-transmitted nematode infections and certainly not global elimination [9]. Among the major reasons why annual deworming may not be successful as an isolated intervention:
A recent Cochrane analysis has questioned the benefits of mass treatments and deworming based on lack of consistent evidence for its beneficial impact on nutrition, hemoglobin and school attendance or performance [15]. It is likely that some of the factors outlined above, in other words, rapid reinfection and lack of drug efficacies, have a role in the Cochrane findings. However, the Cochrane analysis also did not differentiate between the effects of individual nematode species or their differential drug susceptibilities [8]. As an example, hookworm infection has been linked to

anemia in children and adults in a systematic review, with demonstrated benefits of albendazole (but not mebendazole) on improving anemia [16]. The Cochrane analysis partly blurs these findings by treating all soil-transmitted nematode infections and their treatments as equivalent [8]. Nevertheless, the information to date indicates that there are urgencies to improve the effectiveness and efficiencies of global deworming in order to achieve key milestones and Millennium Development Goals.

Approaches to improve global deworming were recently suggested [9]. They include adding ivermectin to albendazole in areas where high levels of trichuriasis (and strongyloidiasis) transmission occur, and possibly adding new anthelmintics such as tribendimidine or a novel Bacillus thuringiensis-derived Cry5B crystal protein in order to target hookworms [9]. Still another approach to the global control and elimination of soil-transmitted nematode infections includes the development and distribution of anthelmintic vaccines [17]. Hookworm disease accounts for almost two-thirds of the global disease burden from soil-transmitted nematode infections [6], so this infection was selected for initial vaccine development. Currently, the Sabin Vaccine Institute Product Development Partnership is developing a bivalent recombinant protein-based human hookworm vaccine [17]. The vaccine comprises two Necator americanus antigens, Na-GST-1 and Na-APR-1, which are formulated on alum together with a second adjuvant [17]. The Na-GST-1 component is in Phase 1 clinical trials with the expectation that Na-APR-1 will follow [17].

Here we explore the concept of adding Ascaris and Trichuris antigens to the two Necator hookworm antigens under development in order to launch a multivalent ‘Pan-anthelmintic’ vaccine for soil-transmitted nematode infections [9]. We will briefly review the status of existing Ascaris and Trichuris antigens that have undergone preclinical testing and the steps that will be required to perform additional antigen discovery, selection and down-selection to just a single vaccine candidate antigen (one for each nematode) for further preclinical evaluation, process development, manufacture, good laboratory practices toxicology preclinical evaluation and early stage clinical testing.

As14 & As16 ::: Chemically defined antigens ::: Status of Ascaris antigen discovery:

These 14 and 16-kDa larval antigens were discovered by immunoscreening a larval cDNA library with sera from rabbits immunized with infective eggs [22,23]. The antigens are found in both larval and adult Ascaris worms and are present in ES as well as defined parasite structures [22,23]. Mice vaccinated intranasally with recombinant As14 or As16 (expressed in Escherichia coli) and formulated with cholera toxin B subunit exhibited about 60% reduction in the numbers of migrating larvae in mice after challenge compared with control mice [22,23]. Protection was associated with elevations in host antibody as well as IFN-γ and IL-10 in cultured supernatants of activated spleen cells, indicative of a mixed TH1 and TH2 response [22,23]. As14 has also been transgenically expressed in rice as a novel oral vaccine delivery approach [39], while As16 ortholog was cloned from A. lumbricoides and shown to be identical [40].

As24 ::: Chemically defined antigens ::: Status of Ascaris antigen discovery:

This molecule was also cloned from A. suum larval cDNA, using immune sera from pigs infected repeatedly with A. suum eggs [41,42]. This nematode-specific protein was expressed in E. coli and found to elicit a 58% reduction in the recovery of A. suum lung-stage larvae. Protection was associated with high levels of anti-As24 IgG and elevated levels of both IFN-γ and IL-10, again indicating TH1 and TH2 mixed responses [41,42]. Anti-As24 IgG inhibited molting of A. suum lung stage indicating its function in the development of Ascaris larvae [42].

As37 ::: Chemically defined antigens ::: Status of Ascaris antigen discovery:

As37 is an immunodominant A. suum larval surface antigen recognized by pig immune serum [43,44]. The molecule, which contains multiple immunoglobulin-like domains, is also found in adult worms. An A. lumbricoides ortholog has been cloned (Al37) and found to exhibit 91% amino acid homology with As37. The molecule elicited 69% reduction in larvae from the liver and lungs following A. lumbricoides egg challenge [45].

As-Enol-1 ::: Chemically defined antigens ::: Status of Ascaris antigen discovery:

Ascaris suum utilizes exogenous glucose for energy generation through the glycolytic pathway, and enolase is one of the enzymes catalyzing the synthesis of phosphoenolpyruvate. Ascaris suum enolase (As-Enol-1) was first cloned from an A. suum infective larvae-specific cDNA library using a microarray analysis [46], and it was also identified as one of the major secreted proteins in

A. suum adult ES products that can trigger nitric oxide production in macrophages [47]. Specific knockout of As-enol-1 by RNAi resulted in the delay of larval development [48]. Mice immunized with DNA coding for As-Enol-1 exhibited 61% reduction in larvae recovery from lung compared with empty plasmid DNA control, with protection correlated to high levels of specific antibody and lymphoproliferative responses [48].

As-GST-1 ::: Chemically defined antigens ::: Status of Ascaris antigen discovery:
Many parasitic nematode glutathione S-transferases belong to a unique Nu class of enzyme that is involved in heme scavenging and detoxification [49]. As noted above, Na-GST-1 from N. americanus is a protective antigen found in the Human Hookworm Vaccine [17], and the A. suum ortholog exhibits 50% amino acid homology to this enzyme [50].

Approaches to antigen discovery, scoring & ranking ::: Status of Ascaris antigen discovery:
Since five of these six antigens listed in Table 2 are the major antigens recognized by protective immune sera induced by living UV-attenuated eggs, we are now comparing them for protective efficacy while simultaneously determining the requirements for additional antigen discovery. In order to down-select an appropriate antigen(s) for further development, these recombinant antigens will be compared in a mouse challenge model. The recombinant proteins will be produced in an appropriate expression system (i.e., bacteria, yeast or baculovirus) and used as immunogens for immunogenicity studies and vaccine trials in a mouse model. In parallel, a proteomics approach will be undertaken to evaluate additional ES and surface proteins from A. suum for their immunoreactivity to immune serum from the pig model as a backup strategy for identification of vaccine candidates. The goal is to rank the top two candidate antigens with respect to their efficacy in a preclinical challenge model, together with the ability to express these antigens as soluble proteins in high yield (see below). The corresponding antigens from A. lumbricoides will also be cloned and expressed and examined for immunological cross-reactivity.

Worm extracts ::: Chemically defined antigens ::: Status of Trichuris antigen discovery:
Homogenized adult T. muris antigens, either emulsified in Freund's complete adjuvant for subcutaneous immunization, or combined with cholera toxin for oral administration, have been administered to several different mouse strains [57]. While the mucosal response was enhanced by oral vaccination, this was not sufficient for protection against infection in the low responders; in other words, protection was achieved only in the BALB/c high-responder mice [57], a result similarly found in oral vaccination for Trichinella leading to only protective immunity in high-responder mice [57]. In contrast, subcutaneous administration provided protective immunity in multiple strains [57,58]. In vaccinated humanized mice, an immunodominant 66 kDa T. muris antigen was identified from worm homogenates [15].

ES antigens ::: Chemically defined antigens ::: Status of Trichuris antigen discovery:
Stichosome-related adult-stage ES products have been identified as potential sources of protective antigens [59] although it has been noted that some stichosome proteins remain stored in the organ and are not actually secreted [60]. Partial protection has been observed following immunization with adult ES products [56], especially when emulsified with Freund's incomplete adjuvant [52]. The protection elicited in susceptible AKR mice included reductions in parasite egg shedding and was associated with peripheral lymph node responses [52].

Trichinella antigens ::: Chemically defined antigens ::: Status of Trichuris antigen discovery:
Findings of cross-reactivity and cross-immunity between Trichuris and Trichinella
[61] have prompted the study of shared antigens. Using ELISA, immunoprecipitation and immunoblotting, it was demonstrated that T. trichiura-infected mice had cross-reactive antibodies to T. spiralis
[61]. Shared stichocyte antigens have also been noted [51]. Among the defined candidate antigens identified from T. spiralis that provide partial protection are an aminopeptidase [62], serine proteases [63–65], parasite-derived cytokines [66], paramyosin [67], heat-shock protein [65] and 87, 53 and 43 kDa secreted antigens of unknown function [67–69].

Approaches to antigen discovery ::: Chemically defined antigens ::: Status of Trichuris antigen discovery:

As outlined above, there are no defined T. trichiura-specific antigens that have been specifically identified for further vaccine development. Thus, there is still a need to discover and develop specific T. trichiura antigens. One approach could utilize the established T. muris AKR murine model. Since the adult ES products of T. muris induced complete protection in immunized mice against infective egg challenge [52], the immune sera will be used to immunoscreen a T. muris adult stichosome cDNA library and identify secreted proteins that induce the protective immunity. Another approach to identify the vaccine antigen is to take advantage of studies already completed with the major Trichinella antigens highlighted previously. Trichuris muris orthologs of these antigens highlighted above can be cloned and expressed in platforms such as bacteria, yeast or baculovirus. These antigens can then be tested in subsequent immunogenicity studies. In parallel, proteomics approaches are useful to identify the full complement of T. muris stichosome antigens and their reactivity to immune serum from a mouse model. Once promising candidates are identified, protein expression on the T. trichura homologs of these candidates can begin and further antigen down-selection can take place.

Avoiding allergic responses ::: Prospects & challenges for vaccine product development:
A key observation made during the clinical testing of human hookworm candidate antigens was that human populations living in endemic areas acquire IgE responses to selected antigens, especially those from infective larvae [70]. Volunteers with prevaccination IgE can develop urticarial reactions, including generalized urticaria, upon receiving recombinant forms of such antigens [70]. Based on these findings, currently, evidence for prevaccination antigen-specific IgE antibodies among endemic populations will be used to discard or down-select potential candidate vaccines [17]. Larval Ascaris antigens in particular will be closely scrutinized. Therefore, a key step in developing a vaccine antigen pipeline will be preventing allergic responses by first examining sera from populations living in areas endemic for ascariasis and trichuriasis.

Selection/down-selection of vaccine antigens for process development ::: Prospects & challenges for vaccine product development:
Selection of the lead candidate antigens will rely on ranking them through a scoring system that assigns a number ranging from 0 to 3, 4 or 5 for each of the critical categories described below. This approach was used previously to rank the leading candidate hookworm antigens used in the Human Hookworm Vaccine [71]. For ascariasis and trichuriasis, the major criteria will include: level of protective immunity; amino acid homology between A. suum and A. lumbricoides or T. muris and T. trichiura; known or presumed mechanism of action; absence of prevaccination IgE among an endemic population; solubility and stability; and expression yield.

Scale-up expression & process development ::: Prospects & challenges for vaccine product development:
The lead two candidate Ascaris and Trichuris antigens will then be subjected to protein expression at the 10–20L fermentation scale and large-scale protein purification. In anticipation of generating a target product profile that would focus on vulnerable populations living in helminth endemic areas of low- and middle-income countries, it is likely that only low-cost expression systems such as yeast (e.g., Pichia pastoris or Hansenula polymorpha) or bacteria (e.g., E. coli) would be selected [72]. In addition to production yield, solubility and stability, the expressed proteins would be evaluated for immunogenicity and ability to induce protective immunity in the animal models highlighted above. A process will be developed for technology transfer to a current good manufacturing practices manufacturer in the USA, Europe or possibly a developing country manufacturer. Following a good laboratory practices toxicology study, an investigational new drug application would be prepared and filed with the US FDA prior to Phase 1 clinical testing. These antigens would be evaluated initially as monovalent antigens formulated on alum, possibly together with a second adjuvant such as a toll-like receptor agonist used for other vaccines under development by the Sabin Vaccine Institute Product Development Partnership, such as a synthetic lipid A [73], E6020 [74] or a CpG oligodeoxynucleotide.

Protein stability ::: Coformulation ::: Prospects & challenges for vaccine product development:
As proteins differ in their individual characteristics (e.g., size and pH), buffers and excipients that provide stability for one protein may not be suitable for another antigen of interest. As such, extensive buffer screenings may be required to identify a formulation that will be ideal for all of the vaccine antigens. Also to be considered are the necessary storage conditions of these formulations, which could affect longtime storage, shipment and short-term storage in the clinic.

Adjuvant/delivery system ::: Coformulation ::: Prospects & challenges for vaccine product development:
TH2-mediated immune responses appear to be the desired protective response common to all three soil-transmitted nematodes targeted by the vaccine. To ensure that the same adjuvant will be appropriate for all antigens, a well-established and relatively inexpensive option are to use an alum formulation, possibly with a second adjuvant as outlined above.

Immunogenicity/efficacy ::: Coformulation ::: Prospects & challenges for vaccine product development:
A recurring concern with combining multiple antigens in a vaccine is the potential for immune interference. For example, the immune response to one antigen may dominate or interfere with the others in the vaccine. In endemic areas, many people suffering from helminth infections are polyparasitized [75], making a coinfection animal model critical to vaccine development. Currently, multiple animal models are used as surrogates for human Ascaris and Trichuris infections, including rodents, pigs and nonhuman primates, with rodents and pigs used most frequently [23,41,42,45–49,76–78]. Rodent models, in other words, mice and rats, are more attractive than pigs or nonhuman primates for initial immunogenicity and efficacy studies as their small size and ease of handling allow larger numbers of animals to be evaluated concurrently. Additionally, genetically defined inbred strains allow identification of genetic backgrounds responsible for susceptibility and resistance to parasites. Several strains, including AKR, SCID and Nude, have been shown to be susceptible to T. muris, developing patent infections [49]. In contrast, while A. suum larvae migrate through the liver and lungs of C57BL/6-infected mice, to date, mice cannot support the development of adult worms, nor patent A. suum infections [79]. Nonetheless, several A. suum antigens identified by screening with immune serum from pigs have been shown to reduce lung larval burdens in susceptible mice, suggesting that protective immune responses in susceptible mice correlate with protective response in pigs [41,42,44–48]. Since C57BL/6 mice infected with a low dose of T. muris develop a TH1-skewed immune response and a susceptible phenotype [80], this mouse strain could potentially serve as an initial coinfection model for evaluating a pan-helminthic vaccine against Ascaris and Trichuris infections. Furthermore, mice would be invaluable in evaluating regulatory mandated immunogenicity testing of antigen/adjuvant combinations and future potency testing of a clinical vaccine [81]. Ultimately, to thoroughly evaluate a coformulated vaccine, both mice and pigs may be necessary to establish sufficient protection data to advance a potential vaccine candidate into product development. Pigs offer some promise as a laboratory vaccine model [82] although they have not yet been used extensively for evaluating T. suis. Further studies will be undertaken to assess whether studies confirming host protection in pigs will be on the critical path for vaccine development.

Modeling:
It will be useful to build on previous mathematical models of helminth transmission dynamics [25] to assess the level of protective immunity necessary to achieve cost–effectiveness relative to current deworming approaches, as previously done for the Human Hookworm Vaccine [74]. Modeling would also help to assess the potential of the vaccine to achieve control and elimination targets by interrupting helminth transmission [13].

Expert commentary:
A strategy for the discovery, expression, isolation and preclinical development is being advanced for lead candidate antigens to prevent both ascariasis and trichuriasis.
Ultimately, these antigens will be evaluated for protection alongside the two antigens comprising the Human Hookworm Vaccine. In so doing, it may be necessary to produce a tetravalent vaccine comprising two hookworm antigens together with an Ascaris and Trichuris antigen, respectively, or even possibly a pentavalent vaccine if a second Ascaris or Trichuris antigen is required to achieve adequate protective immunity. The costs of such vaccines will have to be carefully looked at, even if only inexpensive bacterial or yeast expression vectors are employed. A cost economic modeling exercise may be required to examine the comparative advantage of a Pan-anthelmintic vaccine over current control approaches that rely on anthelmintic drugs and deworming. Previously, the Human Hookworm Vaccine was shown to be cost-effective and even cost-saving under different scenarios [75].

Five-year view:

The next 5 years will be focused on working to resolve the major gaps and key issues that could hinder or slow a pan-helminthic vaccine development program. One major goal will be spearheading a focused antigen discovery program for both Ascaris and Trichuris and their eventual down-selection as detailed above. Once the antigens are identified and expressed, they will enter a process development, characterization and initial stability assessment program. A main consideration will include designing a formulation that will support stability of not only the candidate Ascaris and Trichuris antigens but also the Human Hookworm Vaccine antigens. Another key endeavor will be to build on previous preclinical studies to establish a suitable animal model for the Pan-anthelmintic vaccine. This model would support both immunogenicity and preclinical efficacy studies of our vaccine candidates, as we move toward an investigational new drug filing and clinical trials. Such studies will be critical for determining if the protective immunity achieved for these helminth antigens in laboratory animals can translate to human medicine.