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Molecular mechanisms of hookworm disease: Stealth, virulence, and vaccines

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Hookworms produce a vast repertoire of structurally and functionally diverse molecules that mediate their long-term survival and pathogenesis within a human host. Many of these molecules are secreted by the parasite, after which they interact with critical components of host biology, including processes that are key to host survival. The most important of these interactions is the hookworm's interruption of nutrient acquisition by the host through its ingestion and digestion of host blood. This results in iron deficiency and eventually the microcytic hypochromic anemia or iron deficiency anemia that is the clinical hallmark of hookworm infection. Other molecular mechanisms of hookworm infection cause a systematic suppression of the host immune response to both the parasite and to bystander antigens (eg, vaccines or allergens). This is achieved by a series of molecules that assist the parasite in the stealthy evasion of the host immune response. This review will summarize the current knowledge of the molecular mechanisms used by hookworms to survive for extended periods in the human host (up to 7 years or longer) and examine the pivotal contributions of these molecular mechanisms to chronic hookworm parasitism and host clinical outcomes. (J Allergy Clin Immunol 2012;130:13-21.)

Key words: *Hookworms, virulence factors, immune modulation, vaccines, proteases,* Ancylostoma *secreted proteins*

Abbreviations used

APR: Aspartic protease
ASP: *Ancylostoma* secreted protein
ES: Excretory/secretory
GST: Glutathione-S-transferase
IDA: Iron deficiency anemia
KI: Kunitz-type protease inhibitor
L3: Third-stage larvae
MIF: Macrophage migration inhibitory factor
MMP: Matrix metalloprotease
NAP: Nematode anticoagulant peptide
NIF: Neutrophil inhibitory factor
TIMP: Tissue inhibitor of metalloproteases

The hookworms *Necator americanus*, *Ancylostoma duodenale*, and *Ancylostoma ceylanicum* infect 576 to 740 million persons worldwide, predominantly in impoverished rural and tropical regions of the world, and together they cause one of the world's most debilitating neglected tropical diseases. Chronic hookworm infection results in long-term pathologic consequences primarily because of continuous intestinal blood loss caused by the feeding activities of these hematophagous parasites. Intestinal blood loss is the major pathologic sequela of human hookworm infection.[1,3] Heavily and even moderately infected subjects with poor underlying iron or protein stores can have hookworm disease, the clinical entity that specifically refers to the microcytic hypochromic anemia or iron deficiency anemia (IDA) that results from hookworms feeding on blood.[1,3] Hookworm-induced blood loss is estimated to be as great as 9.0 mL/d in subjects with heavy infections, with hookworm burdens of 40 to 160 worms sufficient to cause anemia. In school-aged children and adults living in resource-poor areas, where host iron stores are often lower than those in developed countries, there is a well-established relationship between the intensity of hookworm infection, intestinal blood loss, and host anemia. Moreover, children and women in the child-bearing years have the lowest iron reserves and, as such, are the most vulnerable to hookworm-induced anemia.[4,5] The consequences of chronic IDA include not only malnutrition but also the impairment of physical and cognitive development. As such, hookworm infection has a significant effect not only on the health of infected subjects but also on economic productivity and educational achievement in regions where the parasite is endemic.[2]

Although hookworm infection can be treated effectively with medication, reinfection often occurs rapidly after treatment.[6] The

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Terms in boldface and italics are defined in the glossary on page 14.

failure to interrupt transmission, coupled with the widespread prevalence of the disease in resource-poor areas and the potential emergence of drug resistance,[7] suggests that additional control measures are urgently needed.

NATURAL HISTORY OF HOOKWORM INFECTION: MOLECULAR MECHANISMS OF SURVIVAL

Immature hookworm third-stage larvae (L3) are approximately 0.5 mm in length and infect the host by penetrating the skin, a process that takes between 30 minutes and 6 hours depending on the species,[8,9] and invading the circulation, where they are carried to the heart and lungs. The parasites then penetrate the alveolae, migrate to the trachea, and are swallowed, reaching their site of predilection in the gastrointestinal tract (fourth-stage larvae), where they develop into blood-feeding, adult-stage hookworms that live out their parasitic existence for years by attaching onto the duodenal mucosa and consuming host blood to obtain nutrition and sexually reproduce.[10] Pivotal to hookworm survival in the host is a vast repertoire of molecules, mostly proteins, known as excretory/secretory (ES) products, which interact with host proteins and play key roles throughout all aspects of the host-parasite relationship. A comprehensive list of such proteins was previously published.[11]

This review will examine the different types of molecular mechanisms that mediate hookworm survival in the host and that result in disease (Box 1). The role of these molecular mechanisms in the host-parasite relationship will be described, including the more "classical" molecular mechanisms that directly contribute to hookworm-related IDA and malnutrition (eg, protease inhibitors and peptidases associated with blood feeding), as well as more novel and recently discovered mechanisms that have more indirect or as yet undefined roles in the pathogenic process of hookworm infection (eg, molecular mechanisms associated with the establishment of parasitism and the worm's survival within the host). These latter molecular mechanisms constitute the most recent research into the pathogenesis of hookworm disease.

BOX 1. Molecular mechanisms of hookworm disease: A natural history of stealth and virulence (Fig 1)

Key hookworm molecules are critical mediators of the biological processes that determine parasitism and are involved in the following stages of infection within the host:

1. larval activation, host invasion, and tissue migration, including digestion of skin and other tissue macromolecules;
2. nutrient acquisition by anticoagulation and degradation of host serum proteins for food; and
3. neutralization of host defenses through inhibition of host intestinal proteases and immune evasion by modulation of the host inflammation response.[12]

Molecular mechanisms associated with larval activation, skin penetration, and tissue migration

Penetration of the skin of human hosts by hookworm L3 is primarily a chemical process that is mediated by the release of a range of proteolytic enzymes from specialized larval glands. Secretions of *N americanus* larvae possess enzymatic activity belonging to all the known major mechanistic classes of proteases and have the ability to degrade the connective tissue substrates ***collagen***, fibronectin, laminin, and elastin. Furthermore, larval skin penetration can be significantly neutralized only by pepstatin A, an inhibitor of aspartic proteases (APRs), implicating the importance of this class of enzymes in the infection process.[13] The molecule most prominently implicated in this process is the APR *Na*-APR-1 because of its ability to digest skin macromolecules and its presence in larval parasites.[14] ***Hyaluronic acid*** is a major component of the extracellular matrix and is associated with cell adhesion by ligand binding with the CD44 cell-surface receptor.[15] *Ancylostoma* species larvae have been shown to exhibit hyaluronidase activity, which might facilitate their migration through host dermal layers by interrupting cellular adhesion mediated by hyaluronic acid.[16] However, the best characterized protease in larval ES is *Ac*-MTP-1, an astacin-like zinc metalloprotease from the dog hookworm *Ancylostoma caninum*.[17,18] *Ac*-MTP-1 aids larval migration through the skin and

GLOSSARY

COLLAGEN: An insoluble fibrous protein of vertebrates that is the chief constituent of the fibrils of connective tissue (as in skin and tendons) and of the organic substance of bones and yields gelatin and glue on prolonged heating with water.

CR3 RECEPTOR: Also known as CD11b/CD18, an adhesion molecule that is also a receptor for iC3. Deficiency of CD18 in type 1 leukocyte adhesion deficiency results in a lack of β_2-integrin adhesion molecules.

EUKARYOTE: Organisms composed of 1 or more cells containing visibly evident nuclei and organelles.

FIBRINOGEN: A plasma protein that is produced in the liver and is converted into fibrin during blood clot formation.

FLUKE: A flattened trematode worm.

GLUTATHIONE-S-TRANSFERASE: An enzyme that functions to detoxify various xenobiotics by conjugating them with glutathione.

HYALURONIC ACID: A viscous glycosaminoglycan that occurs especially in the vitreous body, the umbilical cord, and synovial fluid and as a cementing substance in the subcutaneous tissue.

HYDROLYZE: To undergo the process of splitting a bond and the addition of a hydrogen cation and the hydroxide anion.

INTESTINAL BRUSH BORDER: An area in the small intestine comprised of microvilli that contain enzymes important for digestion. This area is also where nutrient absorption takes place.

MATRIX METALLOPROTEASE (MMP): Proteases that are involved in degrading the extracellular matrix and in tissue repair. They are induced by proinflammatory cytokines. MMPs are produced by the airway epithelium, and levels are increased in the bronchoalveolar lavage fluid of asthmatic subjects.

PHYLOGENETIC: Relating to the evolutionary history of a kind of organism or a group of genetically related organisms.

SERINE: A nonessential amino acid ($C_3H_7NO_3$) that occurs especially as a structural part of many proteins and is a precursor of glycine.

TRYPSIN: A crystallizable proteolytic enzyme most active in a slightly alkaline medium that is produced and secreted in the pancreatic juice in the form of inactive trypsinogen and activated in the intestine.

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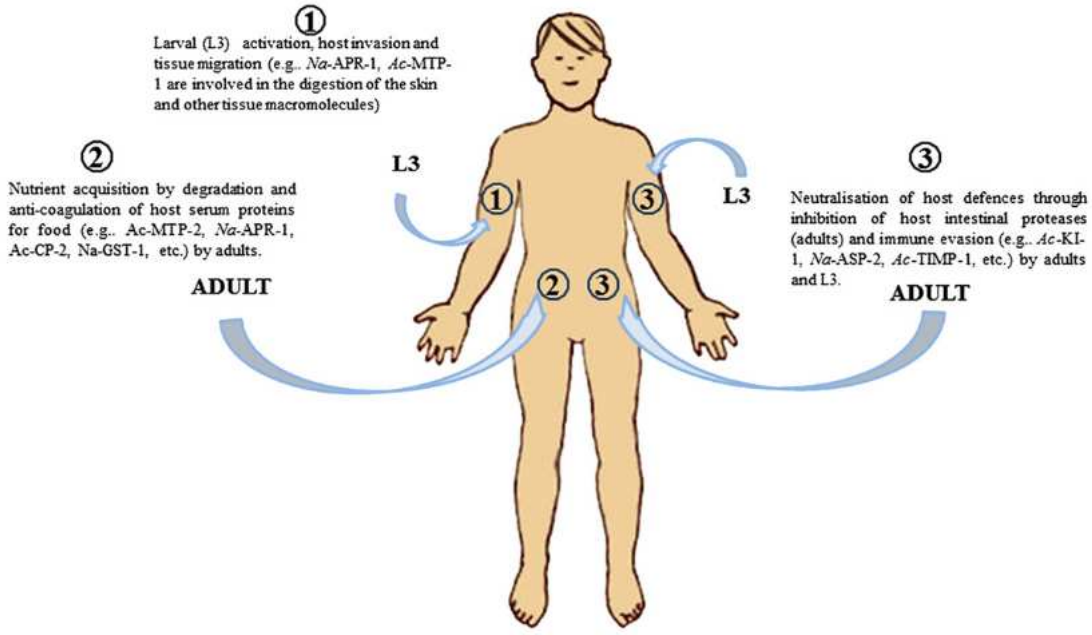

FIG 1. Molecular mechanisms of hookworm disease. The key hookworm molecules that determine parasitism can be divided into 3 categories by the stage or stages of infection in the host. *Stage 1*, Larval activation, host invasion, and tissue migration, including digestion of skin and other tissue macromolecules; *stage 2*, nutrient acquisition by means of anticoagulation and degradation of host serum proteins for food; and *stage 3*, neutralization of host defenses through inhibition of host intestinal proteases and immune evasion by means of modulation of the host inflammation response.

other tissues by degrading fibronectin, laminin, and collagen. The metalloprotease inhibitor 1,10-phenanthroline inhibits larval skin penetration, and antiserum against recombinant *Ac*-MTP-1 can block migration of larvae through tissue *in vitro*,[18] highlighting the importance of this enzyme in the infective process.

Ancylostoma secreted proteins (ASPs) (Box 2) are members of the SCP/Tpx/Ag5/PR-1/Sc7 (SCP/TAPS) family of proteins,[19] which are characterized by the Pfam domain PF00188. This family includes mammalian sperm-coating glycoprotein; glioma pathogenesis-related protein; a lizard toxin that blocks ryanodine receptors; venom allergens from biting/stinging insects, which are potent allergens; plant pathogenesis proteins of the PR-1 subfamily, which are synthesized during pathogen infection or other stress-related responses; and fungal proteins that are loosely associated with fruiting body hyphal walls.[19 21] The functions of most of these proteins are largely unknown, but in hookworms ASPs are very abundant in ES products from L3 parasites undergoing the transition to the parasitic stage, which occurs on activation by host serum.[22,23]

In a comprehensive study, Datu et al[24] demonstrated an enormous "expansion" of ASPs after serum stimulation of *A caninum* L3, both in terms of their transcriptional upregulation and numbers of distinct molecules, showing clearly that ASPs are one of the major molecular groups involved in the early phase of the transition to parasitism in *A caninum*. Transcripts of ASP family members are upregulated in male versus female *Ancylostoma* species hookworms[25] and are predominantly represented in *A caninum* versus *N americanus*.[26] Adult hookworms secrete at least 4 well-characterized ASPs, each of which localizes to a unique organ (the intestine, esophageal glands, cephalic glands, or cuticle) of the parasite,[27] although a proteomic analysis of adult *A caninum* ES products has shown that such worms secrete more than 30 distinct ASPs.[28] The crystal structure of ASP-2 from *N americanus* has revealed a protein conformation similar to that adopted by some chemokines,[29] and the protein was later found to induce neutrophil recruitment,[30] prompting speculation about an immunomodulatory function for this molecule. The only hookworm ASP for which a function has been definitively shown is the *A caninum* neutrophil inhibitory factor (*Ac*-NIF), a protein that binds to the ***CR3 receptor*** integrin and inhibits neutrophil recruitment and adhesion,[31 33] further supporting an immunomodulatory role for at least some hookworm ASPs.

Molecular mechanisms associated with nutrient acquisition by hookworms

After L3 enter the gut, they form a preliminary buccal capsule as fourth-stage larvae and begin feeding on human blood, followed by transition to fifth-stage larvae and adulthood.[39,40] Blood feeding by adult hookworms causes many of the pathologic manifestations associated with chronic infection, most notably IDA.[10] The first step in the blood-feeding process involves attachment of the parasite to the intestinal mucosa by teeth (*Ancylostoma* species) or cutting plates (*Necator* species) and is followed by mechanical disruption of the resulting "mucosal plug" lodged within the worm's buccal capsule; digestion is thought to be aided by metalloproteases, namely the astacin-like metalloprotease *Ac*-MTP-2.[41]

Hookworms also secrete a range of anticoagulants, known as anticoagulant peptides (NAPs), to facilitate ingestion of blood released from ruptured capillaries.[42] In *A caninum* 6 NAPs have been shown to inhibit coagulation factor VIIa/tissue factor complex, and a further 3 are inhibitors of factor Xa.[43 49] *Aca*NAP10 from *A caninum*, *Ace*AP1 from *A ceylanicum*, and *Adu*NAP4

BOX 2. Molecular mechanisms of larval penetration as vaccine targets: ASPs, the first generation of hookworm vaccines

ASP-2 of *N americanus* (*Na*-ASP-2) is a 21.3-kDa protein that is secreted by infective hookworm larvae on entry into the host.[22,23,27,34] Vaccination of hamsters and dogs with recombinant ASP-2 resulted in reduced worm burdens and fecundity after challenge infection.[35] Furthermore, sera from vaccinated animals inhibit migration of infective hookworm larvae through tissue in an *in vitro* assay. Studies of populations living in hookworm-endemic areas have also shown that anti–ASP-2 antibodies are associated with a reduced risk of contracting heavy hookworm infections.[35] On the basis of these results, *Na*-ASP-2 was chosen as the lead hookworm vaccine candidate.[36] In a phase I study conducted in hookworm-naive adults living in the United States, *Na*-ASP-2 adjuvanted with Alhydrogel (Biosector, Ballerup, Denmark) was well tolerated and immunogenic.[37]

As reported in this issue of the *Journal*, in a phase I clinical trial conducted in healthy adults living in an area endemic for *N americanus*, vaccination with a single (10 μg) dose of the *Na*-ASP-2 hookworm vaccine resulted in the development of generalized urticarial reactions. Subsequent analysis showed that the urticarial reactions were associated with increased prevaccination levels of IgE antibodies specific for *Na*-ASP-2, which were most likely present as a result of previous hookworm infection. A survey of adults and children from the same hookworm-endemic area revealed that a significant proportion had increased levels of IgE to *Na*-ASP-2. Our findings have major implications for the development of vaccines against not only hookworm but also all helminths given their common propensity to induce strong T_H2 immune responses. It is possible that intrinsic structural or biological properties of the *Na*-ASP-2 molecule were responsible for the observed immediate-type hypersensitivity reactions. An intriguing possibility is that human subjects have targeted a restricted range of helminth antigens to enable the host to defend itself without inducing self-harm or tolerance.[38]

BOX 3. GST molecular mechanisms of vaccine targets: The next generation of hookworm vaccines

Recently, a promising new generation of hookworm vaccine antigens have been developed that target the parasite enzymes required for blood feeding, hemoglobin digestion, and heme detoxification. The candidate antigen furthest developed in this group is the *N americanus* GST-1 (*Na*-GST-1). In Syrian Golden hamsters immunized with recombinant *Na*-GST-1 or its *A caninum* orthologue *Ac*-GST-1 and subsequently challenged with infective larvae, increased levels of IgG and IgG_1 against *Na*-GST-1 were associated with significant protection when compared with animals injected with adjuvant alone.[71,72,74] It is hypothesized that antibodies raised against *Na*-GST-1 neutralize its ability to detoxify the heme that results from the enzymatic digestion of ingested host hemoglobin, resulting in death of the parasite.[1,2,72,75,76] In contrast to other free-living eukaryotic parasites, hookworms do not synthesize hemoglobin *de novo* and, in the absence of the symbiont that produces it, rely exclusively on capturing hemoglobin and other proteins from their hosts by feeding on host blood, tissue, or fluid. However, once exogenous hemoglobin is consumed and digested by the parasite, the resulting heme byproduct must be transported by a carrier within cells caused by its insolubility and toxicity when oxidized to its ferric form.[1,2,72,75,76,77] Until the discovery of the nematode-specific Nu class of GSTs that includes *Na*-GST-1, there were no known enzymes in the nematode proteome that could putatively bind and detoxify heme.[77-79]

from *A duodenale* are known to inhibit both factors VIIa/tissue factor complex and Xia *(Ac)*,[48] factors Xa and VIIa/tissue factor complex *(Ae)*,[50] and factors Xa and Xia *(Ad)*.[51] NAPs have been localized to the amphidial, cephalic, and esophageal glands of the parasite,[47,52] and putative NAP-coding transcripts have been detected in hookworm gut cDNA libraries,[53] supporting the hypothesis that these molecules play a role in the maintenance of the liquid state of host blood during its transition through the parasite's digestive tract.[45] In addition, a platelet inhibitor from *A caninum* is known to inhibit coagulation by preventing platelet adhesion to ***fibrinogen*** and collagen through blockage of cell-surface glycoproteins.[54,55]

The process of hemoglobin release through red blood cell lysis in the hookworm gut is poorly understood. A hemolytic detergent-soluble protein that forms pores in erythrocyte membranes has been identified from extracts of *A caninum*; however, its precise molecular identity remains unknown.[56] Saposins from the hematophagous liver ***flukes*** *Fasciola hepatica* and *Clonorchis sinensis* are involved in membrane remodeling through interactions with lipidases[57] and saposin-like proteins.[58 60] Transcriptomic analyses of human and canine hookworm digestive tissues have revealed several saposin-like proteins.[53] Two of these, *Ac*-slp-1 and *Ac*-slp-2, are localized to vesicles within *A caninum* intestinal cells. Although the hemolytic activity of these proteins has not been confirmed,[61] structural studies on *Ac*-SLP-1 and the *N americanus* homologue *Na*-SLP-1 have suggested membrane interactive functions.[62]

A large repertoire of hookworm intestinal aspartic, cysteine, and metalloproteases are involved in the digestion of free hemoglobin. In *N americanus*[63] and *A caninum*[64] hemoglobin is digested by using a hierarchic cascade of mechanistically distinct proteases that are expressed in and released from the ***intestinal brush border*** of the worm, similarly to the hemoglobin digestion pathways occurring in ***phylogenetically*** distant blood-feeding parasites (eg, the blood fluke *Schistosoma mansoni*[65] and the malaria parasite *Plasmodium falciparum*[66]).

The cathepsin D–like APRs from *N americanus* and *A caninum*, respectively, termed *Na*-APR-1 and *Ac*-APR-1,[67] cleave the intact hemoglobin tetramer at many sites, showing a promiscuous subsite specificity but a preference for hydrophobic P1 and P19residues.[63,67] Hemoglobin hydrolysis by APR-1 then allows further proteolysis of globin fragments by other proteases. Moreover, proteases of specific species of hookworm will preferentially cleave hemoglobin from their respective hosts, despite high levels of sequence homology across species.[64] *N americanus* *Na*-APR-2, which resembles mammalian pepsin more closely than cathepsin D,[68] localizes primarily to the intestinal microvillar surface in adult worms and hydrolyses intact hemoglobin, although it shares only 25% of its cleavage sites with *Na*-APR-1.

Several hookworm cysteine proteases have been predominantly implicated in digestion of blood.[53,69] Indeed, a number of these proteases, including *Ac*-CP-2, a cathepsin B–like cysteine protease from *A caninum*, and *Na*-CP-3, an *N americanus* cathepsin B–like protease,[63] have been shown to degrade intact hemoglobin. Metalloproteases are thought to play a role in the nutrient acquisition by parasites caused by their presence in the parasite tissue lining the intestinal tract,[53,63] as well as the ES component.[28] A neprilysin-like zinc metalloprotease from *N americanus* (*Na*-MEP-1) has been shown to ***hydrolyze*** host globin fragments after cleavage by *Na*-APR-1.[63] In addition, it has been suggested that aminopeptidases and proteases with exopeptidase functionality aid the blood digestion process in hematophagous parasites by removing terminal amino acids and dipeptides for transport into intestinal cells.[63]

The degradation of hemoglobin liberates both iron-containing heme and hematin, which can be toxic to hookworms because of the generation of harmful oxygen radicals.[70] ***Glutathione-S-transferases*** (GSTs) (Box 3) have been postulated to detoxify

the oxidative iron contained in heme and hematin by forming homodimers to bind these molecules. GSTs that bind heme with high affinity have been isolated from both human and dog hookworms.[71,72] X-ray crystallography studies of *N americanus* GSTs show these molecules are capable of homodimerization in solution, thus creating binding pockets that are accessible to heme.[73]

Molecular mechanisms associated with host defense and immune modulation

Hookworm disease is a chronic infection of human subjects in which adult worms can survive for years in the small intestine.[80] The parasite has evolved several remarkable strategies to ensure its long-term survival in the host. The deployment of broad-spectrum Kunitz-type protease inhibitors (KIs) has been suggested as a mechanism by which hookworms can neutralize host proteases that would otherwise cleave parasite-derived molecules.[81] Kunitz protease inhibitors are ubiquitous among ***eukaryotes***, contain single or multiple cysteine-rich Kunitz domains, and exhibit inhibitory activity against 1 or more ***serine*** proteases, including pancreatic ***trypsin***.[82] For example, recombinant hookworm KI-1 (AceKI-1) from *A ceylanicum* exhibits potent inhibition of trypsin, pancreatic elastase, chymotrypsin, and neutrophil elastase. This broad-spectrum activity appears to be unique among Kunitz inhibitors, suggesting a dual function for AceKI-1 in both protection from intestinal proteases and modulation of the immune system.[81] Jin et al[83] have described an *Ascaris* family serine protease inhibitor from *A duodenale* (AduTIL-1), which contains 2 trypsin inhibitor–like domains shown to have differing inhibitory activities against a range of intestinal proteases. Finally, a large KI has been isolated from *A caninum* (AcKPI-1) that contains 12 tandem Kunitz domains and is most architecturally similar to extracellular matrix proteins involved in cellular remodeling, although the exact function of this protein has not been determined.[84] No anticoagulant activity has been detected for AceKI-1[81] or AduTIL-1,[83] demonstrating that although they are also serine protease inhibitors, these molecules perform biologically disparate roles to the NAPs described in section 2.

Malnutrition, one of the pathologic manifestations of hookworm disease, is considered mostly to occur as a result of IDA but can also manifest as a consequence of intestinal malabsorption caused by parasitic infection.[85,86] It has been suggested that the neutralization of digestive proteases by hookworm-derived, broad-spectrum protease inhibitors, although protecting the parasite from the proteolytic environment of the small intestine, contributes to malnourishment and impairment of physical growth by interfering with the intestinal absorption of nutrients.[87] In support of this theory, hamsters immunized with AceKI-1 and then challenged with *A ceylanicum* hookworms were partially protected against hookworm-associated growth delay without a measureable effect on anemia, suggesting that the prevention of growth delay is due to downstream effects of antibody-mediated neutralization of protease inhibitors and not directly related to parasite death.[87]

To avoid elimination from the human host, hookworms have devised a suite of strategies by which they evade or skew the immune system to promote their survival. This manifests in "suppression" or a subversion of the host immune system,[88,89] thereby providing a more favorable environment for the parasite itself but also minimizing immunopathogenesis. Studies have shown that the hookworm evades the immune system by inducing apoptosis of T lymphocytes (for review, see Taylor et al[90]). Host pathology around the worm is minimized by the parasite's inhibition of the local inflammatory response. Although the literature describing manipulation of the immune system by hookworms is extensive, only a small number of proteins have been implicated in this immunomodulatory response. Two of these belong to the ASP family described in section 1. *Na*-ASP-2 has been shown to induce neutrophil recruitment; however, its receptor on the immune cell surface has yet to be determined.[30] *Ac*-NIF inhibits CD11b/CD18-dependent leukocyte function by binding to the α domain of β_2-integrin on the cell surfaces of T cells.[33] Also, *Ac*-NIF inhibits the adhesion of activated neutrophils to endothelial cells and their release of hydrogen peroxide.[31 33] Studies have showed that recombinant NIF bound to the I domain of CD11b/CD18 blocks the interaction between neutrophils and fibrinogen and prevents neutrophil adherence to the endothelium.[33] Hence localized inflammation is minimized and the parasite is protected from a potentially deleterious immune response. An orthologue of macrophage migration inhibitory factor (MIF) has been identified from *A ceylanicum* (AceMIF) that binds to the mammalian MIF receptor CD74 and is hypothesized to modulate immune responses at the site of worm attachment in the small intestine.[91]

In mammals ***matrix metalloproteases*** (MMPs) are involved in remodeling of the extracellular matrix. Many are secreted by immune cells, as well as the gut epithelium, at sites of inflammation. All are regulated by tissue inhibitors of metalloproteases (TIMPs) through interaction with a netrin domain.[96,97] Adult hookworms have been shown to secrete TIMP homologues or netrin-like proteins that might modulate inflammatory responses, specifically at the site of attachment to the intestinal mucosa. *Ac*-TMP-1 and *Ac*-TMP-2 are TIMPs that have been identified from ES products of adult *A caninum*,[98,99] and *Ac*-TMP-2 has been localized to the esophageal glands of the parasite,[99] supporting the hypothesis that *Ac*-TMPs are released from the worm to act at the attachment site. *Ac*-TMP-1 is one of the most abundant proteins secreted by *A caninum*, with a release rate of 40 ng/h,[98] and although no MMP inhibitory function has been reported for this molecule, it has been shown to inhibit an endogenous metalloprotease, *Ac*-MTP-1.[41] Furthermore, a recent study has demonstrated that dendritic cells that have been stimulated with *Ac*-TMP-1 release inflammatory cytokines and promote the development of regulatory $CD4^1$ and $CD8^1$ T cells,[100] suggesting an immunosuppressive role for this protein. *Ac*-TMP-2 inhibits a range of human MMPs,[99] but its role in immune manipulation is yet to be defined. A protein that adopts a netrin-like fold similar to TIMPs (AceES-2) has been isolated from *A ceylanicum* ES,[101,102] and although it exhibits no significant MMP inhibitory activity,[102] vaccination with AceES-2 protected against anemia in hamsters on challenge infection, suggesting parasite attenuation as a result of the induction of an anti–AceES-2 immune response.[101] However, whether this molecule acts as an immunomodulatory agent remains to be elucidated.

A manifestation of host immunosuppression by hookworms is the dampening of immune-mediated pathology that can cause asthma, inflammatory bowel disease, and other allergic and autoimmune disorders (reviewed in Yazdanbakhsh et al[103]), and although it has been shown that experimental infection with hookworms and administration of hookworm ES can ameliorate the effects of these disorders (Boxes 4 and 5), the specific bioactive molecules within this protein mixture have yet to be

BOX 4. Hookworm molecules as therapeutic molecules

Hookworms achieve long-term survival in their human host by evolving multiple mechanisms to (1) ensure the constant and free flow of blood from the host through a series of powerful anticoagulants and (2) suppress the host immune response and promote a tolerizing regulatory response to modulate inflammation. As such, there is a growing awareness that the molecules used to ensure long-term parasitism might be used in therapeutic contexts to protect the human host from the deleterious effect of unrelated pathologies. Several examples of these therapeutic molecules are listed below:

- recombinant nematode anticoagulant protein c2 targeting blood coagulation factor VIIa/tissue factor[92];
- a recombinant NAP (rNAP5) with antithrombotic efficacy in canine models of thrombosis[93];
- a neuroprotective effect after focal ischemia of an NIF[94]; and
- an NIF that prevents neutrophil-dependent lung injury.[95]

BOX 5. Molecular mechanisms of immune suppression as vaccine targets for autoimmune diseases: Celiac disease

After the epidemiologic evidence of a causal association between the disappearance of helminths from societies with advanced sanitary infrastructure and the apparent increase in the incidence of autoimmune and allergic diseases (the hygiene hypothesis),[103] a number of interventional clinical trials have been undertaken, with inconsistent results.[105,107,108] Recently, 2 clinical trials have been reported in which the effect of experimental human hookworm *(N americanus)* infection on the pathology of celiac disease has been evaluated.[105,107,108] In these studies it was found that basal production of IFN-γ and IL-17A from duodenal biopsy culture was suppressed in hookworm-infected patients with celiac disease compared with that seen in uninfected control subjects. Increased levels of $CD4^+CD25^+$ forkhead box protein 3 (Foxp3)–positive cells in the circulation and intestinal mucosa were associated with active celiac disease.[108] Hookworm infection suppressed an increase in circulating $CD4^+CD25^+Foxp3^+$ cell numbers that was observed in the control subjects during a short-term (1 week) oral gluten challenge period. When duodenal biopsy specimens from hookworm-infected participants were restimulated with the immunodominant gliadin peptide QE65, robust production of IL-2, IFN-γ, and IL-17A was detected, even before gluten challenge, while participants were strictly adhering to a gluten-free diet.[105] Intriguingly, IL-5 was produced in response to stimulation with QE65 only after hookworm infection. Thus hookworm-induced T_H2 and IL-10 cross-regulation of the T_H1/T_H17 inflammatory response might be responsible for the suppression of these responses during experimental hookworm infection.[105,108]

BOX 6. Key concepts and therapeutic implications

- Hookworms are intestinal parasites that feed on host blood, resulting in chronic IDA and protein malnutrition.
- Key molecular mechanisms by which hookworms survive in the host and cause disease include those that mediate:
  - larval entry into the host and migration through tissue until establishment of patency in the small intestine;
  - ingestion and digestion of host blood for use as a source of energy; and
  - escape from host defenses through inhibition of intestinal proteases and modulation of specific and systemic immune responses.
- ASPs are the predominant ES products released by hookworm larvae on skin penetration and host invasion; these play a role in larval migration and evasion of the host immune response to initial infection.
- Molecular mechanisms that enable hookworms to use blood as an energy source include:
  - NAPs;
  - saposin-like proteins that might mediate hemolysis;
  - aspartic and cysteine proteases that cleave hemoglobin in an ordered cascade; and
  - proteins, such as GSTs (eg, GST-1), that detoxify free heme.
- Evasion of host defenses is mediated by several KIs of intestinal proteases and the modulation of the host immune system by TIMPs and netrin-like proteins that:
  - promote development of regulatory $CD4^+$ and $CD8^+$ T cells that inhibit the host inflammatory response and
  - secondarily might reduce immune-mediated pathology associated with allergic and autoimmune diseases.
- Identifying critical hookworm molecules involved in immunomodulation host immune responses might yield therapeutic agents for diseases, such as asthma, inflammatory bowel disease, and celiac disease.
- Molecules involved in the hookworm blood digestion pathway, such as APR-1 and GST-1, might be ideal targets of new vaccines.
- Vaccines targeting ASPs are problematic due to the tendency of hookworms to induce the host immune system to produce IgE to these proteins, resulting in allergic reactions on vaccination in the context of previous exposure to the parasite.

identified.[104,106] It is tempting to speculate that immunomodulatory virulence factors, such as hookworm TIMPs, might indeed be these elusive molecules, and the development of recombinant proteins derived from these agents might unlock novel therapeutics for allergic and autoimmune disorders.

CONCLUSION

The continued unraveling of the molecular mechanisms involved in the pathogenesis of hookworm disease have not only increased our understanding of the pathobiology of a complex multicellular parasite but also added more detail to the roadmap of the hookworm's survival inside a host and has highlighted areas of vulnerability during its intramammalian journey (Box 6). The majority of these "weak spots" are potentially susceptible to immune attack, and some have been exploited to create antihookworm vaccines. Most of this effort has been directed at targeting molecular mechanisms involved in blood feeding and nutrient acquisition, in which recombinant versions of critical components of this process (eg, the apical hemoglobin digestive protease *Na*-APR-1[109]) are being developed as vaccines that will induce protective antibodies to neutralize their parasite-derived counterparts and impair worm survival.[2,110,111] Vaccines are also being developed that target other factors involved in the hookworm's blood digestion pathway, such as the metabolic heme detoxifier *Na*-GST-1,[72] and a vaccine that targets the abundantly secreted larval-stage ASP *Na*-ASP-2.[37,74]

By no means is the list of molecular mechanisms described herein a definitive one. Advances in hookworm proteomics[28,112] have made it possible to characterize the wealth of proteins present in the parasite secretome in a biologically relevant context, and this will undoubtedly uncover crucial new players in every aspect of the pathogenesis of hookworm infection. Characterization of the potential of these proteins as vaccines will offer up new candidate antigens in the fight against this debilitating disease.

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