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Chapter 9

Solving the Riddle of the Lung-Stage Schistosomula Paved the Way to a Novel Remedy and an Efficacious Vaccine for Schistosomiasis

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Additional information is available at the end of the chapter

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1. Introduction

The field of schistosomiasis vaccine has suffered from several entrenched dogmas, which have delayed progress.

The first dogma states that the main mechanism of innate and acquired immunity-related parasite attrition is antibody-dependent cell-mediated cytotoxicity (ADCC). ADCC has been shown to effectively mediate killing of 3-, 18- or 24 hr-old schistosomula in human, mouse, and rat models. However, this phenomenon is of no in vivo relevance as larvae of this age are still in the epidermis, impervious to host immune attacks. Intact, healthy older larvae, pre-adults and adult schistosomes are entirely invisible to the immune system, and thus, are never threatened by ADCC in vitro or in vivo. Concurrently, the immune effectors "hunt" for larvae in the pulmonary capillaries, proposed by von Lichtenberg et al. in 1977 [1] as a plausible mechanism for resistance to infection, was entirely neglected, and never mentioned or referred to.

Second dogma is to consider parasite surface membrane antigens of great importance as vaccine antigens, because they reside at the host-parasite interface, and were shown to induce robust immune responses. However, schistosome surface membrane molecules are hidden, inaccessible to host antibodies, and accordingly, induced immune effectors are unable to interact with the parasite.

Third, stressing that Th1 immune responses are the pillars for acquired immunity to larval infection in mice. This dogma is entrenched notwithstanding the numerous reports documenting the importance of exclusive type 2 immune responses in rat, monkey, and human schistosomiasis, and despite that larval antigens induce principally Th1 relat-

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ed cytokines and antibodies that are not entirely protective in mice. Enhancement of these Th1 responses during mouse immunization, via the use of Th1-biased constructs and adjuvants, consistently elicited only partial resistance, with candidate vaccine antigens discarded one after the other.

This review aims to dispel these dogmas and opens a new avenue for the development of a remedy and vaccine for schistosomiasis.

2. Body

2.1. Schistosomiasis

Schistosomiasis, also known as bilharziosis, bilharzia, or snail fever, is endemic in 74 countries of the Developing World, infecting between 391 and 600 million people worldwide. with close to 800 million, mostly children, at risk [2,3]. The disease burden is estimated to exceed 70 million disability-adjusted life-years [4]. The causative agents are flatworms, dioecious (separate sex) trematodes, of the family Schistosomatidae, with *Schistosoma mansoni* and *Schistosoma haematobium* responsible for the largest numbers of human infections. The infective stage, the cercaria, invades host skin, and stays in the blood- and lymph-free epidermis for a minimum of 40 and up to 72 hr, impervious to innate immunity effectors [5,6]. The parasite develops into a schistosomulum, exchanging the classical outer membrane for a double lipid bilayer covering [7], and releasing in the process of physiological and biochemical changes numerous enzymes and other molecules, which may interact with the innate immunity receptors on keratinocytes and Langerhans cells [4,8-11]. The innate immunity cells proceed to activation of the acquired immune system, and produce cytokines that shape the amount and direction of the adaptive immune responses to the developing parasite [4,8-10]. Schistosomula then penetrate dermal blood vessels, and remain intravascular for their life span, and therefore, schistosomes of the genus *Schistosoma* are known as blood flukes [12]. The schistosomula travel via the pulmonary artery reaching the lungs within 4-6 and 6-10 days for *S. mansoni* and *S. haematobium*, respectively [13], painstakingly negociate the lung capillaries, then migrate to the hepatic portal vessels, where they start to actively feed, mature, and copulate. It is important here to recall that developing (and mature) schistosomes live in the blood vessels and capillaries, and hence, released, excreted, or secreted molecules are blood-borne products, which likely access macrophages, dendritic cells, and B cells in the spleen rather than the lung tissue draining lymph nodes. It is documented that developing schistosomula products elicit predominant T helper (Th) Th1 and Th17 immune responses, dominated by interferon-gamma (IFN-g) and interleukin (IL)-17, and IgG2a and IgG2b antibodies [14-19]. The male carrying the female in the gynaecophoric groove, the schist, then migrates to the mesenteric venules (*S. mansoni*) or the vesical capillaries around the urinary bladder (*S. haematobium*). Hundreds of eggs are deposited daily. Eggs exit the host via the intestine with the feces (*S. mansoni*) or the urinary bladder, with the urine (*S. haematobium*) to continue the life cycle in compatible snails [12,20]. Numerous eggs are retained in the host tissue, inciting intense immune responses to the parasite egg antigens,

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characterized by granulomas formation around the eggs and progressive liver (*S. mansoni*) or urinary bladder (*S. haematobium*) tissue damage and fibrosis [21], and dominated by Th2 cytokines [14,15].

Skewing of the immune responses of patently infected hosts towards the Th2 axis may be the reason whereby infection with schistosomes increases the severity of subsequent infection with other pathogens namely *Plasmodium falciparum*, *Leishmania*, *Toxoplasma gondii*, *Mycobacteria*, *Entamoeba histolytica*, *Staphylococcus aureus*, or *Salmonella* [22, and references therein]. Schistosomiasis also may predispose individuals to infection with human immunodeficiency (HIV) and hepatitis C (HCV) virus, and increases resulting disease persistence and severity [2,23,24]. Individuals with schistosomiasis haematobium are at great risk for HIV infection, and for cancer development [24,25], afflictions all requiring robust Th1 responses for control and elimination [26].

2.2. Control

2.2.1. Molluscicides

Schistosome species require 2 hosts, a definitive mammalian and an intermediate freshwater snail host, for completion of the life cycle. *Schistosoma mansoni* and *S. haematobium* eggs release the miracidium, which must find a snail of the genus *Biomphalaria* and *Bulinus*, respectively for asexual reproduction, whereby infection of a compatible snail with a single miracidium results in production of thousands of infective cercariae [12,20]. The prevalence of schistosomiasis is linked to compatible snail distribution. Accordingly, breaking the dreadful cycle may well be accomplished via elimination of the obligatory intermediate snail host.

Snails of the *Biomphalaria* and *Bulinus* spp. live under water, and all molluscicidal measures must take into account vegetation, fish, ducks, and other animals habitat, as well as water quality for drinking and irrigation, rendering control of snails with chemicals, such as acrolein, copper sulfate, and niclosamide, a particularly costly approach [2,27].

2.2.2. Sanitation and health education

People acquire schistosomiasis while farming, fishing, bathing, washing, and performing recreational activities in the vicinity of water bodies contaminated with infected snails. Accordingly, safe water supply, improved sanitation, and health education are mandatory in order to effectively reduce (and eventually eliminate) the very significant global burden of disease due to schistosomiasis and other helminthic parasites, yet are unavailable in numerous countries due to poor economical, social, and political conditions [2-4].

2.2.3. Praziquantel

Praziquantel (PZQ), a pyrazino-isoquinoline derivative, FW 312.41, is practically now the single cost-affordable, and relatively safe and effective oral drug for mass treatment of hundreds of millions of individuals afflicted with schistosomiasis mansoni, schistosomiasis hae-

matobium, and schistosomiasis japonicum as well [2-4,21]. Due to its hydrophobic nature and small size, PZQ inserts itself into the parasite outer lipid bilayer membrane, leading to its vacuolization, blebbing, and disruption [28]. Several reports documented the ability of PZQ to bind and polymerise actin [29-34]. Actin-based membrane skeleton (the fence) lies beneath the inner leaflet of the plasma membrane [35], and accordingly PZQ-parasite actin binding and subsequent polymerization may explain the severe muscle contraction and the paralysis worms undergo immediately upon exposure in vitro to PZQ. This mode of action, if proven to be true, suggests that PZQ treatment might not be entirely safe for children, persons with cardiovascular afflictions, and pregnant women [36].

Even if absolutely and entirely safe, PZQ treatment is not 100% proof and does not prevent reinfection or diminishes prevalence [37], necessitating periodic examination and repeated treatments, and thus, exacerbating the possiblity of the emergence of parasite resistance to the drug, a threat that has already materialized in several settings [38,39].

2.2.4. Vaccine status – Documented target: The lung-stage schistosomula

The hope for development of a schistosomiasis vaccine stems from the strong, and reproducible, protective immunity obtained after immunization of experimental hosts with radiation-attenuated (RA) cercariae [1,40], and the documented human resistance to infection (endemic normals), or reinfection after chemotherapy [41-43]. Sera and spleen cells of RA-vaccinated, and worm antigens-protectively immunized mice were used for identification of the potential protective target antigens. These studies resulted in identification, cloning, gene expression and assessment of protective potential of a plethora of molecules, among which *S. mansoni* irradiation associated vaccine antigen, IrV-5 [44], glutathione-S-transferase, GST [45], triose phosphate isomerase, TPI [46,47], paramyosin [48], fatty acid binding protein, Sm 14 [49], the surface membrane antigen Sm23 [50], the calcium-dependent cysteine protease, calpain [51], enolase [52,53], and calreticulin [54-56]. Reactivity of serum antibodies and peripheral blood mononuclear cells of resistant humans with fractionated worm molecules succeeded in identification of *S. mansoni* glyceraldehyde 3-phosphate dehydrogenase [57-61] as a potential candidate vaccine antigen. These molecules were used in conjunction with different adjuvants, namely complete (CFA) and incomplete (IFA) Freund's adjuvant for mouse immunization, leading to only hardly significant ($P < 0.05$) of some 30-35% reduction of challenge worm burden. Except for Sm23, these molecules are all cytosolic, and the poor protective capacity was ascribed to their inability to interact with the host immune system effector molecules.

Sm 23, an outer *S. mansoni* surface membrane antigen was among the molecules thought to be a target of protective immunity and of potential importance due to its residence at the host-parasite interface [50]. The full length molecule in a plasmid or recombinant form failed to lead to highly significant, consistent, or reproducible protection despite of their use in combination with a number of different adjuvants [62-64]. The *S. mansoni* integral surface membrane proteins tetraspanins (TSP)-1 and TSP-2 encoding cDNA were cloned, sequenced, and expressed and shown to elicit protection levels of 57% and 64% (TSP-2) and 34% and 52% (TSP-1) for mean adult worm burdens and liver egg burdens, respectively,

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over two independent trials in CBA/CaH mice [65]. The protection levels were not reproduced using *S. japonicum* counterpart, whereby mice immunized with the recombinant protein of a single TSP-2 subclass showed no protection, while immunization with a mixture of seven recombinant TSP-2 subclasses provided a moderate protection [66]. A recent study used *S. mansoni* TSP-2 extracellular loop 2 region in conjunction with alum and CpG as adjuvants leading to extremely variable protection levels against challenge infection within cohorts of highly inbred C57BL/6 mice [67]. An *S. mansoni* stomatin-like protein, a tegument protein located at the host-parasite interface, engendered in immunized mice a partial protection of 30-32%, associated with specific IgG1 and IgG2a antibodies and elevated production of IFN-g and tumor necrosis factor (TNF), while no IL-4 production was detected, suggesting a Th1-predominant immune response [68]. A schistosomular tegument preparation (Smteg) was used for C57BL/6 mice immunization, subcutaneously, on days 0, 15, and 30, resulting in significant antibody production, increased percentage of CD4 + IFN-g+ and CD4 + IL-10+ cells in spleen and increased production of IFN-g and IL-10 by spleen cells, but failed to reduce parasite burden, female fecundity and morbidity [69].

Sm23-, tetraspanins-, and other tegument-associated molecules- based protection was ascribed to specific antibody interaction with the molecules residing at the host-parasite interface, followed by binding to effector cells, which are able to elicit vigorous complement, and antibody-dependent cell-mediated cytotoxicity (ADCC) [65,67]. Indeed, ADCC was shown to be effective in killing a substantial proportion of 3-, 18- and sometimes 24-hr-old schistosomula [70-76], a phenomenon of limited in vivo relevance since larvae of that age still reside in the epidermis, impervious to any immune attack. On the other hand, older larvae, migrating schistosomula, pre-adut and adult worms surface membrane antigens are inaccessible to antibody binding [77,78], and thus, not threatened by ADCC. According, surface membrane, like cytosolic, antigens may not be considered for effective vaccination against schistosome infection.

No vaccine antigens appeared available, while we were striken by the paradox of lung-stage schistosomula being documented as the target of innate and RA vaccine immunity [1,40], and obtaining nutrients essentially via the tegument, while their surface membrane are entirely hidden, inaccessible to specific antibody binding.

3. The enigma of the lung-stage schistosomula

3.1. Lung-stage schistosomula strive without feeding in lung capillaries while surface membrane molecules are entirely hidden, inaccessible to specific antibody binding

Migrating schistosomula usually do not ingest erythrocytes because of their undersized mouth [79], and import sugar, fatty acids, and other nutrients directly across their tegument and into their internal tissues via membrane-spanning transporters [80-82]. Paradoxically, no specific antibody is able to bind to lung-stage larvae surface membrane antigens, as judged by several serological tests, namely indirect membrane immunofluorescence [1,40,77,78,83-89]. We were unable to visualize the presence of the glucose transporter

SGTP4 on the surface membrane of intact in vitro cultured or ex vivo 5 -7 day-old schistosomula, using specific antibodies and indirect membrane immunofluorescence [90]. Entire failure of larval surface membrane antigen detection does not give support to the contention that the tegument is bounded externally by a single lipid bilayer, overlain by a laminate secretion containing numerous proteins and molecular complexes [82,91], and was previously ascribed to shedding of antigenic molecules [86], masking by host proteins [85,88], or intrinsic biochemical modifications of the outer membrane [77,83,84,87, 89].

3.2. Attempts to expose the lung-stage and adult worm surface membrane antigens to specific antibody binding

In an attempt to overcome the lack of exposure of *S. mansoni* and *S. haematobium* lung stage larvae apical membrane antigens to specific antibody binding, we started by manipulating potential cues for increased surface antigenic expression, such as lack of glucose and amino acids, and extremes of pH or HCO_3^- concentration. All such trials failed to alter the negligible *S. mansoni* larval reactivity with RA vaccine and infection sera in membrane indirect immunofluorescence (IF). It was then thought that incubation of ex-vivo lung stage schistosomula in strongly hydrophobic medium might induce exposure of outer surface membrane hydrophobic sites, where antigenic molecules could be sequestered. Lung-stage schistosomula were, therefore, incubated with sera diluted in sterile corn oil. It is of note that corn oil essentially consists of poly- (linoleic acid, 45.9 to 55.5 %), and mono- (olecic acid, 28.4 to 36.9 %) unsaturated fatty acids, and contains 1 to 2% unsaponifiables particularly rich in sterols and tocopherols. *Schistosoma mansoni* [92] and *S. haematobium* (Figure 1) ex-vivo lung-stage schistosomula could readily bind specific antibodies of RA vaccine or infection sera in the fluorescent antibody test, following incubation in corn-oil in a concentration- and time-dependent manner.

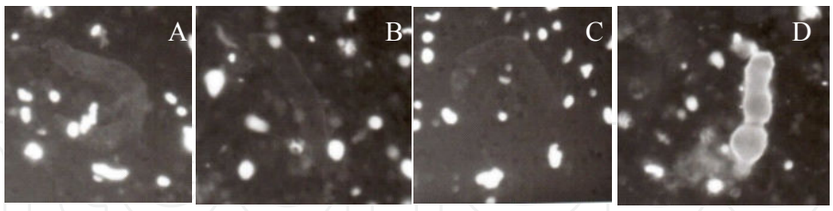


Figure 1. Assessment of the serological reactivity of *S. haematobium* lung-stage schistosomula by IF. Seven days ex vivo larvae incubated with control (A,C) or RA vaccine (B,D) serum diluted 1:50 in RPMI medium, before (A,B) or following incubation for 6 hr with 90% oil (C,D) were tested in IF and photographed under ultraviolet microscopy. x 200.

Since incubation of lung-stage parasites in corn oil led to exposure of antigenic sites in a concentration and time-dependent fashion, it was suggested that corn oil lipids may mediate efflux of cholesterol or phospholipids from the worm outer membrane, leading to changes in lateral diffusion of surface antigens that resulted into a dramatic increase in the avidity of antibody binding. Treatment with the membrane-impermeable, cholesterol-extracting drug methyl-b-cyclodextrin (MBCD), followed by visualization of surface membrane cholesterol

by staining with filipin III, a fluorescent polyene antibiotic, widely used for the detection and the quantitation of cholesterol in biomembranes, allowed us to examine the role of cholesterol in surface membrane antigen sequestration of *S. mansoni* and *S. haematobium* ex vivo lung-stage larvae. Evaluation of cholesterol content and distribution by filipin staining demonstrated that MBCD efficiently extracts cholesterol from the surface membrane of *S. mansoni* and *S. haematobium* lung-stage larvae [93,94]. Treatment of *S. mansoni* ex-vivo lung-stage larvae with MBCD consistently and reproducibly led to considerable binding of specific antibodies to the outer membrane surface. The data, thus, suggest that depletion of cholesterol from the outer membrane of *S. mansoni* lung-stage schistosomula corrects their failure to bind antibodies in the fluorescent antibody test. However, *S. haematobium* larvae treated with 2.5, 5.0, or 10 mM MBCD were consistently negative following incubation with either control or RA serum in the fluorescent antibody test. Hence, the results indicated that almost complete depletion of cholesterol from the surface membrane of *S. haematobium* larvae did not modify the negligible binding of specific antibodies by the surface membrane antigens. This means that *S. haematobium* and *S. mansoni* ex vivo lung-stage larvae differ regarding the contribution of cholesterol to the sequestration of surface membrane antigens [93,94].

3.3. Solving the schistosomula lung-stage enigma and predicting the existence of a parasite tegument-associated neutral sphingomyelinase (nSMase)

The data together strongly supported the contention that antigenic molecules persist on the surface of ex-vivo lung-stage schistosomula [89-91], yet are entirely inaccessible to specific antibody binding, in part due to cholesterol sequestration. Surface membrane antigens of *S. mansoni* and *S. haematobium* ex vivo lung schistosomula were, however, readily exposed to specific antibody binding following incubation in corn oil as mentioned above. It was hypothesized that corn oil unsaturated fatty acids (FA) might activate parasite surface membrane-bound neutral sphingomyelinase (nSMase), leading to hydrolysis of surface membrane sphingomyelin (SM), and subsequent decrease in outer bilayer lipid rigidity and permeability. At variance from MBCD, incubation of lung-stage schistosomula with unsaturated FA, such as corn and olive oil, or arachidonic acid (ARA), led to exposure of the, otherwise concealed, surface membrane antigens of *S. mansoni* and *S. haematobium* alike. In that respect, *S. haematobium* appeared more sensitive than *S. mansoni*, requiring lower unsaturated FA concentrations and shorter incubation periods [95, and references therein].

Evaluation of cholesterol amount and distribution by the filipin staining method indicated that unsaturated FA did not appear to elicit exposure of schistosomular surface membrane antigens via cholesterol extraction, as previously suggested. While a definite proof needs and remains to be established, some lines of evidence support the hypothesis that unsaturated FA might elicit larval surface membrane antigen exposure via their ability to stimulate a putative tegument-associated nSMase, with subsequent SM hydrolysis, and access of antibodies [95,96].

We have been able to demonstrate that 1- intact healthy ex vivo schistosomula, displaying equilibrium in SM synthesis and hydrolysis, allow molecules of less than 600 Da to access their surface membrane molecules. 2- Ex vivo larvae exposed to conditions condu-

cive to nSMase inactivation (pH less than 7.2, high concentrations of nitric oxide or hydrogen peroxide), do not allow neither antibodies nor even very small molecules (less than 600 Da) to access their surface membrane molecules [96]. Sphingomyelin appears critical in maintaining the rigidity and impermeability of the parasite outer lipid bilayer, likely via the ability of hydroxyl and amide groups in the interfacial region of sphingolipids to form with water molecules a tight netwok of hydrogen bonds [96, and references therein]. Recently, quasi-elastic and inelastic neutron scattering experiments on *S. mansoni* and *S. haematobium* worms and larvae have been performed. The obtained experimental findings suggest that the larva-medium interaction is triggered by the hydrogen bond network. Furthermore, the strength of that hydrogen bond network-based interaction appeared to be higher for *S. mansoni* larvae than adult worms and for *S. mansoni* than *S. haematobium* [Federica Migliardo, Unpublished Observations]. 3- Inhibition of ex vivo larvae SM synthesis or moderate nSMase activation (exposure to low concentrations of unsaturated FA, especially ARA) allows specific antibody access and visualization of surface membrane antigens. Exposure of ex vivo larvae to conditions conducive to excessive nSMase activation (hypoxia, high concentrations of unsaturated FA, notably ARA) lead to their irreversible attrition [96, and references therein].

3.4. Evidence for the existence of parasite tegument-associated nSMase and its activation by arachidonic acid

In 2006, we predicted, and provided evidence for, the existence of a schistosome tegument-associated Mg^{2+}-dependent nSMase, which is able to hydrolyze some SM molecules, thus allowing nutrients, but not host antibodies, to access proteins at the host-parasite interface [96]. Excessive activation of the elusive nSMase using the unsaturated FA, ARA led to larval and adult worm surface membrane antigens exposure and eventual attrition [95-97]. The identification and sequence of *S. mansoni* putative nSMase was reported by Berriman et al. in 2009 [98; Accession number XP_002578732.1]. The molecule of 70.99 kDa was shown to consist of 631 amino acids (aa) with an N-terminal, 300 aa Mg^{++}-dependent exonuclease/endonuclease/ phosphatase (EEP) superfamily catalytic domain, and predicted 7 transmembrane regions at aa locations 355-377, 392-414, 435-457, 461-483, 527-549, 564-586 and 595-617 (genedb. org/ gene/ Smp_162880). The *S. mansoni* putative nSMase showed about 52% homology with the amino terminal 300 aa of *Mus musculus* and human nSMase 1, which consists of 419 and 423 aa, respectively, an exonuclease/endonuclease/phosphatase superfamily domain at the N-terminus, and two transmembrane domains at the C-terminus.

Using primers based on the published sequence of *S. mansoni* nSMase, El Halbousy, Tallima, and El Ridi (personal communication) succeeded in cloning and sequencing 836 bp near the 5′ end of *S. haematobium* nSMase-encoding mRNA. The predicted aa sequences corresponded to aa18- aa277 in the *S. mansoni* counterpart with 96% identities and 98% positives (Figure 2), and contained the conserved domains characterizing the EEP superfamily.

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SHnSM  1    FPSSTVRKEDRVNAIAAXLSVGDFDVILLQEIWLESDYRKLRNILDEKYPYSNYFYCNLI  60
            FPSSTVRKEDRVNAIA+  LSVGDFDVILLQEIWLESDYRKLRNILDEKYPYSNYFYCNLI
SMnSM  18   FPSSTVRKEDRVNAIASKLSVGDFDVILLQEIWLESDYRKLRNILDEKYPYSNYFYCNLI  77

SHnSM  61   GTGMCIFSKWTIECVFTHPFTTNGYPHLIHQADYYCGKGIGLARITSKEGFRINFYVTHL  120
            GTGMCIFSKWTIECVFTHPFT NGYPHLIHQADYYCGKGIGLARITSKEGFRINFYVTHL
SMnSM 78    GTGMCIFSKWTIECVFTHPFTANGYPHLIHQADYYCGKGIGLARITSKEGFRINFYVTHL  137

SHnSM  121  IARYELDRMLDKYNGHRISQLVEIMEFVRMTSTGSDAIIITGDFNLESNTSAIELFSTSL  180
            IARYELDRMLD+YNGHRISQLVE+MEFVRMTSTGSDAIIITGDFNLESNTSAIELFSTSL
SMnSM 138   IARYELDRMLDRYNGHRISQLVEVMEFVRMTSTGSDAIIITGDFNLESNTSAIELFSTSL  197

SHnSM 181   KLSDAWLNNTVALKNTNITELESEGCTCDRADNPYRNQLWTNTYGNGERLDYIFYRSGPS  240
            KLSDAWLNNTVALKNTNIT+LESEGCTCDRADNPYRNQLWTNTYGNGERLDYIFYRSGPS
SMnSM 198   KLSDAWLNNTVALKNTNITDLESEGCTCDRADNPYRNQLWTNTYGNGERLDYIFYRSGPS  257

SHnSM 241   IIDSFHIPSYAKLVCDSCWF  260
            IIDSF+IPSYAKLVC+SCW
Sbjct 258   IIDSFYIPSYAKLVCNSCWL  277
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Figure 2. Homology between predicted amino acid sequences of *S. mansoni* (SMnSM) and *S. haematobium* (SHnSM) neutral sphingomyelinase.

A systematically improved high quality genome and transcriptome of the human blood fluke *S. mansoni* was recently reported by Protasio et al. (unpublished observations), whereby sequence for *S. mansoni* putative nSMase was now shown to encode 431 (CCD60196.1) and not 631 (XP_002578732.1) aa and 100% identity with the latter sequence only for the first 345 aa, as only 2 transmembrane domains were identified near the carboxyl end, between aa 325 and 375.

It is of note that the whole-genome sequence of *S. haematobium* was recently published [99], and reported the coding sequence (1038 bp) of an *S. haematobium* putative neutral sphingomyelinase (http://www.schistodb.net; Sha_103241), encoding 345 aa, with 3 transmembrane domains at the carboxyl end. Blasting of our SHnSM sequences with Sha_103241 revealed 94-97% homology. However, the sretch of SHnSm aa33-116 (highlighted in Figure 2), which contained EEP superfamily signature sites and showed complete homology with the *S. mansoni* counterpart, was lacking in Sha_103241 published sequence.

Antibodies specific to *S. mansoni* nSMase were generated in mice, immunized with peptides based on the molecule predicted aa sequence, synthesized as multiple antigen peptide (MAP) constructs. We were able to confirm the presence of the enzyme in adult male and female *S. mansoni* and *S. haematobium* tegument as judged by enzyme-linked immunosorbent assay (ELISA) and membrane and cytoplasmic IF. We were also the first to measure nSMase enzymatic activity in Triton X-100-solubilized surface membrane (Sup 1) and whole worm soluble (SWAP) molecules of male and female *S. mansoni* and *S. haematobium*. Neutral, but no acidic, sphingomyelinase activity was readily detectable by the Amplex Red Sphingomyelinase Assay, and increased with incubation time and protein amount. The nSMase

activity of Sup 1 and SWAP of male and female *S. mansoni* and *S. haematobium* adult worms was significantly ($P < 0.05$ - < 0.0001) increased following exposure to 125 or 250 mM linoleic acid, ARA, docosahexaenoic acid, or phosphatidyl serine, with ARA consistently showing the highest nSMase activating potential [100].

4. Arachidonic acid as a remedy for schistosomiasis

Since incubation of *S. mansoni* and *S. haematobium* ex vivo larvae [95,96] and adult worms [97] with ARA leads to exposure of surface membrane antigens and eventual attrition, it was rational to propose ARA for chemotherapy of schistosomiasis. ARA could be schistosomicidal per se, and additionally would expose the parasite surface membrane molecules to host antibodies-mediated attack, thus eliciting drug and immune system synergy.

In our studies, pure ARA from Sigma was used for in vitro and preliminary in vivo studies, while ARA from Martek was used for in vivo studies [101,102]. We have demonstrated that 5 mM pure ARA (Sigma) leads to irreversible killing of ex vivo larval, juvenile, pre-adult and adult *S. mansoni* and *S. haematobium* worms, within 1- 5 hr, depending on the parasite age, and the fetal calf serum concentration. ARA-mediated worm attrition was prevented by nSMase inhibitors such as $CaCl_2$ and GW4869. Electron microscopy studies revealed entire disruption of the outer lipid bilayers, the strength of which correlated with ARA concentration. These consistent and reproducible findings indicate that ARA is schistosomicidal per se [101].

ARA-mediated *S. mansoni* and *S. haematobium* worm in vitro attrition was reproduced in vivo whereby a series of 20 independent experiments, using BALB/c or C57BL/6 mice or Syrian hamsters, indicated that oral administration of 300-2500 mg/kg ARA in a pure form (Sigma), included in infant formula (Nestle), or capsules (X Factor, Molecular Nutrition), on 2 consecutive days, consistently led to between 50 and 80% decrease in *S. mansoni* or *S. haematobium* worm burden [101,102]. ARA-mediated attrition in vivo appeared to be associated with high titers of serum antibodies to tegumental antigens, because it was significantly higher when treatment was started at 8, rather than 5 or 6, week post infection with *S. mansoni*. Immune responses to adult worm tegumental antigens are certainly powerful in adults and children with patent or chronic schistosomiasis, and ARA is already marketed for human use in USA and Canada for proper development of newborns, and muscle growth of athletes. Accordingly, it is recommended to start pre-clinical and clinical studies in human volunteers for development of ARA as a safe and cost-effective remedy to schistosomiasis, especially that no ARA-related adverse effects were seen in any experiment in mice or hamsters [101-103].

5. Larval excretory-secretory products as vaccine candidates

Schistosome cytosolic and surface membrane antigens are entirely hidden, inaccessible to the host immune system effectors. As spelled out by Patrick Skelly [104], “schistosomes

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have achieved invisibility", and if it were not for the parasite "scent", the excretory-secretory products (ESP), a schistosomiasis vaccine would be as good as over.

Excretory-secretory products of cercariae, in vitro cultured and ex vivo lung-stage schistosomula, and adult worms of *S. mansoni, S. japonicum* [8,17,105-111], and *S. haematobium* as well [99] have been identified in several studies, and were found to be dominated by actin, enolase, aldolase, GST,TPI, glyceraldehyde-3 phosphate dehydrogenase (SG3PDH), thioredoxin peroxidease (TPX) = peroxyredoxin, proteases, and calcium-binding proteins, namely calpain.

Cercarial ESP would activate innate immune cells in the epidermis and dermis [8-11,111-117], but would not be targeted by antibody or complement products in the blood- and lymph-free epidermis. In the dermis, larval ESP may interact with host innate effectors, such as natural antibodies, serum lectins and complement components, resulting into inflammation that would facilitate the parasite entry into dermal blood capillaries. Schistosomula then migrate via the venous system in relatively large vessels, whereby ESP are quickly "washed" away by neutrophils and monocytes. In contrast, lung-stage larvae ESP likely stagnate in the narrow, convoluted, and notably thin-walled lung capillaries and liver sinusoids. These sites are the most strenuous of the journey, an occasion for the parasite to get rid of the less fit "members", and an explanation for the lung-stage and pre-adult larvae being documented as the target of innate and acquired resistance to schistosomiasis [1,118]. Adult worms reside in vessels, which are neither narrow nor thin-walled, and accordingly, the large ESP amounts they release are rapidly pinocytosed or phagocytosed, and transported away from the worms for stimulation of rather innocuous immune responses in the spleen, the principal target for intravascular antigens.

6. The plausible mechanism of innate and immune attrition of invading larvae

Migrating larvae ESP presented by blood monocytes and dendritic cells trigger Th lymphocytes in host spleen for production of predominantly Th1 (IFN-g and TNF) and Th17 (IL-17) cytokines [14,15,17-19], especially if the host was previously RA-vaccinated [16,118] or immunized with vaccine candidate antigens in conjunction with Th1 adjuvants [62-68]. Additionally, larval ESP- antibody complexes may activate immune effector cells via FcR binding. Stimulated monocytes produce nitric oxide and reactive oxygen products [119], which are lethal to near-by schistosomules; however, these toxic molecules inhibit the parasite tegument-associated nSMase activity, leading to outer membrane entire impermeability [95,96,120,121]. Neutrophils recruited and activated by Th17 cytokines likely ensnare larvae in their extracellular traps, yet secrete proteases and other enzymes that might all be ineffective because of the worm tight lipid bilayer and inaccessibility of surface membrane molecules [119,122,123].

Eosinophils also produce extracellular traps, and their basic molecules would be severely toxic to captured schistomula [124,125]. Basophils release cytotoxic basic molecules, and

most importantly, pharmacologic mediators able to modulate endothelial integrity, thus encouraging larvae to escape extravascularly to a certain demise [126]. Indeed, that attrition mechanism was shown to be of great importance in resistance to challenge infection in the RA model [118]. However, eosinophils and basophils are recruited and activated by type 2 cytokines [125,126], and thus, are prevented from active participation in the hunt for developing larvae [1,19] following infection of naive and antigens/Th1 adjuvant-immunized mice. Nevertheless, attempts to vaccinate mice against schistosomiasis uniformly aim at enhancing Th1 immune responses, in spite of the documented role of Th2 cytokines, namely IL-4, in protection of RA immunized mice [127-130]. Also in rats, sterile resistance to *S. mansoni* is associated with production of IL-4, IL-5, and IL-13, while susceptibility is accompanied with elevated expression of IFN-g [131,132]. In humans, a good association was previously observed between serum IgE production and resistance to infection after chemotherapy [41-43]. Indeed, Fallon et al. [133] reported that "adult resistance and child susceptibly to reinfection after chemotherapy have been described for all 3 *Schistosoma* species that most commonly infect man. For all 3 parasite species, the immunological correlates of this age-dependent resistance are associated with type 2 responses". These findings were entirely confirmed in recent studies [134-136].

7. The road to a sterilizing schistosomiasis vaccine

Larval ESP are innumerable, among which we have selected those eliciting the weakest Th1 and Th17 responses, namely SG3PDH in a recombinant (rSG3PDH) form and a TPX MAP construct [19,110]. Nevetheless, it was critical to use an adjuvant that would skew the larval immune responses towards the Th2 axis. In contrast to incomplete Freund's adjuvant, alum, polyinosinic-polycytidylic acid, and peptidoglycan, the Th2 master cytokine, thymic stromal lymphopoietin (TSLP), succeeded in directing the ESP-mediated immune responses towards a Th2-biased profile in prototypical Th1 (C57BL/6) and Th2 (BALB/c) mice [110,137]. Thereafter, we have immunized outbred, akin to humans, mice with rSG3PDH and TPX MAP in conjunction with the type 2 cytokines, TSLP, IL-25, or IL-33 [138, and references therein]. Results of 8 independent experiments indicated that these formulations elicited IgM, IgG1, and IgA specific antibodies, and an increase in ex vivo spleen cells release of IL-4, IL-5, and IL-13 correlated with consistent, reproducible, and highly significant ($P < 0.0001$) reduction of 62% to 78% in challenge *S. mansoni* worm burden [138]. Similar studies using *S. haematobium* are now in progress.

8. Conclusion

Improved selection of larval ESP, singly or in a mixture, and type 2 adjuvant is expected to result into a sterilizing vaccine against schistosome infection. Concurrent development of ARA, a nutrient, a component of our cell membranes, for chemotherapy of infection in un-

immunized individuals, will likely lead to full control, and eventual elimination of schistosomiasis.

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