Schistosomiasis is a severe parasitic disease caused by blood-dwelling flukes, namely *Schistosoma mansoni* and *Schistosoma haematobium* which are responsible for the great majority of about 300 million human infections, with 800 million, largely children, at risk of the infection in 74 countries in Africa, the Middle East, and South America. Developing and adult worms are unscathed by the surrounding immune effectors and antibodies because the parasite is protected by a double lipid bilayer armor which allows access of nutrients, while binding of specific antibodies is denied.

Scope of review: Fluorescence recovery after bleaching, extraction of surface membrane cholesterol by methyl-(-)-cyclodextrin, inhibition and activation of sphingomyelin biosynthesis and hydrolysis, and elastic incoherent and quasi-elastic neutron scattering approaches have helped to clarify the basic mechanism of this immune evasion, and showed that sphingomyelin (SM) molecules in the worm apical lipid bilayer form with surrounding water molecules a tight hydrogen bond barrier. Viability of the parasite and permeability of the outer shield are controlled by equilibrium between SM biosynthesis and activity of a tegument-associated neutral sphingomyelinase (nSMase).

Major conclusions: Excessive nSMase activation by polyunsaturated fatty acids (PUFA), such as arachidonic acid (ARA) leads to disruption of the SM molecules and associated hydrogen bond network, with subsequent access of host antibodies and immune effectors to the outer membrane and eventual parasite death.

General significance: ARA was predicted and shown to be a potent schistosomicide in vitro and in vivo in experimental animals and in children. Additionally, it was advocated that schistosomiasis vaccine candidates should be selected uniquely among excretory–secretory products of developing worms, as contrary to cytosolic and surface membrane antigens, they are able to activate the effector functions of the host antibodies and toxic molecules. This article is part of a Special Issue entitled “Science for Life” Guest Editor: Dr. Austen Angell, Dr. Salvatore Magazì and Dr. Federica Migliardo’. © 2016 Elsevier B.V. All rights reserved.

1. Introduction: Schistosomiasis

Schistosomiasis is a severe parasitic disease caused by flat (*Platyhelminthes*), blood dwelling worms of the genus *Schistosoma*. *Schistosoma mansoni* and *Schistosoma haematobium* are responsible for the great majority of about 300 million human infections, with 800 million, largely children, at risk of the infection in 74 countries in Africa, the Middle East, and South America [1]. Infection is initiated via human and other mammals’ skin exposure to the infective stage, the cercariae released by the intermediate snail host, which strives in freshwater bodies. Upon epidermis invasion, the larvae, now termed schistosomula, undergo a series of physiological and biochemical transformations ending in replacement of the conventional trilaminate membrane by an unusual two closely-apposed lipid bilayers, a likely adaptation for survival in the blood capillaries among immune cells and molecules. Perfectly well harnessed schistosomula exit the blood- and lymph-free epidermis to the dermis, en route to the dermal blood or lymph capillaries and lifelong residency in the bloodstream. The migrating schistosomula reach the lung and then the liver portal veins where they copulate, and the male carrying the female in the ventral gynecophoric canal or schist, hence the genus name, reach their final destination in the venous plexus of the lower intestine (*S. mansoni*) or urinary bladder (*S. haematobium*), living for years, if not decades, entirely unscathed by the surrounding immune effector cells and molecules. The paired worms daily deposit hundreds of eggs, which use their spine and hydrolytic enzymes to
make their way out of the blood capillary to the wall and then the lumen of the intestine or urinary bladder, leading to severe disruption of tissues and organ integrity. The eggs aim to exit the host with feces or urine, and search for the snail intermediate host. The infecting larva, named miracidium, reproduces asexually in the snail tissue generating thousands of infective cercariae, to continue the life cycle and transmission of infection (Fig. 1). The greatest harm, however, is due to the inflammatory immune reactions directed to the schistosome eggs that fail to exit, and remain trapped into the host tissues, principally liver and urogenital organs [2,3].

The major reason for the establishment of the parasite is the inability of the host immune system to eliminate the migrating larvae and adult worms because the access of host antibodies to the surface membrane antigens is denied. Consequently, major mechanisms of parasite attrition, namely antibody-dependent, complement- or cell-mediated cytotoxicity (ADCC) are prohibited [4]. Indeed, worms live in the blood vasculature without evidence of a single cell attached to their surface [5].

2. The worm surface controversy

Hockley and McLaren [6–8] were the first to report that the trilaminate outer membrane of invading cercaria of S. mansoni is replaced within 3 h of vertebrate host skin invasion by a heptalaminated membrane, which originates from multilaminate membrane-bounded vacuoles passed from subtegumental cells into the tegument where they enlarge, join to the outer membrane and open to the exterior. The multilamellar vacuoles were not described as secretory [6–8]. The multilaminate membranous bodies were very numerous in the tegument of developing and adult worms, appearing to be involved in the repair of damaged outer membrane. The heptalaminate membrane covers a syncytial tegument connected to subtegumental cells, and lined by a conventional basal membrane [7]. Thorough electron microscopy studies definitely established that the outer heptalaminated membrane of adult S. mansoni and other blood flukes consist of two closely apposed lipid bilayers 18 nm thick, apparently different in lipid composition because the outer lipid bilayer was only visualized after uranyl acetate staining. Of note, patches of diffuse material were seen on the external surface of the double lipid bilayer membrane of some S. mansoni specimens. The occurrence and distribution of the diffuse material indicated that it likely originated from the host and did not represent a true glycoalyx [8]. The recent purification of a serine protease-like enzyme present on the surface of S. mansoni and its identification as a host (mouse) carboxylesterase 1C lend support to that suggestion [9]. Conversely, the non-blood flukes were reported to be covered by a single lipid bilayer approximately 8 nm in thickness and a distinct, worm-derived glycoalyx [8].

The difference in biochemical properties of the two lipid bilayers on the surface of schistosomes was a major argument to propose that the outer heptalaminated membrane is actually a normal plasma membrane overlain with membrane-like secretions, arising from syncytium multilamellar vacuoles, which were described as “secretory” vesicles [10]. Analyses with whole cercariae and 1 and 3 h schistosomula using the S. mansoni glucose transporter protein SGTP4 as a marker supported the currently accepted model of adult tegument biogenesis stating surface tegumental membranes are derived from the migration of multilamellar vesicles from within cytons to the surface. No evidences were reported to advocate the outer membrane actually consists of a single lipid bilayer covered with secretions, which resemble a membrane [11]. Yet, the organization of the tegument surface was relentlessly advocated to be consisting of a single plasma membrane overlain by a membrane-like secretion, the membranocalyx [12,13]. If the worms were displaying their secreted, likely immunogenic, materials and intramembranous particles at the very host–parasite interface, one may not understand why host cells are hardly seen adhering to the surface of in vivo or ex vivo schistosomes [5,12,14].

Fig. 1. Lifecycle of Schistosoma parasites as they pass through human hosts and freshwater snails. Source: The Open University, Environment: Journeys Through a Changing World, U116, Block 3, Figure 1.13.
3. Elucidating the schistosome major immune evasion mechanism

3.1. Evidence

Several pioneering reports documented that newly developed and lung-stage larvae, like adult worms, are resistant to antibody-mediated damage in vitro, and display loss of antibody-mediated macrophage adherence independently of antigen mimicry, membrane turnover, or release of immune-modulatory molecules and proteases [15–17]. Mechanically transformed cercariae cultured in vitro for 3 h were readily killed by lymphokine-treated peritoneal macrophages or the macrophage cell line IC-2, whereas 7 and 10 day old lung schistosomula and 4 to 6 week old liver-stage parasites were refractory to macrophage effects. Notably, schistosomules isolated from the portal circulation 2 to 3 weeks after infection showed no evidence of surface-bound immunoglobulin in a quantitative immunofluorescence assay, and antisera from chronically infected mice or mice vaccinated with irradiated cercariae failed to react with the surface of these parasites in vitro in indirect membrane immunofluorescence (IF). The reduced surface antigenicity of developing and mature *S. mansoni* was ascribed to antigen shedding rather than host molecule acquisition [18,19]. Additionally, schistosomula of *S. mansoni* were shown to become fully resistant after 24–48 h of culture to damage by human blood eosinophils in the presence of human anti-schistosomular sera or human complement. This resistance was attributed to failure of antibodies and complement component C3 to bind to the surface of the cultured larvae. The insusceptibility to antibody, complement, macrophage, and eosinophil immune attack was not ascribed to antigen mimicry or shedding but rather to undefined biochemical and biophysical properties of the surface membrane structure [4,20].

3.2. Biochemical studies helped understand the basis of the immune evasion mechanism

Data obtained following incubation of *S. mansoni* cercariae and newly developed larvae with merocyanine 540, which binds to membranes with predominantly loosely packed lipids, indicated that surface membrane lipid phase of the parasite changes from gel to liquid-crystalline immediately after skin penetration. The relatively unrestricted lateral diffusion properties of larvae surface membrane lipids were supported by fluorescence recovery after photobleaching results with 5-((octadecanoyl)-amino fluorescein and further suggested that the parasite outer lipid bilayer possesses the ability to reseal small holes [21,22]. More extensive studies used quenching with trypan blue to locate 5-((octadecanoyl)-amino fluorescein and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylinodocarbocyanine perchlorate in the parasite double lipid bilayer, and revealed that the external leaflet of the outer monolayer of schistosomula contains a considerable proportion of immobile lipid molecules. The equivalent leaflet in adult worms is organized into domains of different lipid compositions and structures, gel or liquid-crystalline phase; yet, the inner leaflet and lipid bilayer are entirely immobile. The immobility of the surface membrane lipid molecules was proposed as the basic mechanism of resistance of developing and adult worms to the effect of antibody-mediated attrition mechanisms [23,24].

On another, yet related note, developing schistosomula were shown to incorporate host fatty acids and glycolipids into their surface membranes [4,25–27]. Additionally, recent studies indicated that the tegument of adult *S. mansoni* is enriched in parasite-specific phospholipid species, namely phosphatidylcholine that contains the unusual fatty acid, 5-octadecenoic acid [28].

Several potential cues for increased surface antigenic expression of *S. mansoni* ex vivo lung-stage schistosomula, such as lack of glucose and amino acids and extremes of pH or HCO3 concentration, failed to alter the negligible larval reactivity with control, infection, or irradiated cercaria-vaccine serum in IF tests. In contrast, incubation of larvae overnight at 37 °C/5% CO2 in 90% corn oil for 6 h led to surface membrane changes, which allowed specific and strong antibody binding that was not associated with severe disorganization of the tegument structure, pits and holes. The data together indicated that the lung-stage worms’ concealment of antigenic molecules in the outer membrane could be reversed in vitro after exposure to corn oil, in a concentration- and time-dependent manner [29].

Cholesterol helps to pack phospholipids in plasma membranes, lessens lipids lateral diffusion and gives more rigidity and impermeability to the membranes [30]. In schistosomes, the cholesterol to phospholipid molar ratio in the surface double lipid bilayer reaches 1:1 as in mammalian cell membrane rafts [30–33], thus supporting the hypothesis that states that cholesterol is critical for the rigidity and impermeability of the double lipid bilayer of developing and adult *S. mansoni* [24]. Methyl-β-cyclodextrin (MBCD), a membrane impermeable, hydrophobic oligosaccharide specifically extracts cholesterol from plasma membranes, without affecting sphingolipids or disrupting the integrity of the lipid bilayer [34 and references therein]. The polyene macrolide, filipin [45,65,85,10R,12R,14R,16S,27-octahydoxy-3′-[(1R-hydroxy-hexyl)17,28R-dimethyl-oxacyclooctacosa-17,19E,21E,23E,25E-pentaen-2-one] interacts with unesterified cholesterol in a stereochemically and stoichiometrically defined manner to produce filipin–cholesterol complex, which causes alterations in filipin absorption and fluorescence spectra [35], explaining filipin wide use for the detection, localization, and quantification of cholesterol in biological membranes [36]. These biochemical techniques were used to assess the role of outer membrane cholesterol in denying specific antibody binding to the surface membrane antigens of lung-stage schistosomula [37,38]. Ex vivo *S. mansoni* lung-stage larvae treated with 0 mM MBCD (intact controls) showed intense filipin cholesterol fluorescence over the entire surface, while *S. haematobium* schistosomula treated in parallel showed less intense fluorescence, which, moreover, was limited to the rim of the larva. Additionally, MBCD failed to completely extract the cholesterol from the surface membrane of all treated *S. mansoni* larvae even when MBCD concentration was raised to 10 mM. Conversely, 2.5 mM MBCD led to severe depletion of cholesterol from the surface membrane of a substantial majority of test *S. haematobium* larvae. These data indicate that lung-stage schistosomula of *S. mansoni* and *S. haematobium* are heterogeneous in terms of surface membrane cholesterol content [38]. Of interest, our studies leading to calculation of the cholesterol content per worm and approximate estimation of worm surface area revealed that outer membrane cholesterol content is significantly (P < 0.0001) higher in *S. mansoni* than *S. haematobium* adult worms, and is about twice higher in males than females for both species [39]. These differences in surface membrane cholesterol content between *S. mansoni* and *S. haematobium* and between males and females may play a role in dictating their abode preference and mode of living, respectively.

Following cholesterol complete depletion, as monitored by filipin staining, antibodies specific to schistosome surface membrane antigens readily bound to lung-stage larvae of *S. mansoni* but not *S. haematobium* [38]. In addition, total depletion of cholesterol from the surface membrane of adult *S. mansoni* and *S. haematobium* using 40 mM MBCD led to exposure of 70% of *S. mansoni* and only 50% of *S. haematobium* adult worms to specific antibody binding in the IF test [39]. The results indicated that cholesterol is an essential, but not the sole, factor in preventing access of specific antibodies to the surface membrane antigens of larval and adults *S. mansoni* and *S. haematobium*.

While *S. haematobium* lung-stage schistosomula did not bind specific antibodies in IF following removal of surface membrane cholesterol, the intact larvae incubated in the presence of polyunsaturated fatty acids (PUFA) as in corn or olive oil, or 10–20 μM arachidonic acid (ARA), and fixed in 0.1% formaldehyde or 1% paraformaldehyde were strikingly reactive in IF test. As PUFA, especially ARA, are documented activators of membrane-associated, magnesium-dependent, neutral
sphingomyelinases (nSMase) [40–44], we proposed that the principal target of action of the polyunsaturated oleic acid and ARA is a parasite tegument-associated nSMase, which is able to hydrolyze apical membrane sphingomyelin (SM) to pro-apoptotic ceramide and free phosphocholine (Fig. 2), and surmised that SM is the lipid molecule instrumental in concealing the parasite surface membrane molecules [43,44].

A series of experiments were performed to examine the validity of our hypothesis. First, exposure of S. mansoni and S. haematobium larvae to 50% olive or 80% corn oil or 20 μM ARA led to release of detectable amounts of phosphorus in the medium, likely from the freed phosphocholine. The level of inorganic phosphorus in the supernatants increased with increase of the number of treated larvae and was highest following ARA treatment [44]. Second, MgCl₂, a potent nSMase activator, was essential for exposure of the surface membrane antigens of S. mansoni and S. haematobium lung-stage larvae to specific antibody binding. Third, CaCl₂, a potent inhibitor of nSMase activity at mM concentrations [40,41] prevented 50% olive oil and 20 μM ARA induction of larval IF reactivity with specific antibodies [43].

Fourth, extraction of ex vivo S. mansoni or S. haematobium lung-stage schistosomula in the absence of CO₂ and maintaining the medium at pH 7.4–7.6, the optimal pH for nSMase activation, led to exposure of surface membrane antigens to specific antibody binding as assessed by IF, provided and only when the larvae were pretreated with 0.1% formaldehyde or 1% paraformaldehyde (Fig. 3A–D). Slight pH-mediated nSMase activation did not allow antibody binding, but likely permitted access of methylene hydrate (HCHO + H₂O; HO–CH₂–OH) polymers of 2–8 molecules to interact with and cross-link proteins at the host–parasite interface, disrupt outer double lipid bilayer integrity and, thus, allow antibody binding to the surface membrane antigens [44–46]. Fifth, extraction of ex vivo larvae of S. mansoni and S. haematobium from lung pieces at pH 7.4–7.6 followed by incubation in the presence of the potent nSMase inhibitor, manumycin [47] or glutathione [40,41] prevented reactivity of larvae in the IF test despite prior fixation with 0.1% formaldehyde (Fig. 3E, F). Finally, the importance of SM in securing the impermeability of lung schistosomula was obtained via exposure of larvae to the SM biosynthesis inhibitors myriocin or γ-cycloserine [48, 49]. This treatment allowed ready access of specific antibodies to the surface of treated larvae even without fixation with formaldehyde. The effect of SM biosynthesis inhibition was most drastic for S. haematobium schistosomules, regarding changes in morphology and intensity of reactivity in the IF test (Fig. 3G, H).

Equilibrium in parasite tegument-associated nSMase activity, thus, allows small molecules of <600 Da, like glucose and fatty acids, to interact with their respective transporters in the surface membrane while the access of antibody molecules (>150,000 Da) is denied. Vigorous nSMase activation following worms exposure to PUFA leads to disintegration of the outer membrane SM molecules and eventual parasite attrition [43,44]. The SM molecules likely maintain the rigidity and impermeability of the surface membrane outer lipid bilayer via the ability of hydroxyl and amide groups in the interfacial region of sphingolipids to form with surrounding water molecules a tight net.

3.3. Biophysical studies documented the basis of the immune evasion mechanism

The above described evidences have found a definite confirmation by the experiments performed by biochemical methods. On the other hand, biophysical methods have revealed themselves a very effective tool to investigate the molecular mechanisms related to the infection process. The combination of biochemical techniques with spectroscopic techniques, such as neutron scattering, that are not traditionally used for such studies, is the unconventional approach we adopted to address some fundamental aspects: i) the nature of the interaction between Schistosoma and medium, ii) the strength of the interaction between Schistosoma and medium and iii) the different degrees of resistance shown by larvae and adult worms of S. mansoni and S. haematobium.

In a complementary way in respect to the biochemical techniques, the biophysical approach allows to look at the infection process at a molecular scale, by focusing on the features of the interaction between Schistosoma and medium which is mediated by the parasite apical lipid membrane. Following this philosophy, elastic incoherent (EINS)
spectra, by the temperature dependence of the diffusion coefficient and by the Q dependence of the translational linewidth; and ii) the study of the rigidity of parasites and the determination of the schistosome resistance by the dependence of the EINS intensity and the mean square displacement on temperature and by the evaluation of the pseudo-force constant.

From the theoretical point of view, QENS and EINS allow to point out different kinds of motions depending on the instrumental energy resolution of the used spectrometer: the elastic contribution, i.e. the scattered intensity collected within a fixed energy window determined by the instrumental energy resolution and centered at \( \omega = 0 \), decreases with temperature showing a sharp change when the system relaxation time becomes shorter than the instrumental resolution time. Concurrently, this induces an increase of the quasi-elastic contribution due to neutrons scattered out from the instrumental energy resolution window. QENS, under specific circumstances, allows following molecular motions in an even very complex hydrogenated systems, taking advantage by the very high scattering cross-section of the hydrogen atoms that implies a direct access to relevant information about hydrogen-bonded interactions and dynamical properties. In addition, the different contributions to the motions revealed by QENS can be evaluated particularly in the case of the decoupling approximation. In such a case, it is possible to separate the translational, rotational and vibrational contributions. In our study, since the rotational contribution behaves as a flat background in the explored energy windows, only the translational contribution is present and it is described by a Lorentzian function with a linewidth, which is proportional to the diffusion coefficient and the exchanged wavevector Q.

The Q behavior of the translational linewidth is in agreement with the random jump diffusion model, where the motion of the molecule is supposed to be characterized by jumps between a time \( \tau \), called “residence time” among different sites [54]. The evaluation of the Q \( \leftrightarrow 0 \) limit of the translational linewidth furnishes the values for the diffusion coefficient of \( 1.80 \times 10^{-5} \text{ cm}^2/\text{s} \) and \( 2.62 \times 10^{-5} \text{ cm}^2/\text{s} \) for S. mansoni larvae, S. mansoni adult worms and S. haematobium adult worms, respectively. The obtained values show that larvae diffuse more slowly than adult worms and that there is a remarkable difference in the diffusion of the two species of schistosome, S. mansoni worms being characterized by a lower mobility in respect to S. haematobium worms. This is in line with the evidences that a remarkable proportion of immobile lipid molecules is found in the external leaflet of the outer monolayer of schistosomula and in the inner leaflet and lipid bilayer in adult worms, as described in the previous section, and that the degree of the lateral diffusion decrease induced by the outer double lipid cholesterol to the schistosome membranes is different for developing and adult worms, i.e. higher for S. mansoni lung-stage larvae than S. mansoni adult worms and higher for S. mansoni adult worms than for S. haematobium adult worms [21,22,38,39].

In order to determine the nature of the interaction between schistosomes and medium, the measurements on S. mansoni lung-stage larvae have been performed at two temperature values, i.e. \( T = 300 \text{ K} \) and \( T = 320 \text{ K} \), this increase induces the death of larvae, by providing for the diffusion coefficient of the values \( 1.80 \times 10^{-5} \text{ cm}^2/\text{s} \) at \( T = 300 \text{ K} \) and \( 2.40 \times 10^{-5} \text{ cm}^2/\text{s} \) at \( T = 320 \text{ K} \), respectively. These values allowed evaluating the activation energy value, which is equal to 9.4 kJ/mol and to get the confirmation that the barrier between Schistosoma and surrounding environment is based on a hydrogen-bonded network, which is responsible for the prevention of the free access of host molecules to the schistosomula apical membrane antigens [44].

The QENS measurements have been repeated after 24 h, so inducing hypoxic conditions to both larvae and worms. In such conditions, we obtained the values for the diffusion coefficient of \( 2.28 \times 10^{-5} \text{ cm}^2/\text{s} \) and \( 2.93 \times 10^{-5} \text{ cm}^2/\text{s} \) for S. mansoni larvae and S. mansoni adult worms, respectively. The comparison among the diffusion coefficient values obtained under normal and hypoxic conditions highlights an increase of the mobility of both larval and adult worms, these latter diffusing faster.
than larvae, so confirming that the activation of the parasite tegument-associated neutral nSMase by hypoxia and the consequent excessive SM hydrolysis induces the collapse of the hydrogen bond network [55].

The different degrees of the rigidity of \textit{S. mansoni} lung-stage larvae, \textit{S. mansoni} and \textit{S. haematobium} adult worms, which are related to the different resistance properties of parasites, have been determined by the EINS data analysis.

The EINS intensity and the derived mean square displacement behavior as a function of Q points out a more marked dependence on Q, particularly from a distance of 3.14 Å where the trend deviates from the linear one, for \textit{S. haematobium} adult worms in respect to \textit{S. mansoni} adult worms and for \textit{S. mansoni} adult worms in respect to \textit{S. mansoni} lung-stage larvae. The Q dependence is a measure of the rigidity, these two quantities being related by an inverse proportionality, therefore less rigid – more flexible – systems show a more strongly changing behavior as a function of Q. These properties can provide a molecular explanation to the evidence that the degree of the resistance and impermeability increase induced by cholesterol is higher for \textit{S. mansoni} lung-stage larvae than \textit{S. mansoni} adult worms and higher for \textit{S. mansoni} adult worms than for \textit{S. haematobium} adult worms [21, 22,38,39]. In addition, the EINS evidences can be related to the differences observed in the mechanism of resistance of developing and adult worms to the effect of antibody-mediated attrition mechanisms. Furthermore, the different sensitivities observed for the two schistosome species to nSMase activators, with a higher effect for \textit{S. haematobium} [38,43], as well as the different effects of SM biosynthesis inhibition for \textit{S. haematobium} and \textit{S. mansoni} schistosomula, which are related to the SM role in securing the impermeability of lung schistosomula, can be justified by the observed differences in the schistosome rigidity.

In line with the QENS evidences for \textit{Schistosoma} under hypoxic conditions, i.e. repeating the measurements after 24 h, the rigidity of both \textit{S. mansoni} larvae and adult worms is decreased, with a greater effect for adult parasites. Analogously, the temperature increase has the effect to make \textit{S. mansoni} larvae more flexible as a consequence of the hydrogen-bonded barrier collapse induced by temperature.

The QENS and EINS findings allowed, therefore, to conclude that the interaction between \textit{Schistosoma} and medium is based on a strong hydrogen-bonded network with a higher strength, that implies a higher rigidity and then a higher resistance, for \textit{S. mansoni} larvae than for \textit{S. mansoni} adult worms and for \textit{S. mansoni} adult worms than for \textit{S. haematobium} adult worms.

4. Outcome

4.1. A new schistosomicide

We have, thus, predicted, and provided evidence for the existence of a parasite tegument-associated nSMase in 2005 and 2006 [43,44]. In 2009, the nucleotide and amino acid sequencing of \textit{S. mansoni} nSMase was documented by Berriman et al. [56]. A systematically improved high quality genome and transcriptome of the human blood fluke \textit{S. mansoni} was subsequently reported by Protasio et al. in 2011 (unpublished), whereby sequence for \textit{S. mansoni} putative nSMase was shown to encode 431 (CCD60196.1) and not 631 (XP_002578732.1; obsolete version) amino acids (aa) and 100% identity with the latter sequence for the first 345 aa, and only 2 transmembrane domains were identified near the carboxyl end, between aa 325 and 375. These new findings fully explained the results that we have obtained in western blotting and immunostaining of \textit{S. mansoni} tegument-associated nSMase and cloning and sequencing of partial length \textit{S. haematobium} nSMase [57,58]. The full length of \textit{S. haematobium} nSMase sequence was published in 2012 (KGB40808.1) [59]. We were the first to measure nSMase enzymatic activity in Triton X-100-solubilized surface membrane and whole worm soluble molecules of male and female \textit{S. mansoni} and \textit{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. Like nSMase family members, the schistosome nSMase activity was significantly \((P<0.01)\) decreased following exposure to the nSMase specific inhibitor GW4869, and significantly \((P<0.05\text{--}0.0001)\) enhanced by exposure to PUFA, especially ARA [57]. Thus, death of schistosome larvae and adult worms following in vitro exposure to ARA high concentrations \((>20 \mu M \text{ and } 5 \text{ mM})\), respectively, is essentially due to hydrolysis of the apical membrane SM molecules and disruption of the protective hydrogen bond barrier.

Based upon these findings, it was straightforward to predict that ARA has schistosomicidal potential. Repeat experiments demonstrated that ARA leads to irreversible death of larval and adult \textit{S. mansoni} and \textit{S. haematobium} in vitro, even in the presence of 100% fetal calf serum. Death was accompanied by severe and irreversible disintegration of the outer surface membrane. Most importantly, experiments in outbred and inbred mice and outbred hamsters using pure ARA or ARA incorporated in Nestle milk documented ARA highly significant schistosomicidal activity against \textit{S. mansoni} and \textit{S. haematobium} infection, associated with safety as side effects and toxicity were not observed [60,61].

Capsules of pure ARA were provided by DSM (DSM Nutritional Products, Columbia, MD) and used, alone or in combination with praziquantel (PZQ), for treatment of Egyptian children infected with \textit{S. mansoni}. Of note, PZQ is currently the most effective drug for treatment of human schistosomiasis and has, therefore, been extensively used in mass treatment campaigns in numerous endemic countries. Because of its small molecular weight (312.4 g/mol) and hydrophobic nature (2-cyclohexylcarbonyl-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1-a]quinolin-4-one), PZQ accesses and interacts with the schistosome outer double lipid bilayer membrane, causing its blebbing and vacuolation. The subsequent spastic paralysis could not be explained by excessive influx of calcium ions and was suggested to likely be due to PZQ binding to, and polymerization of, tegument-associated actin [62] and references therein.

Light (<100 eggs per gram stool, epg) and moderate (100–400 epg) infection with \textit{S. mansoni} were detected in children residing in Menoufiya Governorate, located in the Nile Delta, approximately 90 km north of Cairo, Egypt. Arachidonic acid proved as efficacious as PZQ in treatment of Menoufiya schoolchildren with low infection intensity (78% and 85% cure rate, respectively). Combination of ARA and PZQ led to 100% cure rate of schoolchildren with moderate intensity infection. Biochemical, hematological, and immunological parameters were either unchanged or ameliorated following ARA or PZQ therapy [63].

Treatment with ARA or PZQ alone displayed limited efficacy in cure of \textit{S. mansoni}-infected children residing in areas of high endemicity, where schoolchildren were exposed to repeated mass PZQ administration campaigns, as in Motoubis area, Kafr El Sheikh Governorate, located in the Nile Delta, approximately 145 km north of Cairo, Egypt [64]. Cure rates in children with light infection did not exceed 50% and 60% respectively. Cure rates in children with high infection intensity (>400 epg) were alarmingly low, around only 20%, following administration of PZQ or ARA alone. Cure rates improved uniquely when PZQ was combined with ARA, reaching 83% and 78% for children with light and heavy infections, respectively, indicating that reduced worm sensitivity to chemotherapy is not due to infection-induced immunosuppression in children or development of parasite specific resistance to PZQ. It is more probable that continuous PZQ treatment in the endemic areas results in selection of parasites with tightest outer lipid bilayer barrier consequent to higher percentage of cholesterol and SM and/or less active tegument-associated nSMase [62,64]. This speculation is supported by previous findings on the lack of PZQ-induced tegumental disruption in \textit{S. mansoni} isolates obtained from humans resistant to PZQ cure [65]. Accordingly, it is necessary that PZQ intercalation in the outer membrane combines with ARA activation of tegument-associated nSMase, in order
to facilitate worm attrition [62]. Therefore, use of ARA alone was recommended for therapy of travelers to endemic regions and light infections in children residing in areas of low endemicity. For regions of high endemicity, especially where mass PZQ treatment campaigns were applied, combination of PZQ and ARA was indicated [62,64].

Based on nSMase activation is not restricted to schistosomiasis and ARA. Thus, Wu et al. [66] reported that PUFA limit breast cancer cell growth via activation of surface membrane-associated nSMase. Withanolide D, a pure herbal compound isolated from Withania somnifera mediated leukemia cells apoptosis via activation of plasma membrane associated nSMase2 [67,68]. The cytotoxic activity of propanoxadiol on different human cancer cells was ascribed to up regulation and activation of nSMase 2, leading to disappearance of SM from the plasma membrane outer leaflet [69]. In addition, the mechanism of curcumin-mediated apoptosis of human leukemia HL60 cells and their HL60/VCR multidrug-resistant counterparts was identified as fast activation of membrane-associated nSMase, followed by inhibition of sphingomyelin synthase activity [70]. Membrane bound, magnesium-dependent membrane-associated nSMase, followed by inhibition of sphingomyelin metabolism, while Romero-Ramirez et al. [73] designed protective efficacy is attributable to the fact surface membrane molecules of healthy intact larval and adult schistosomes, such as Sm23, glucose transporters, and tetraspanins, unfailingly expressed on surface membranes, Parasitol. Today 2 (1986) 318–319. Recently, the advocates of tetraspans and other surface membrane antigens as potential vaccine candidates had to admit that the protective efficacy is attributable to the surface membrane molecules are secreted in exosome-like extracellular vesicles of 30 to 150 nm in size within the excretory–secretory products (ESP) of cultured worms [78]. Extracellular vesicles were also found to be excreted–secreted by the liver fluke, Fasciola hepatica [79].

Since surface membrane and cytotoxic antigens are not accessed by the immune effectors, potential schistosomiasis vaccine candidates are to be looked for uniquely among larval and developing worms ESP, such as fatty acid binding protein (Sm14), calpain and other proteases, glutathione-S-transferase (GST), and glycolytic enzymes, namely aldolase, enolase and glyceraldehyde-3-phosphate dehydrogenase (SG3PDH) [80–82]. In support, vaccine candidates that are now considered for pre-clinical Phase I and II clinical trials are the ESP, GST [83], Sm14 [84], and calpain [85]. Of note, the ESP SG3PDH was readily detected on the surface membrane of S. mansoni healthy in vitro cultured (5 day-old) and ex vivo lung-stage (6 day-old) schistosomula provided prior activation of worm tegument-associated nSMase and fixation with formaldehyde [86]. Recently, SG3PDH in conjunction with the type 2 immunity-inducing ESP, S. mansoni cysteine peptidases cathepsin B1 and cathepsin L were shown to reproducibly elicit highly significant (P < 0.0001) reduction of approximately 70% of challenge S. mansoni and S. haematobium worm burden and worm egg counts in outbred mice and hamsters [87].

References

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