Schistosoma mansoni glyceraldehyde 3-phosphate dehydrogenase is a lung-stage schistosomula surface membrane antigen

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Abstract. We have previously reported that *Schistosoma mansoni* larvae emerging from host lung at pH 7.5–7.8 and then fixed with diluted formaldehyde (HCHO) readily bind radiation-attenuated cercariae (RA) vaccine serum antibodies, as assessed by indirect membrane immunofluorescence (IF). Here we show that *S. mansoni* schistosomula emerging from lung pieces under 5% CO₂ (pH ≤7.3) readily bind RA vaccine serum antibodies, provided they have been incubated for 12 h at pH 7.5–7.8 in foetal calf serum-free RPMI medium, and fixed with diluted HCHO. *Ex vivo* larvae exposed during incubation to GW4869, a specific inhibitor of tegument-bound, neutral sphingomyelinase (nSMase) displayed significantly diminished binding of RA vaccine serum antibodies, thus suggesting that nSMase activity at pH ≥7.5 leads to exposure of lung-stage larvae surface membrane antigens to specific antibody detection. More importantly, *ex vivo* larvae readily bound antibodies directed to dipeptidic multiple antigen peptide constructs, based on *S. mansoni*-specific sequences in *S. mansoni* glyceraldehyde 3-phosphate dehydrogenase (SG3PDH). Lung-stage schistosomula IF reactivity was diminished following antiserum absorption with recombinant SG3PDH. The data together indicate that intact *ex vivo*, as well as, 5-day-old *in vitro*-grown larvae express SG3PDH on their surface membrane. The findings are discussed in relation to the importance of surface membrane proteins as candidate vaccine antigens.

Schistosomiasis is a parasitic disease caused by the platyhelminth worms *Schistosoma mansoni*, *Schistosoma haematobium* and *Schistosoma japonicum*, affecting 207 million people in the developing world, with 779 million, mostly children, at risk of the infection (Steinmann et al. 2006). Schistosomes develop and live in the blood vessels of their vertebrate hosts. The developing and adult schistosomes are covered by the tegument, a 2- to 4-µm thick syncytium. The innermost membrane of the tegument is a conventional basal membrane, whereas the outer membrane on the syncytium surface consists of two, tightly apposed bilayers, each of which is composed of outer and inner leaflets (Hockley 1973, Hockley and McLaren 1973). The disease can be eradicated via elimination of transmission if an effective vaccine is available. Many roadblocks hinder the development of such vaccine, among which our inability to identify an appropriate vaccine antigen and the major mechanisms of immune resistance to the infection. There is consensus, however, about the lung-stage schistosomula being the main targets of natural and RA-mediated protective immunity (Coulson 1997). 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 (Dean 1977, Dessein et al. 1981, McLaren and Terry 1982, Foley et al. 1986, Pearce et al. 1986, Chiang and Caulfield 1989, Kusel and Gordon 1989, Coulson 1997, El Ridi et al. 2003, 2004b, Tallima and El Ridi 2005, Tallima et al. 2005, El Ridi and Tallima 2006, Kusel et al. 2007). Entire failure of 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 (Braschi et al. 2006), and was previously ascribed to shedding of antigenic molecules (Pearce et al. 1986), masking by host proteins (McLaren and Terry 1982, Chiang and Caulfield 1989), or intrinsic biochemical modifications of the outer membrane (Dean 1977, Dessein et al. 1981, Foley et al. 1986, Kusel and Gordon 1989, Kusel et al. 2007). Our recent findings have supported the latter hypothesis, whereby extraction of outer membrane cholesterol by the impermeable cholesterol-binding drug, methyl-β-cyclodextrin readily induced exposure of surface membrane antigens in lung-stage larvae (El Ridi et al. 2004b, Tallima and El Ridi 2005). Surface membrane antigen exposure could be even more readily elicited via manipulating the balance between sphingomyelin (SM) biosynthesis and breakdown towards SM hydrolysis, suggesting that SM is responsible for preventing antibody access to surface membrane antigens (Tallima et al. 2005, El Ridi and Tallima 2006). Accordingly, the way was paved to identify the surface membrane antigens of the lung-stage larvae. In this report we show that the vaccine candidate, *Schistosoma mansoni* glyceraldehyde 3-phosphate dehydrogenase, SG3PDH (El Ridi et al. 2001a, b, 2004a, Tallima et al. 2003, Vepřek et al. 2004 and references therein) is a lung-stage schistosomula surface membrane antigen.

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MATERIALS AND METHODS

Animals and parasites. Cercariae and animal hosts were obtained from the Schistosome Biological Materials Supply Program, Theodore Bilharz Research Institute, Giza, Egypt. Lung-stage schistosomula were recovered from male Syrian hamsters 6 days after percutaneous infection with 2,000 cercariae of *S. mansoni*. Infection and perfusion were performed under anaesthesia, as described (El Ridi and Tallima 2006) following the recommendations of the current edition of the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, USA. Lung pieces were incubated in RPMI medium-heparin supplemented with 10% foetal calf serum (FCS, BioWhittaker, Verviers, Belgium) for 4 h at 37°C in a humidified atmosphere containing 5.0% CO₂ (pH ≤ 7.3), to allow the schistosomula to emerge (El Ridi et al. 2003, 2004b, Tallima and El Ridi 2005, Tallima et al. 2005, El Ridi and Tallima 2006). The larval suspension was then poured through a Nitex 132-mesh nylon screen, treated for 10 min at 4°C with sterile 0.16 M ammonium chloride/0.017 M Tris buffer, pH 8.0, to lyse the erythrocytes, isolated from contaminant lung cells by centrifugation over 40% percoll (Pharmacia, Uppsala, Sweden) in RPMI medium, as described (El Ridi et al. 2003, 2004b, Tallima and El Ridi 2005), and then incubated for 12 h, at 37°C and pH 7.5–7.8, in RPMI medium containing 20%, 2%, or 0% FCS. In some experiments, the FCS-free RPMI medium was supplemented with 0, 7.5, 10, or 20.0 µM of the nSMase specific inhibitor GW4869 (Sigma, St Louis, MO, USA) dissolved in dimethyl sulfoxide, at a concentration of 1.0 mg/1.0 ml (Luberto et al. 2002, Marchesini et al. 2003, Kolmakova et al. 2004, Wu et al. 2005). At the end of the incubation period, larvae consistently showed high viability, motility, and body contractility.

Cercariae-derived schistosomula were prepared as described by Lazdins et al. (1982), and cultured at 37°C under 5% CO₂ for 4–6 days in 10–90% FCS in RPMI medium, or medium 169 (Harrop and Wilson 1993) supplemented with 300 U/ml penicillin, 300 µg/ml streptomycin, and 160 µg/ml gentamicin (all from BioWhittaker). The viability of schistosomula was assessed by determining the percentages which excluded Trypan blue, and appeared healthy, transparent, motile, and contractile. Approximately 90% of larvae cultured in 50% FCS-50% RPMI medium were viable as assessed by the Trypan blue dye exclusion test, and actively motile and contractile. Accordingly, larvae were cultured in 50% FCS for 4 days, and then in FCS-free RPMI medium at 37°C, under atmospheric air, for 24 h. At the end of the incubation period, more than 80% of the 5-day *in vitro* schistosomula were motile and excluded Trypan blue; approximately 30–50% were elongated, while the rest were generally 20 to 40% smaller than those developed in hamsters, in agreement with Basch (1981).

Antigens and antisera. Serum was obtained from naïve BALB/c mice (control serum), or BALB/c mice immunized by the tail exposure method, twice at 4-week interval, with ultra-violet (UV) radiation-attenuated cercariae of *S. mansoni* (RA vaccine serum). Cercariae used for immunisation were attenuated by exposure to 330 µW/cm² UV light from an S-68 Mineralight Lamp (Ultra-Violet Products, San Gabriel, CA, USA)

for 3 min, as described previously (Tallima et al. 2005, El Ridi and Tallima 2006).

The coding sequence for SG3PDH was obtained and expressed, and the recombinant protein (rSG3PDH) was purified by metal affinity chromatography (HiTrapTM Affinity Column, Pharmacia) as detailed previously (El Ridi et al. 2001b). Antigen aliquots were kept until use at –76°C and thawed only once. The amino acid (aa) sequence of SG3PDH-derived peptides, selected based on lowest homology to the human enzyme (El Ridi et al. 2001a, 2004a, Tallima et al. 2003) is as follows: peptide A, LKNTVDVSV-OH (aa 24–33); B1, KVNGKLISVHCERDP-OH (aa 67–81); B, ERDPANIPWDKD-OH (aa 78–89); C, ENSYEKSMVSV-OH (aa 138–148); D, GKGASYEEIKAAVK AAAS-OH (aa 251–268); and E, ITHMHKVDHA-OH (aa 329–338). Tetrabranch, dipeptidic multiple antigen peptide constructs (D-MAP) A-B, B1-C, and D-E, synthesized at Research Genetics (Huntsville, AL, USA), were used to generate in BALB/c mice potent antisera against the peptide immunogens and rSG3PDH, as described (El Ridi et al. 2004a).

Peptide P5, based on amino acids 178–192 (MTLTYTLNPTLWPI) of an *S. mansoni* glucose transporter protein 4 (SGTP4) extrafacial domain (Skelly et al. 1998) was synthesized at Harvard Biopolymers Laboratories, Harvard Medical School (Cambridge, MA, USA), and used to prepare an antiserum in BALB/c mice, following procedures previously described (El Ridi et al. 2001a, 2004a, Tallima et al. 2003, Vepřek et al. 2004).

Indirect membrane immunofluorescence (IF). Larvae were fixed for a total of 10 min in 50 volumes 0.1% formaldehyde (HCHO) or 1.0% paraformaldehyde in FCS-free RPMI medium. Larvae were washed twice in RPMI medium supplemented with 5% FCS (RPMI medium-5% FCS), and incubated in 100 µl RPMI medium-5% FCS containing 1.0 µl control or test serum, overnight at room temperature. In some experiments control and test sera were previously absorbed with 25 µg/µl bovine serum albumin (BSA) or rSG3PDH, overnight at room temperature. Larvae were then washed four times in RPMI medium-5% FCS, incubated with 1:100-diluted anti-mouse IgG (Fab-specific) fluorescein isothiocyanate (FITC)-labeled goat immunoglobulins F(ab')₂ fragment of affinity isolated antibody, adsorbed with bovine, horse, and human serum proteins (Sigma), washed four times in RPMI medium, and inspected by alternate light and UV microscopy (Olympus Inverted Microscope Model IX70, Olympus, Tokyo, Japan).

RESULTS

Membrane immunofluorescence reactivity of 5-day-old *in vitro* cultured schistosomula

Mechanically transformed schistosomula grown *in vitro* for 5 days in the presence of 50% FCS and 5% CO₂ and then fixed with 0.1% HCHO do not bind antibodies from control or RA vaccine serum (data not shown). Yet, when 4-day-old larvae were cultured for a further 24 h period in RPMI medium-50% FCS but in the absence of CO₂, with medium pH reaching 7.6 ± 0.1, strong IF reactivity with RA vaccine, but not con-

Fig. 1. Membrane immunofluorescence reactivity of 5-day-old *in vitro* grown schistosomula. Larvae were incubated for the last 24 h of culture at pH 7.5–7.6 and then tested by IF with control (A, IF intensity 0+), RA vaccine (B, IF intensity 1/2+, 2+ and 3+), anti-SGTP4 peptide 5 (C, D), anti-D MAP B1-C (E), or anti-D MAP D-E (F) serum, and examined under light (C) and UV (A, B, D–F) microscopy. Scale bar (A–F) = 100 μ m.

Table 1. Membrane immunofluorescence reactivity of 5-day-old *in vitro* cultured schistosomula.

Serum	Total number of larvae	Percent of larvae showing IF intensity of*				
		0+	1/2+	1+	2+	3+
Control	105	97.1	2.8	0.0	0.0	0.0
RA vaccine	112	0.0	12.5	13.3	13.2	60.7
Anti-Peptide 5	136	35.2	19.8	15.4	23.5	5.8
Anti-D MAP B1-C	138	14.5	0.0	38.4	34.7	12.3
Anti-D MAP D-E	128	9.3	25.0	35.9	25.0	4.6

*Representative of three independent experiments; IF intensity as depicted in Fig. 1.

control, serum was evident. Larvae also bound antibodies of anti-SGTP4 peptide P5 (anti-Peptide 5), anti-D MAP B1-C, and anti-D MAP D-E sera, with IF intensity of 0+ to 3+ (Fig. 1, Table 1). Larval IF reactivity diminished following absorption of anti-D MAP B1-C or D-E sera with rSG3PDH. It is of note that larvae did not bind antibodies of five individual BALB/c mouse anti-D MAP A-B sera (data not shown).

Membrane immunofluorescence reactivity of *ex vivo* lung-stage schistosomula

Ex vivo lung-stage larvae emerging from lung pieces under 5% CO₂ did not bind antibodies from control or test sera whether fixed with 1% paraformaldehyde or 0.1% HCHO. When larvae isolated from lung cells were then maintained for 12 h at pH 7.5–7.8 in the presence of 20% or 2% FCS, only about 36% and 44%, respectively of *ex vivo* schistosomula showed 1/2+ to 1+ IF reactivity with RA vaccine serum, while they were entirely negative with control, anti-SGTP4 peptide P5, or any anti-D MAP sera in three independent IF experiments. However, when *ex vivo* larvae were incubated for 12 h at pH 7.5–7.8 in FCS-free RPMI medium, approximately 80–100% thereof were IF positive when tested with RA vaccine, anti-SGTP4 peptide P5, anti-D MAP B1-C, or anti-D MAP D-E sera (Figs. 2, 3). Schistosomular IF intensity was greatly diminished following absorption of anti-D MAP B1-C or anti-D MAP D-E sera with rSG3PDH (Fig. 2F), but not BSA. No reactivity was evident in repeated IF tests with five individual

Fig. 2. Membrane immunofluorescence reactivity of *ex vivo* lung-stage schistosomula. *Ex vivo* larvae incubated for 12 h at pH 7.5–7.8 in FCS-free medium were tested by IF with control (A), RA vaccine (B), anti-SGTP4 peptide 5 (C), anti-D MAP B1-C (D), anti-D MAP D-E (E), or rSG3PDH-absorbed anti-D MAP B1-C (F) serum, and examined under UV microscopy. Scale bar (A–F) = 150 μ m.

BALB/c mouse anti-D MAP A-B sera (data not shown), despite the strong antisera recognition of the peptide immunogens and rSG3PDH (El Ridi et al. 2004a).

It is of note that when *ex vivo* larvae were incubated for 12 h at pH 7.5–7.8 in FCS-free RPMI medium supplemented with 7.5, 10, or 20 μ M GW4869, a specific inhibitor of tegument-bound nSMase, IF reactivity to RA vaccine, anti-D MAP B1-C, and anti-D MAP D-E sera significantly ($P < 0.0001$, Chi-square test) diminished regarding percent of positive larvae and IF intensity (Fig. 3).

DISCUSSION

Our previous studies using lung-stage schistosomula (Tallima et al. 2005, El Ridi and Tallima 2006) and adult worms (Tallima et al. 2007) of *Schistosoma man-*

soni and *S. haematobium* pointed to sphingomyelin (SM) as the major player in preventing antibody access to the larval surface membrane antigens, likely owing to the tight barrier of hydrogen bonds SM readily forms with water molecules (El Ridi and Tallima 2006). This suggestion is supported by several pieces of evidence obtained in the present study. (i) Lung-stage larvae emerging from lung pieces in the presence of 10% FCS and 5% CO₂ (pH 7.2–7.3) certainly have the balance of SM metabolism tilted towards biosynthesis rather than breakdown, since nSMase activity requires a pH ≥ 7.5 (El Ridi and Tallima 2006 and references therein); such larvae do not bind even RA vaccine serum antibodies in IF. (ii) Larvae emerging in the presence of 10% FCS and 5% CO₂ (pH 7.2–7.3), and then incubated at pH 7.5–7.8 in FCS-supplemented RPMI medium, likely

Fig. 3. Effect of nSMase activity inhibitor on membrane immunofluorescence reactivity of *ex vivo* lung-stage schistosomula. *Ex vivo* larvae were incubated for 12 h at pH 7.5–7.8 in FCS-free medium supplemented with 0, 7.5, 10 or 20 μM GW4869 and then tested by IF with control, RA vaccine, or anti-D MAP B1-C serum. Each column represents mean percent of larvae showing IF intensity of 0+ (□), 1+ (▤), 2+ (▥) and 3+ (■), in two independent experiments.

display equilibrium between SM biosynthesis and breakdown. Only a small proportion of such larvae showed binding of RA vaccine serum antibodies, while the glucose transporter, proven to be present on the parasite outer membrane (Skelly et al. 1998), was not detected by IF using antiserum to an SGTP4 extrafacial domain. (iii) *Ex vivo* larvae incubated for 12 h at pH 7.5–7.8 without FCS certainly display higher level of SM hydrolysis than biosynthesis, and as a result, the great majority of larvae were shown to bind RA vaccine antibodies and to readily express SGTP4. (iv) *Ex vivo* larvae incubated for 12 h at pH 7.5–7.8 without FCS but in the presence of the specific nSMase inhibitor GW4869 (Luberto et al. 2002, Marchesini et al. 2003, Kolmakova et al. 2004, Wu et al. 2005), likely failed to activate nSMase and hydrolyze SM. The majority of these larvae failed to bind either control or RA vaccine serum antibodies (Fig. 3).

Tilting SM metabolism of lung-stage larvae towards SM breakdown via manipulation of pH and concentration of lipid nutrients allowed us to document the presence of SG3PDH in the surface membrane of *in vitro* grown, as well as, *ex vivo* lung-stage larvae. *In vitro* cultured and *ex vivo* lung-stage schistosomula did not differ greatly in respect to IF reactivity or intensity with anti-SG3PDH sera, suggesting that SG3PDH expression was not sensitive to the impossibility of reproducing the host environmental factors *in vitro* (Chai et al. 2006). We have used antibodies to peptides based on the amino acid sequence of SG3PDH, and not to the recombinant molecule, to ascertain the specificity of the antigen-antibody reaction and avoid possible artifacts resulting

from the binding of antibodies directed to traces of bacterial proteins. The molecule appeared to be present on the surface of the majority of *ex vivo* lung-stage larvae examined and not only on a small proportion as previously reported (El Ridi et al. 2003).

In contrast to D MAP B1-C or D-E, antibodies to peptide A and B in D-MAP A–B (El Ridi et al. 2004a) failed to bind to the surface membrane of *in vitro* cultured or *ex vivo* lung-stage larvae, indicating that SG3PDH epitopes might be differentially expressed on the parasite surface. Additionally, epitopes expressed on the larval outer membrane did not appear to be all available on the surface of the native recombinant molecule since absorption with rSG3PDH diminished, but failed to abolish, binding of anti-D MAP B1-C and D MAP D-E antibodies to the worm surface.

It is of note that glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected on the surface of 3 h *in vitro* schistosomula (Goudot-Crozel et al. 1989), and among adult worms surface membrane-associated molecules, such as amino acids, glucose, or copper ion transporter proteins, alkaline phosphatase, and tetraspanins (Braschi et al. 2006, Braschi and Wilson 2006). Braschi and Wilson (2006) diagram places SG3PDH just below the surface membrane inner bilayer of adult *S. mansoni* worms. The findings of our study do not support such a location in lung-stage larvae, since SG3PDH was detected in the surface membrane of intact *in vitro* cultured and *ex vivo* schistosomula, which did not suffer at any time the slightest exposure to solvents, detergents, chaotropic agents, high or low osmotic pressure or temperature, shearing, or vortexing.

Goudot-Crozel et al. (1989) have reported that the predicted amino acid sequence of *S. mansoni* GAPDH does not contain a typical transmembrane domain or a phosphatidylinositol site, yet shows two clusters of hydrophobic residues near the amino terminus that may represent a signal peptide for the translocation of the protein through membranes. Additionally, several reports have described GAPDH to be also surface membrane-bound in bacteria (Alvarez et al. 2003, Seifert et al. 2003) and numerous eukaryotic cell types (Wernstedt et al. 1975, Daum et al. 1988, Xu and Becker 1998, Campanella et al. 2005), likely interacting with other proteins, such as ion channels protein subunits (Dhar-Chowdhury et al. 2005), glucose transporter proteins (Heard et al. 1998), or actin (Poglazov and Livanova 1986). It is important to clarify the significance of SG3PDH occurrence on the surface of 3 h (Goudot-Crozel et al. 1989), lung-stage, and adult (Braschi et al. 2006, Braschi and Wilson 2006) *S. mansoni* worms, as several reports documented that apart from its glycolytic function, GAPDH has multiple non-glycolytic roles, and is fully capable of functioning in a membrane-bound state (Wernstedt et al. 1975, Daum et al. 1988, Muronetz et al. 1996, Heard et al. 1998, Xu and Becker 1998, Alvarez et al. 2003, Seifert et al. 2003, Campanella et al. 2005, Dhar-Chowdhury et al. 2005).

Advocating the surface membrane location of SG3PDH or any other molecule such as SGTP4 (Skelly et

al. 1998), or the tetraspanins (Loukas et al. 2007) should not be construed to indicate they are particularly important as vaccine antigens, owing to the fact these antigens are concealed, hidden, entirely inaccessible to specific effector antibodies in intact parasites *in vivo*. Notably, proteins “exposed” at the adult schistosome surface and revealed by biotinylation by Braschi and Wilson were only “exposed” to molecules of 435–560 Da (Braschi and Wilson 2006). In our study, putative tilting of SM metabolism balance towards breakdown does not render the peripheral membrane molecules available to antibody binding, it only permits access of small OH-CH₂-OH (HCHO) polymers, which then cross-link surface membrane proteins, allowing for their visualisation by IF (El Ridi and Tallima 2006). The surface lipid bilayer would be permeable to antibody ($\geq 150,000$ Da) binding and effector activity only in developing and adult parasites suffering excessive loss of tegument integrity. Such worms are certainly poised to die without the contribution or help of antibody-dependent killing. Nevertheless, it is vital to identify the worm surface membrane antigens, as elucidation of the molecular organisation at the parasite-host interface will help in developing effective drugs and vaccine strategies.

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