Schistosoma Mansoni: the Identification of Some Highly Immunogenic Surface Antigens of the Lung Stage Larvae as Promising Vaccine Candidates Against Schistosomiasis

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ABSTRACT

Background: *Schistosomiasis is a global health problem caused by several species of schistosome blood flukes. It is endemic in 74 developing countries; 200 million people are infected worldwide, causing an estimated 200.000 deaths / year. Chemotherapy, although effective, it does not prevent re-infection, and in addition, partial drug resistance may occur. This is why the development of long - lasting immunity through vaccination may be the real solution to control the spread of the disease.* ***Objective:*** *The molecules on the surface or associated with the tegument of the lung stage (**7-days schistosomules**) of **Schistosoma mansoni** (**S. mansoni**) are the major target in the present study as potential vaccine candidates.* ***Materials and methods:*** *Nonidet P-40 (NP-40) extracted soluble surface proteins of 7-days schistosomules were subjected to 12.5% Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE). The separated surface proteins were electrotransfered onto a nitrocellulose membrane (PVDF), then, western blotting was performed. The non specific binding sites on the membrane were blocked by 1% non-fat dry milk in phosphate buffered saline (PSB), then, the membrane was incubated with pooled sera (primary antibody) collected from **S. mansoni** chronically infected patients and absorped to **E. coli** lysate. Anti-rabbit IgG conjugated with alkaline phosphatase was used as the secondary antibody.* ***Results****: A number of different immunogenic extracted surface antigens of 7-days schistosomules have been identified by the antibodies in the sera of **S. mansoni** chronically infected patients of different molecular weights, 18 kDa, 28 kDa, 33 kDa, 38 kDa, 40 kDa, 42 kDa, 45 kDa, 50 kDa, 62 kDa, 66 kDa, 81 kDa and 116 kDa.* ***In conclusion****: Progress in schistosome genome research has offered a unique chance to move rapidly from genome sequences to vaccine development. Proteins bound to the surface of parasites are potential vaccine candidates, or they can be used for diagnosis. In the present study, twelve surface proteins of the lung stage larva of **S. mansoni** with different degrees of immunogenicity are identified; any one of these proteins could be a promising vaccine candidate for **S. mansoni**.*

INTRODUCTION

The persistence of adult schistosomes in the bloodstream for decades means they must deploy unique and effective immune evasion strategies at their interface with the host[1]. This species is transmitted by Biomphalaria snails found in Africa, the Arabian Peninsula and South America[2].

Treatment of the disease is based on the anti-helminthic drug, praziquantel, but chemotherapy cannot prevent re-infection, and there is evidence supporting the development of drug-resistant parasites[3], limiting the success of strategies based on chemotherapy alone and emphasizing the need for a complementary approach. Vaccination combined with drug treatment is considered to be the most cost-effective strategy against schistosomiasis[4]. Identification of effective vaccine antigens and drug targets has been hindered by the complexity of the parasite life cycle and an incomplete comprehension of its immune evasion strategies [5, 6].

The first attempts to develop a schistosome vaccine began half a century ago. By analogy with successful microbial and viral vaccines, they involved the vaccination of mice with crude worm extracts or purified components, followed by a cercarial challenge[7]. The results were equivocal with 20, 30, and even 50% reduction in worm burden recorded, but there was a lack of consistency, even in the same laboratory. It seemed apparent that crude extracts were inadequate vaccines. Perhaps there were a few key antigens that needed to be identified, but this begs the question about the mechanisms that a vaccine was intended to elicit[6].

The lung schistosomulum seems to be the main target of immune mechanisms, in response to the radiation-attenuated vaccine in the mouse model[8].

It is important to keep in mind that proteins which are exclusive to the adult orm may not be necessary to induce immunity in the mouse model, since radiation attenuated cercariae do not develop into the adult stage. In addition, it also seems that antigens specific to the cercaria may not be important, since attenuated 7-day schistosomula administered intradermally, are capable of inducing high levels of protection[9].

Therefore, genes that are differentially expressed in the transition from cercaria to schistosomulum intra-host lung stage may be relevant for the parasite's survival in the host and therefore may be potential vaccine candidates[10].

Excretory-secretory (E-S) products released from intact, viable, elongated, and contractile schistosomula are ideal potential vaccines, as such molecules can readily play a central role in the induction of local primary and memory immune response effectors (specific antibodies and leukocytes-derived inflammatory cytokines) that would directly target, surround, and pursue the larvae while negotiating the lung capillaries[11].

EST database was interrogated for the occurrence of transcripts in lung schistosomula encoding the proteins of interest released from adult worms by the enzyme treatments as Sm29, Sm200, Sm25 and Calpain. Nearly all transcripts were represented in both larvae and adults in roughly similar numbers. Based on the transcript evidence, it was concluded tentatively that the most exposed tegument surface proteins of adults are also likely to be present on the surface of the migrating lung schistosomulum[1].

MATERIALS & METHODS

This study was carried out in the Medical Biochemistry Department, Faculty of Medicine, Al Minia University.

The preparation of NP-40 soluble surface proteins extract of 7-days schistosomules[12]. Schistosomules obtained from Theodore Bilharz Research institute were mixed (not homogenized to extract surface antigens only) with NP-40 buffer containing 20 mM Tris, pH 7.4, 0.1% NP-40 and 2 mM phenyl methyl sulfonyl fluoride (PMSF), then, vortexed every 5 min for 1 hr. The pellet was spun down and the supernatant was collected and stored at -20ºC.

Sera were obtained from chronically infected patients with ***S. mansoni*** subjected to endoscopic sclerotherapy for treatment of esophageal varices from Tropical Medicine Outpatient Clinic, Zagazig University Hospitals.

SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE)[13].The resolving gel (12.5%) and stacking gel (5%), the gel was left 30 min for polymerization, 10 µl of each one of the sera was mixed with an equal volume of 2 X SDS(sodium dodecyl sulfate) gel loading buffer {1.25 ml 1 M Tris, pH 6.8, 4.0 ml 10% SDS,2.0 ml glycerol,20 mg bromophenol blue, 1.0 ml β–mercaptoethanol and deionized distilled water (dd H_2O) up to10 ml}, the samples were boiled for 5 min , then, loaded onto the gel. Electrophoresis was carried with constant current of 20–25 mAmp until the dye arrived at the gel front. The gel was stained in 0.2% Commassie blue with rocking for 1-2 hrs, then, destained till acceptable back ground was obtained and left to dry.

Absorption of anti- *E. coli* antibodies to *E. coli* lysate[14].A single colony of ***E. coli*** strain***Y1090*** was grown in 100 ml LB medium (10 g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl and distilled H_2O up to 1L, pH 7.0) to saturation. The cells were harvested by centrifugation at 8000 rpm for 10 min at 4ºC, then, suspended in 3 ml of Tris-Cl, pH 8.0, 10 mM EDTA, pH 8.0. The suspension was frozen and thawed several times, then, sonicated at full power for six periods of 20 sec each at 0ºC. The extract of the cells was centrifuged at 11000 rpm for 10 min at 4ºC, the supernatant was transferred to another tube and the lysate was stored at -20ºC. To use the lysate, antiserum was diluted 1:10 in TNT buffer (10 mM Tris- HCl, pH 8.0, 0.15 M NaCl/L and 0.05% Tween-20) containing 1% gelatin, 3% bovine serum albumin, then, 0.5 lysate was added to each 1 ml of the antiserum. The mixture was incubated at 15ºC over night, then, kept stored at 4ºC with 0.05% sodium azide till the time of use.

Western blot analysis[15].After electrophoresis, the gel was placed in a sandwich form with a piece of PVDF membrane in a western transfer buffer (3.02 g Tris-base, 18.8 g glycine, 200 ml ethanol and dd H_2O up to a liter); the transfer was carried out at 75-100 volts for 1 hr. The membrane was blocked in 5% non fat dry milk in PBS, washed 4 times in PBST (0.05% Tween-20 in PBS), then, incubated with the pooled sera collected from chronically infected

patients with ***S. mansoni*** after absorption of anti- ***E. coli*** antibodies to ***E. coli*** **lysate** (the primary antibody) for 3 hrs at room temperature (RT), then washed 4 times in PBST. The secondary antibody (Anti-rabbit IgG conjugated with alkaline phosphatase) was incubated with the membrane for 1 hr at RT, then, washed 4 times in PBS. The substrate color reagent (Vector Laboratories) was dissolved in 0.1 M Tris- HCl, pH 9.5 and incubated with the membrane until good signals were developed. The membrane was washed with H_2O to stop the reaction and left to dry in air.

RESULTS

Identification of some immunogenic extracted surface proteins of 7-days schistosomules using sera obtained from ***S. mansoni*** chronically infected patients is based on the antibodies raised against surface proteins of the lung stage larvae of ***S. mansoni*** in the obtained sera which were used to identify some antigenic epitopes of these surface proteins by SDS–PAGE and western blotting techniques. The NP–40 detergent was used to extract surface proteins of 7-days schistosomules which were separated by 12.5% SDS–PAGE and stained with 0.2% Commassie blue (Fig .1**)** , then, subjected to electrotransfer onto PVDF membrane which was probed with sera of chronically infected patients with ***S. mansoni*** after absorption of anti- ***E. coli*** antibodies to ***E. coli*** **lysate** to avoid the false positive signals that may be obtained in the western blotting, also, the non-specific binding sites were blocked by blotto (5% non fat dry milk in PBS). Several antigens with different molecular weights (18 kDa, 28 kDa, 33 kDa, 38 kDa, 40 kDa, 42 kDa, 45 kDa, 50 kDa, 62 kDa, 66 kDa, 81 kDa and 116 kDa) have been identified by the sera's specific antibodies; the identified antigens show different degrees of immunogenicity (Figure 2).

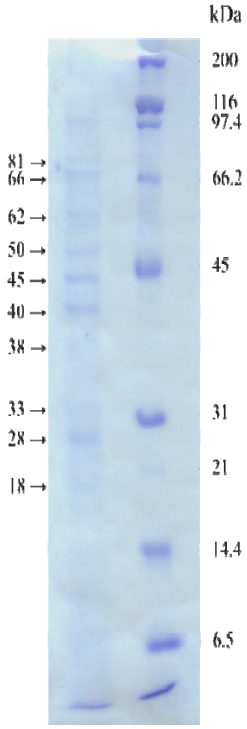

Fig.1 A commassie stained 12.5% SDS-PAGE after electrotransfer of the NP-40 extracted surface proteins of 7-days schistosomules onto PVDF membrane. The molecular weight marker is indicated on the right side of the gel, the arrows point at traces of some of the separated proteins that remain after the transfer.

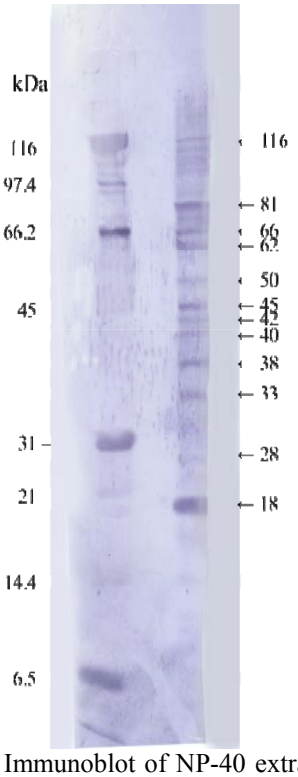

Fig.2 Immunoblot of NP-40 extracted surface proteins of 7-days schistosomules probed with sera of chronically infected patients with ***S. mansoni*** after being absorped to ***E. coli* lysate**. The molecular weight marker is indicated on the left side of PVDF membrane and the arrows point at the identified proteins with different molecular weights and with different degrees of immunogenicity.

DISCUSSION

In the past 10 years, resistance to the anti-schistosome 'drug of choice' (Praziquantel) has been observed[16] and just one new potential drug has emerged[17]. Also, there is a desperate need for new approaches to control this parasite, including vaccines[18].

It is widely hoped that a vaccine may be developed with the potential to reduce morbidity and mortality, but even if vaccine development succeeds, it is not seen as a likely panacea[19]. Schistosome research has revealed that this is a complex parasite with capacity for immune evasion[20] and modulation[21], which makes it harder to develop an anti-schistosome vaccine.

Since the last decade, schistosome membrane-bound antigens have been frequent targets of vaccine studies. Sm16[22] and Sm13[23] are examples of antigens that contain a signal peptide, one of the essential features of membrane-bound antigens.

The immunological properties of these surface antigens have also been investigated. [24] characterized the immunoreactivity antibody titers varied widely in infected patients**.** [23] found that sera from schistosomiasis patients specifically recognized Sm13.

Potential vaccine candidates should include proteins that are preferentially surface-exposed or exported and are expressed in intramammalian stages[25]. Some recent studies using advanced molecular techniques have allowed the isolation of surface-exposed proteins [26, 27], but their protective properties remain unknown.

People become infected when the free-swimming larva, the cercaria, enters through the skin and becomes the schistosomulum. Schistosomula are susceptible to immune responses during their first few days in the host before they become adult parasites[18].

The development of newly transformed ***S. mansoni*** schistosomula over the first 5–7 days as they enter the vasculature and progress to the lungs represents what many believe to be a critical window of opportunity for vaccine-mediated protection [16, 28]. At this stage the parasite presents a distinct suite of proteins on its tegument and has not yet become fully cloaked in host-derived molecules[29].

Moreover, juvenile schistosomula are more susceptible to antibody-dependent cellular cytotoxicity[30] than are older schistosomula and adult worms. Therefore, it is reasoned that the highly expressed proteins at this stage, particularly secreted and membrane proteins, are worthy targets for the development of vaccines and new drugs.

The schistosome tegument, a unique double lipid bilayered syncitium that covers the external surface of the intra-mammalian developmental stages, represents the point of interaction between the parasite and mammalian host tissues. This structure is pivotal for parasite survival within the host and is therefore a primary target of anthelmintic drugs[31] and vaccines [32,16].

Some of these surface exposed proteins are proving to be efficacious vaccines [33, 34], yet the expression profiles of only some of these genes have been explored, and have involved using arrays covering only 3,000 genes[35].

In the present study, NP-40 soluble extract of surface proteins of 7-days schistosomules was electrophoresed using 12.5% SDS-PAGE, then, the separated proteins were transferred onto PVDF membrane which was incubated with sera collected from patients with chronic ***S. mansoni*** infection, sera was absorped to ***E. coli*** **lysate** for removal of anti- ***E. coli*** antibodies to avoid the non-specific interactions between the extracted surface proteins of 7-days schistosomules and anti- ***E. coli*** antibodies which will result in misleading signals. A number of antigens with different degrees of immunogenicity were identified; their roughly estimated molecular weights according to the corresponding marker were approximately, 18 kDa, 28 kDa, 33 kDa, 38 kDa, 40 kDa, 42 kDa, 45 kDa, 50 kDa, 62 kDa, 66 kDa, 81 kDa and 116 kDa.

When 6-day-old ex vivo larvae were isolated from mouse or hamster lung cells and used for generation of E-S products, which were shown to elicit strong immune responses and significant ($P<0.05$) protection against challenge infection in BALB/c mice. Proteomic analysis of E-S molecules following 10x concentration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis identified peptides related to innumerable host and about 15 ***S. mansoni***-specific proteins[11].

Some important proteins playing roles in different metabolic pathways are also expressed as tegumental proteins, the data reported[36] indicate that intact ex vivo, as well as, 5-day-old in vitro-grown larvae of ***S. mansoni*** express Schistosoma mansoni glyceraldehyde 3-phosphate dehydrogenase (SG3PDH) on their surface membrane.

The obtained data provide a framework by which to select targets

for vaccine and drug design based on genes that are critical for the development of ***S. mansoni*** larvae during their first few days in the mammalian host.

This study is considered as an initial screen for potential vaccine antigens to be followed by murine protection studies and functional analysis of the identified proteins. Also, the discovery of target proteins that represent the week points in the worm's defenses.

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الشستوسوما مانسونای: تعیین بعض الأنیجینات ذات الأستجابات المناعیة العالیة من السطح الخارجی من الطور الصدری للطفیل کأمصال واعدة للطفیل

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یمثل مرض البلهارسیا مشکلة صحیة عالمیة یسببها العدید من أجناس طفیل الشستوسوما. یعد المرض متوطنا فی ٧٤ من الدول النامیة وحوالی ٢٠٠ ملیون مصاب حول العالم ومایقرب من ٢٠٠٠٠٠ حالة وفاة کل عام. العلاج الدوائی للمرض علی الرغم من فاعلیتة الأ أنة لایمنع عودة الأصابة بالمرض مع ظهور المقاومة الجزئیة من الطفیل للعلاج لذا أصبحت المناعة الطویلة بالأمصال هی الحل الحقیقی لمنع أنتشار المرض. *الهدف من البحث*: البروتینات علی سطح أو المرتبطة بغلاف الطور الصدری لطفیل الشستوسوما مانسونای هی الهدف الرئیسی لهذة الدراسة بأعتبار هذة البروتینات أمصال مقترحة للطفیل. الطرق: تم أستخلاص بروتینات سطح الطور الصدری للطفیل وبأستخدام الفصل الکهربائی للبروتینات ثم نقل هذة البروتینات الی غشاء نیتروسیلیلوز فی تجربة لطخة ویسترن. غشاء نیتروسیلیلوز والمحتوی علی البروتینات المنقولة کهربائیا تمت حضانتة مع مصل الدم المأخوذ من مرضی مصابین بالطفیل المزمن والمحتوی علی الاجسام المضادة لهذة البروتینات السطحیة للطور الصدری للطفیل ثم مع الاجسام المضادة الثانویة. النتائج: تم تعیین عدد من الانتیجینات السطحیة للطور الصدری للطفیل ذات أوزان جزیئیة وأستجابات مناعیة مختلفة (١٨، ٢٨، ٣٣، ٣٨، ٤٠، ٤٢، ٤٥، ٥٠، ٦٢، ٦٦، ٨١، ١١٦ کیلودالتون). الأستنتاج: التطور فی أبحاث جینوم طفیل الشستوسوما منح فرصة ممیزة للأنتقال لتطویر مصل لهذا الطفیل أو تستخدم فی تشخیصة. نظرا للاستجابات المناعیة المختلفة لما تم تعیینة من البروتینات السطحیة للطور الصدری للطفیل فأن أحد هذة البروتینات من الممکن ان یکون مصلا واعدا للطفیل.