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***Schistosoma mansoni* host-exposed surface antigens characterized by sera and recombinant antibodies from schistosomiasis-resistant rats[★]**

Jorge Sepulveda, Jacqueline M. Tremblay, Jon P. DeGnore, Patrick J. Skelly, and Charles B. Shoemaker^{*}

Department of Biomedical Sciences, Tufts Cummings School of Veterinary Medicine, 200 Westboro Rd, North Grafton, MA 01536, USA

Abstract

Antibodies from *Schistosoma mansoni*-infected rats, unlike mice, show a higher titer for schistosome apical tegumental antigens compared with non-apical membrane antigens. These antibodies bind to the surface of living lung stage worms and to formaldehyde-fixed adult worms. We produced a single-chain antibody Fv domain (scFv) phage library displaying the antibody repertoire of rats highly immune to schistosome infection and we selected for scFvs that recognize the host-exposed surface of worms. Five unique rat scFvs (Teg1, Teg4, Teg5, Teg20 and Teg37) were obtained which recognize schistosome surface epitopes. Each of the scFvs recognizes the surface of living schistosomula and lung stage schistosomules and/or the surface of formaldehyde-fixed adult worms. None of these scFvs reproducibly stained living adult worms. This suggests that a change occurs during the transition from lung schistosomules to 4 week adults such that at least some surface antigens, although remaining on the surface in living adult worms, can no longer be immunologically stained. Teg1 and Teg4 scFvs both recognize specific bands on Western blots. No bands were observed for the other three scFvs, suggesting that these scFvs may recognize non-protein or conformationally-dependent epitopes. Teg1 was unambiguously identified as recognizing the *S. mansoni* tetraspanin antigen, SmTSP-2, within the large extracellular domain. Teg4 recognizes a 35 kD band tentatively identified as Sm29 by proteomic analysis. These scFvs can now be used to characterize schistosome epitopes at the host-parasite interface, to target worms in vivo, and to study the mechanisms by which these worms naturally evade immune damage to the tegument within permissive hosts.

Keywords

Schistosome; Schistosomula; Tegument; Antigen; Rat; scFv

[★]Note: Nucleotide sequence data reported in this paper are available in the GenBank™ database under Accession Nos. **FJ648344** to **FJ648348**.

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^{*}Corresponding author. Dr. Charles B. Shoemaker, Department of Biomedical Sciences, Tufts Cummings School of Veterinary Medicine, 200 Westboro Rd, North Grafton, MA 01536, USA. Tel.: +1 508-887-4324; fax: +1 508-839-7911. charles.shoemaker@tufts.edu.

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1. Introduction

Schistosomes are blood flukes that cause the endemic disease schistosomiasis (also called bilharzia) in an estimated 200 million individuals in 74 tropical and subtropical developing countries (World Health Organization, 2002) with nearly 800 million more people at risk of infection (Steinmann et al., 2006). These complex multicellular trematodes can survive for decades inside the vascular system of immune competent permissive hosts. Schistosomiasis is primarily a consequence of immunopathology that is elicited by schistosome eggs and there is no evidence that permissive hosts develop a significant damaging host immune response directed at juvenile or adult worms under normal circumstances (Keating et al., 2006). The host-interactive surface of schistosomes is the tegument, a syncytium that covers the entire worm and which is bounded by an unusual double-bilayer apical membrane at the host/parasite interface and a normal single bilayer basal membrane on the inside (reviewed by Skelly and Wilson, 2006). In permissive hosts, there appears to be little antibody bound to adult worms (Smithers et al., 1969; Brindley and Sher, 1987) or cellular immune responses directed at the worms residing in the vascular system (Keating et al., 2006). The lack of a clear immune response directed at the schistosome tegument is surprising since the parasite has a large, invaginated surface area that performs a variety of essential functional interactions with the host such as nutrient uptake and environmental sensing typically involving proteins.

Many researchers have sought to identify evasion mechanisms to explain the poor host immune response targeting the host-interactive schistosome tegument during chronic infections (reviewed by Skelly and Wilson, 2006). Proposed strategies include minimizing protein exposure at the surface, antigen masking with host antigens, epitope concealment by poorly immunogenic carbohydrates, poor immunogenicity of exposed antigens, induction of blocking antibodies, secretion of a variety of immune modulators and rapid tegument turnover. The inability to identify anti-tegument monoclonal antibodies (mAbs) that recognize epitopes exposed on living juvenile or adult worms (Riengrojpitak et al., 1989) has limited studies on immune evasion and highlighted the effectiveness of the parasites' immune evasion capability. The unique heptalaminate outer tegumental membrane itself may be an adaptation of blood-dwelling trematodes that resists host immune effectors such as complement and antibody-dependent cell-mediated cytotoxicity (ADCC) (Threadgold, 1984; Capron et al., 1987; Skelly, 2004).

Despite the many mechanisms schistosomes may use to evade immune effectors, there is good evidence that the worms can be damaged by anti-tegument antibody responses. For example adult worms were killed or their tegument badly damaged following their transfer from mice into macaque monkeys pre-immunized against mouse antigens (Smithers et al., 1969; Clegg et al., 1970). The schistosomes were assumed to acquire murine antigens in their tegument while resident in the mice, and these antigens provide the target for a damaging antibody response following their transfer to monkeys containing anti-mouse antibodies. This group later showed that immunization of experimental animals with tegumental membrane preparations evoke partial protective immunity that significantly correlated with specific anti-tegument antibody responses (Smithers et al., 1989). Antibody against the tegument has also been shown to play a critical role in enhancing schistosome killing following chemotherapy with praziquantel (Brindley and Sher, 1987; Doenhoff et al., 1988). More recently, antibody recognition of the *Schistosoma mansoni* tegumental membrane antigens, SmTSP-2 and Sm29, were found to be significantly higher in individuals displaying putative resistance to schistosomiasis than in chronically infected people (Cardoso et al., 2006; Tran et al., 2006). Vaccination of mice with these antigens also elicits potent protection against schistosome infection (Tran et al., 2006; Cardoso et al., 2008).

There is also evidence that rats, which are semi-permissive to *S. mansoni* infection, employ an anti-tegment humoral response to kill schistosomes. For example, Fischer rats are quite tolerant of a low dose (<50 cercariae) challenge (Phillips et al., 1975) while almost completely eliminating a high dose (>500 cercariae) challenge approximately 4 weeks p.i. (Knopf et al., 1977; Phillips et al., 1977; Cioli et al., 1978). Resistance to schistosomiasis can be passively transferred, via immune rat serum, to naïve mice even when given 1 week p.i. (Barker et al., 1985). Protective antibodies can be removed by absorption on adult schistosomes, strongly indicating that antibodies to adult surface epitopes mediate at least some killing (Barker et al., 1985). Putative effector mechanisms have been reported to include both complement-mediated and antibody-dependent cell-mediated mechanisms (David and Butterworth, 1977; Butterworth et al., 1982; Capron et al., 1982; Khalife et al., 2000).

The literature shows that schistosomes can be susceptible to antibody-mediated damage to their tegument but not enough is known regarding the identity and nature of the tegumental antigens that are available to the host immune system. Such antigens, properly presented as vaccine immunogens, should be capable of eliciting anti-tegmental antibodies and thus may elicit protective immunity in normally permissive hosts. One approach we have taken is to identify antigens exposed on living mammalian-stage worms which are recognized by antibodies from rats that are actively rejecting schistosome infections. Research on schistosome tegument antigens was greatly aided by recent proteomic studies that identified many of the tegumental proteins (van Balkom et al., 2005; Braschi et al., 2006) including a small subset of those that was shown to be exposed on living worms by surface biotinylation (Braschi and Wilson, 2006). In this study, we prepare a single-chain Fv domain (scFv) library, displayed on phage, representing the antibody repertoire of schistosome immune rats. We then identify and partially characterize a set of five unique scFvs that each recognize the exposed surface of living juvenile schistosomes and formaldehyde-fixed adult worms.

2. Materials and methods

2.1. Parasites

Swiss female mice, 5–7 weeks old, recently exposed to 125 *S. mansoni* cercariae (Puerto Rican strain) were obtained from Dr. Fred Lewis at the Biomedical Research Institute, Rockville, Maryland (USA). All research animal use was approved by the Tufts Institutional Animal Care and Use Committee and the animals were maintained in accordance with institutional and government guidelines. Juvenile and adult schistosomes were collected at various times p.i. by portal vein perfusion with a citrate-saline solution (NaCl 0.85%, sodium citrate 1.5%). Worms were collected over a NYTEX sieve, washed with RPMI and immediately fixed for 4 h to overnight with cold, freshly prepared 4% paraformaldehyde in PBS. Lung stage worms were collected from finely diced, perfused lung tissue that had been incubated in RPMI media for several hours at 37°C using lungs obtained from mice approximately 5–6 days p.i. with 1,000–4,000 cercariae (Lewis and Colley, 1977).

Schistosoma mansoni infected *Biomphalaria glabrata* were obtained from Dr. Fred Lewis and cercariae were shed under light. Fischer CDF male rats, 50–70 gm, were anesthetized with isofluorane gas and infected by placing 1,000 cercariae (1 ml) on the shaved abdomen for 20 min. In some cases, rats were re-infected after 4 weeks in the same way. Adult worms were recovered by portal vein perfusion. Blood was obtained from the tail vein and serum prepared by standard procedures.

Cercariae were transformed to schistosomula and cultured for several days in RPMI as previously described (Skelly et al., 2003) or for longer periods in Basch medium (Basch, 1981).

2.2. Schistosome extracts

Tegument preparations were prepared by sucrose-gradient centrifugation of a freeze/thaw extraction method previously described (Roberts et al., 1983; Brouwers et al., 1999). Briefly, adult worms were washed twice with Hanks balanced salt solution (HBBS, Invitrogen) and frozen in liquid nitrogen. After thawing on ice, worms were extensively washed with ice-cold Tris-buffered saline (TBS; 20 mM Tris-HCl, 0.85% [w/v] NaCl and protease inhibitors (Complete Mini, Roche)). The outer tegumental membrane was removed by vortexing the worms (10×1 s each) in Eppendorf tubes. The supernatant, enriched in outer tegument membranes, was centrifuged at 5,000 g for 30 min. The resulting pellet, called the apical membrane extract, was resuspended in TBS.

A non-apical membrane preparation was prepared from the “stripped” worms remaining after tegument removal. Membranes were suspended by homogenization in ice-cold TBS containing the protease inhibitors (Complete Mini, Roche) in a teflon/glass homogenizer. After addition of 10 vol. of 20% (w/v) sucrose, the suspension was centrifuged at 4°C at 1,000 g for 10 min. The supernatant was subsequently centrifuged at 105,000 g for 1 h at 4°C. The resulting pellet was resuspended in TBS or sample buffer and referred to as the non-apical membrane extract.

The quality of the enriched apical plasma membrane was tested by ELISA quantification for the known apical tegumental membrane-specific protein, SGTP4 (Skelly and Shoemaker, 1996). SGTP4 was typically approximately 40-fold enriched in apical versus non-apical extracts, indicating a highly selective enrichment for apical membrane proteins in this fraction (Brouwers et al., 1999). Protein concentrations were measured by the Bradford reagent (Sigma-Aldrich) as recommended.

2.3. scFv phage-display library

The preparation and characterization of the scFv phage-display library used in this study has been described previously by Sepulveda and Shoemaker (2008). Briefly, the scFv-display library was prepared using antibody V_H and V_L coding DNA amplified by PCR from the B cells of rats that were twice infected by *S. mansoni* cercariae. The V_H and V_L coding DNAs were cloned into a phage display vector separated by DNA encoding a flexible spacer domain and fused in frame to the phage gene III coding DNA. The library has a complexity of approximately 10^7 with >90% displaying full-length scFvs. For panning, an aliquot of the library containing 10^9 colony forming units (CFU) of phage was brought to log phase growth and infected with helper phage to produce an amplified phage preparation displaying scFvs (Sepulveda and Shoemaker, 2008).

2.4. Selection of schistosome worm surface binding scFvs

Selection for worm surface binding scFvs was carried out by “panning” for scFv-displayed phage that bind to both fixed adult schistosomes and apical membrane extract coated on plastic. This was done by alternating the use of two different panning regimens, one for binding to fixed worms and one for binding to apical membrane antigens. Both panning regimens were repeated multiple times in each panning experiment.

To pan for scFv binding phage to schistosome membrane extracts, Immuntubes (Nunc) were first coated overnight at 4°C with 8 µg/ml adult schistosome apical membrane extract in 1 ml PBS (HyQ PBS (1×), 67 mM (PO₄), HyClone). The tubes were then washed three times with PBS and blocked with 4% non-fat dried milk in PBS (MPBS). A 1 ml suspension of the scFv phage-display library containing $\sim 10^{12}$ CFU in MPBS was prepared and incubated in the coated Immuntube for 1 h at room temperature. The tubes were washed five times with PBS containing 0.1% Tween 20 (PBST) followed by five times with PBS. Bound phage was eluted with 1 ml of 100 mM HCl for 10 min after which the eluate was neutralized with 0.5 ml of 1

M Tris–HCl, pH 8.0. The eluted phage were used to infect a 10 ml culture of log-phase *Escherichia coli* ER2738 cells (Biolabs). A small aliquot of the infected bacteria was used in serial dilutions to titrate the number of phage eluted while the remainder was processed to amplify the phagemid for further selection or analysis (Sepulveda and Shoemaker, 2008).

To pan for scFvs recognizing exposed epitopes on the worm surface, intact adult worms were used as targets. Adult worms were fixed in 4%–paraformaldehyde (PFA) overnight at 4°C and washed at least five times with RPMI media. Five adult worms were used for each panning round. Approximately 10^{11} CFU of phage in MPBS were incubated with fixed worms for 1 h at room temperature. Worms were washed three times with RPMI-10% FCS (Invitrogen) by adding media and removing unbound phage with a pipette. Bound phage were eluted and titered as above.

Clones shown positive by ELISA on apical membrane extracts (Sepulveda and Shoemaker, 2008) following each panning experiment were “fingerprinted” by analysis of their *Bst*NI digestion patterns (Tomlinson et al., 1992). One clone representing each unique fingerprint was sequenced to characterize the scFv coding region.

2.5. Production of recombinant proteins

Selected scFv coding regions were subcloned into the *E. coli* expression vector JSC-His and produced and purified as previously described (Sepulveda and Shoemaker, 2008). The recombinant scFvs are expressed with a hexahistidine tag for purification and an epitope tag (E-tag) for detection. The large extracellular domains of Sm23 and SmTSP-2 were expressed as maltose-binding protein (MBP)-fusion proteins as described by Sepulveda and Shoemaker (2008). Purity and quantity of the recombinant scFv was assessed by Coomassie Blue staining of SDS-PAGE.

2.6. Immunofluorescence (IF)

Prior to staining, adult *S. mansoni* worms were sometimes fixed and blocked immediately after perfusion as for scFv-display library panning (see section 2.4). In other cases, worms were perfused with a formaldehyde solution (see section 2.1) in an effort to fix them prior to their removal from the animal. Where living schistosomes were used, they were maintained in RPMI, 10% FCS prior to use. The scFv or sera preparations were diluted in RPMI as indicated and incubated with worms for 1 h. After three washes with RPMI, the worms were incubated for 1 h at room temperature with one of the following FITC-conjugated antibodies: anti-E-tag antibodies (GE Healthcare) for scFv detection; anti-mouse-IgG (Zymed) for mouse antibody detection; or anti-rat-IgG (Invitrogen) for rat antibody detection. After three RPMI washes, samples were mounted using Vectashield mounting media H-1000 (Fisher), or simply HBSS (Invitrogen), and binding was detected by fluorescence microscopy. At least 50 parasites were observed for each detecting antibody or scFv and those displaying representative staining patterns were photographed.

2.7. Western blotting

Proteins resolved by SDS-PAGE were transferred to BioTrace polyvinylidene fluoride (PVDF) membranes (PALL Corp., USA) at 100 mA overnight. After visualizing the protein markers with Ponceau S, the membrane was blocked with MPBS overnight at 4°C or for 2 h at room temperature. Antibody incubations were for 1 h at room temperature followed by three washes, 5–10 min each, in PBST. The resulting immunoblots were developed using the TMB membrane peroxidase substrate system (3-C) (KPL Inc., USA).

3. Results

3.1. Serum antibodies from schistosome-infected rats recognize host-exposed antigens

Fischer rats were immunized by two *S. mansoni* infections with 1,000 cercariae, 8 weeks apart. To test whether the rats actively rejected the parasites, one animal was sacrificed after four and 8 weeks for perfusion and worm count. After 4 weeks, a large number of worms were recovered, but the worms were significantly smaller and less active than worms recovered from a 4 week infection of mice (Supplementary Fig. S1). After 7 weeks of infection, only a single immature worm pair was recovered from the rat. Five *S. mansoni*-infected rats were re-challenged with cercariae and 4 weeks later one test rat was found to be free of worms by vascular perfusion. Four weeks p.i., sera were collected from the remaining 2× cercariae-infected rats and their spleens were obtained as the source of RNA for an immune rat antibody-display phage library.

The anti-schistosome antibody responses of infected rats ($n = 5$) were compared to those of similarly infected susceptible mice ($n = 5$). Five weeks p.i., at the time rats undergo rejection of schistosomes, rat sera recognizes apical extracts to a substantially greater extent than non-apical extracts (which has some apical contamination). Sera from mice at the same time p.i. recognize both fractions approximately equally and with lower apparent titers (Fig. 1A). Western blots support the selective antibody recognition of apical membrane proteins by *S. mansoni*-infected rats compared with mice, and display a stronger recognition of several antigen species (Fig. 1B). Interestingly, this Western blot specificity appears very similar to that previously observed in mice immunized with apical membrane detergent insoluble complexes (Racoosin et al., 1999).

We next tested 2×-immune rat sera for recognition of host-exposed antigens by IF staining of mammalian-stage schistosomes at various stages of development. The surface of recently transformed, living schistosomula maintained in culture for 5 days were clearly recognized by 2× immune rat sera while non-immune rat sera showed no recognition (Fig. 2A,B). The surface staining always appears as rather evenly spaced patches, often lining up in horizontal rows or “belts” across the width of the worms. Lung stage schistosomules (5 days) were obtained from lung tissue and tested for recognition by 2×-immune rat sera (Fig. 2C). The sera brightly stained the lung schistosomules producing a pattern similar to that seen in the cultured schistosomula.

The 2×-immune pooled rat serum was unable to reproducibly stain living adult worms. The staining was usually not significantly brighter than for non-immune sera except for occasional regions of strong fluorescence that were usually associated with obvious physical damage to the worm. The situation changed when live worms were fixed by formaldehyde prior to staining. As seen in Fig. 3, the surface of 3 week old, juvenile, formaldehyde-fixed, schistosomes were brightly stained. Interestingly, as for the younger worms, the staining occurred in patches of different sizes although no belting patterns were apparent. Additional images of similarly stained juvenile adult worms are shown in Supplementary Fig. S2A–F. As worms mature, unfixed worms stain poorly with 2× immune rat sera (not shown) while fixed worms generally stain with a patchy appearance similar to that of younger adult worms (Fig. 4). Sera from mice that were infected once with *S. mansoni* variably stained both schistosomula and fixed adult worms in a pattern similar to that produced by rats, although the signals were generally much weaker than observed with equivalent rat sera (not shown).

3.2. Selection for recombinant immune rat recombinant antibodies (scFvs) recognizing the schistosome surface

The spleens obtained from 2× immune rats were used as the source of mRNA for construction of a recombinant antibody scFv phage display library as we reported earlier (Sepulveda and Shoemaker, 2008). The rat scFv-display library had been previously validated (Sepulveda and

Shoemaker, 2008) by showing that it contains scFvs recognizing two known *S. mansoni* tetraspanin membrane antigens, Sm23 and SmTSP-2 (Wright et al., 1991; Reynolds et al., 1992; Tran et al., 2006; Sepulveda and Shoemaker, 2008). This library was used in a variety of selection regimens with the goal to identify scFvs binding to the host-exposed surface of living juvenile and adult schistosomes.

Initial panning efforts designed to select scFv-displaying phage binding to living adult worms was unsuccessful. Since we were also unable to obtain reproducible IF staining of adult worms with 2 $\times$ -immune rat sera (above), we suspect that the surface may be too dynamic to permit phage to remain bound throughout the panning process. To prevent this, we next used multiple cycles of panning on formaldehyde-fixed adult worms. This approach was also unsuccessful in selecting for specific worm binding scFvs, most likely because of a high background of phage that become non-specifically trapped by, or bound to, the complex, invaginated surface of adult worms. To minimize a background problem, we tested several regimens in which we alternated selection for phage binding to formaldehyde-fixed adult schistosomes with selections for phage binding to isolated apical membrane extracts. The use of the plastic-coated apical membrane protein was expected to permit a high stringency selection for phage-displayed scFvs recognizing tegumental antigens but would not specifically select for scFvs that bind to host-exposed epitopes. By combining both panning regimens in a selection experiment, we sought to enrich for scFvs that bind specifically to worm-surface exposed epitopes on apical membrane antigens.

In one panning regimen, three successive selections were performed using apical membrane extracts followed by four cycles of selection on fixed schistosomes (see Materials and methods). From this regimen, we obtained three clones that proved to be unique and to show strong, specific recognition of apical membrane extracts by ELISA. These clones were designated scFv Teg1, Teg4 and Teg5. A second round of selection reversed the order of selection, using worms first and then apical membrane extracts. In addition to re-isolating Teg1, Teg4 and Teg5, two additional unique scFvs were identified (Teg20 and Teg37). The nucleotide sequences were submitted to GenBank (accession numbers FJ648344 to FJ648348) and the predicted protein sequences are shown in Supplementary Fig. S3.

Of interest, clone Teg1 encoded a scFv that was identical to scFv T1 that had been previously selected from this library for binding to the extracellular loop of the known apical membrane protein, SmTSP-2 (Sepulveda and Shoemaker, 2008). This scFv was thus unambiguously identified as an anti-SmTSP-2 scFv which recognizes a protein migrating at approximately 28 kD on SDS-PAGE (Sepulveda and Shoemaker, 2008). In fact, SmTSP-2 was chosen as one of the antigens used for validating the immune rat scFv library because it had been reported to localize to the apical surface of worms (Tran et al., 2006).

3.3. Characterization of the five schistosome apical membrane antigen binding scFvs

All five of the Teg scFvs that were identified following panning on apical membrane antigens and fixed adult schistosomes were characterized by ELISA and Western blotting for recognition of apical membrane antigens. ELISA results (Fig. 5) demonstrated that all Teg scFvs strongly recognize apical extracts compared with non-apical extracts. Clear recognition of apical antigens is observed at 1 nM concentrations of Teg1, Teg4 and Teg5, suggesting that these scFvs have good affinity for their targets. Teg4 and Teg5 show the greatest apparent specificity for apical antigens. The S1 scFv recognizing Sm23 (Sepulveda and Shoemaker, 2008), an antigen found throughout adult schistosomes (van Balkom et al., 2005), was also tested in this assay and, as expected, was found to equally recognize apical and non-apical membranes in the same ELISA.

Western blots were performed as the first step to identifying the antigens recognized by the schistosome scFvs selected for affinity to both apical membrane extracts and fixed intact adult worms. Teg1 was previously shown to recognize the 28 kD SmTsp2 antigen (Sepulveda and Shoemaker, 2008). As shown in Fig. 6, Teg4 recognizes a distinct antigen of approximately 35 kDa in Western blots of apical membrane extracts. None of the remaining three scFvs, Teg5, Teg20 or Teg37, recognized distinct antigen species on Western blots.

Preparative SDS-PAGE was performed on apical membrane extract and the 38 kDa region of the gel recognized by Teg4 was excised and subjected to MS/MS proteomic analysis. Four schistosome proteins were clearly identified by this analysis: actin; venom allergen-like protein 6; 14-3-3 epsilon; and Sm29 (Supplementary Table S1). Of these four proteins, only Sm29 is reported to be specifically localized to the apical membrane of the tegument (Cardoso et al., 2008). Unlike the other proteins, Sm29 is known to migrate as a discrete band at 35 kDa on SDS-PAGE and to be immunogenic following schistosome infection (Cardoso et al., 2008). Based on these data, we tentatively identify the antigen recognized by Teg4 as Sm29.

3.4. Recognition of the schistosome host-exposed surface by the Teg scFvs

The five scFvs, obtained from the B cells of 2× schistosome-infected rats and selected for host-exposed epitopes on apical membrane antigens, were each tested for their ability to bind to the surfaces of living schistosomula and juvenile and mature adults. All of the scFvs recognize living schistosomula with Teg1, Teg4 and Teg5 producing the strongest fluorescence (Fig. 7A). Interestingly, the pattern of recognition of the different Teg scFvs all appeared to have the same patchy and belted appearance on the surface of schistosomula (Fig. 7B) that was observed using the polyclonal 2×-immune rat sera (Fig. 2A). This was most obvious with the Teg1 and Teg4 scFvs which produce the strongest staining of schistosomula (see also Supplementary Fig. S4A–C).

As with the 2×-immune rat sera, none of the scFvs were able to stain living adult schistosomes. Four of the five Teg scFvs (Teg4, Teg5, Teg20 and Teg37) clearly recognize formaldehyde-fixed 6–8 week adults as visualized in Fig. 8A where the focal plane was on the edge of the worms. When the worms stained by these four scFvs were viewed at the worm surface (Teg5 shown in Fig. 8B), the staining for each had a similar patchy appearance as observed on fixed adult worms stained with polyclonal sera from the 2×-immune rats (Fig. 2C). The patchy staining was not uniform (unlike the belted pattern in schistosomula), but rather distributed in small, interspersed textural patches with no apparent regularity. In contrast to the other scFvs, the Teg1 scFv produced intermittent patches of bright fluorescence, clearly distinct from the pattern produced by the other four Teg scFvs. This scFv stained brightly at apparently damaged sections of the worms suggesting that the Teg1 epitope was exposed only in regions where the tegument had been disrupted.

4. Discussion

We believe that we report here the first identification of recombinant antibodies that bind to epitopes on the host-exposed surface of living schistosomes. The antibodies, expressed as scFv domains, derive from rats that are in the active process of rejecting a schistosome infection. Rats are semi-permissive hosts for *S. mansoni* and reject most of the parasites approximately 4 weeks following a bolus infection (Knopf et al., 1977; Phillips et al., 1977; Cioli et al., 1978). Following this infection, the rats rapidly reject subsequent challenge employing a protective response that has been associated with antibody effectors (Horta and Ramalho-Pinto, 1984; Mangold and Dean, 1986; Cetre et al., 1999; Khalife et al., 2000). Clearly, antibodies recognizing host-exposed epitopes must be considered as candidates for immune effectors and antigens containing host-exposed epitopes are strong candidates for vaccine targets. This study reports five scFvs that we believe are excellent reagents in the search for promising vaccine

targets, for dissecting the role of antibodies in schistosome immunity and for improved understanding of the host/parasite relationship. These five scFvs likely represent only a fraction of the worm surface binding antibodies present following schistosome infection of rats and we thus expect the scFv library to remain a useful resource for further studies of the host-exposed schistosome tegument.

In this study, we employed the Fischer rat model of schistosomiasis which has previously been shown to be semi-permissive to *S. mansoni*. We reproduced earlier studies demonstrating that Fischer rats reject large infections of *S. mansoni* shortly after the fourth week of infection. After 4 weeks, the worms that were recovered from the rat by liver perfusion were much smaller and less robust than worms recovered from mice at the same time p.i.. We also found that rats develop a higher titer antibody response against tegumental antigens by 5–6 weeks p.i. than do mice. This more robust surface antibody response may participate in the worm rejection or it may simply be a consequence of increased worm damage and death caused by some other effector mechanism. Following a second infection, the titer of tegumental antibodies was further boosted and this serum was potent in its ability to immunologically stain living lung stage schistosomula and formaldehyde-fixed adults. The spleens from the twice infected rats were thus a good source of B cells for preparing a scFv-display library to select antibodies recognizing worm surface epitopes.

Five different, unique scFvs were selected from the immune rat scFv-display library for their ability to bind to both apical membrane extracts and intact, formaldehyde-fixed adult worms. The antigen recognized by one of the scFvs, Teg1, was unambiguously identified as SmTSP-2. This antigen was previously shown to be localized to the apical tegument by proteomics (van Balkom et al., 2005; Braschi et al., 2006) and IF (Tran et al., 2006), although these studies did not investigate exposure of SmTSP-2 epitopes on living schistosomes. Our studies demonstrate that SmTSP-2 is clearly exposed on living larval and lung-stage schistosomes. Exposure of SmTSP-2 on adult worms was ambiguous as IFA staining with Teg1 was observed only in discrete bright patches that appear to be regions of damaged tegument.

Another selected scFv, Teg4, produced the brightest staining of both larval and adult schistosomes. This scFv recognizes an antigen migrating at approximately 35 kD on Western blots following SDS-PAGE of apical extracts. It is intriguing that two different mAbs were previously identified from *S. mansoni*-infected rats that each recognized a schistosome surface antigen estimated at ~38 kD. One of these rat mAbs was found to protect against cercarial challenge and the other had blocking activity (Dissous et al., 1982; Grzych et al., 1984). This antigen was never identified and it is quite possibly the same antigen recognized by Teg4 scFv. In our studies, proteomic MS/MS analysis of the excised 35 kD antigen recognized by Teg4 identified Sm29 as the major membrane protein species in the fraction. The identity of Sm29 is consistent with other results indicating this protein to be a schistosome apical membrane antigen (van Balkom et al., 2005; Braschi et al., 2006; Cardoso et al., 2008). Western blots of anti-Sm29 antisera recognize a band having similar mobility as the Teg4 antigen on SDS-PAGE (Cardoso et al., 2008). The Teg4 scFv, however, did not recognize recombinant Sm29, expressed in *E. coli*, on Western blots (not shown). While these results may indicate that Teg4 recognizes a different apical protein than Sm29, it is also possible that Teg4 recognizes a conformational or non-protein epitope on Sm29 that is not reproduced within bacterial expressed recombinant Sm29. Sm29 protein sequence is strongly suggestive of a glycosylphosphatidylinositol (GPI)-anchored protein. When isolated from schistosomes, Sm29 migrates much slower on SDS-PAGE than would be expected based on its protein MW of 29 kD, further suggesting that it becomes post-translationally modified (Cardoso et al., 2008). Interestingly, we have recently identified several scFvs from the 2× infected rat library that do recognize recombinant Sm29, confirming that Sm29 is an immunogen following

schistosome infection in rat, but these scFvs do not appear to recognize host-exposed schistosome epitopes.

Two recent findings are strongly consistent with our finding that SmTSP-2 and Sm29 are present on the exposed surface of schistosomes. Both of these antigens are among a small group of antigens found to be exposed to surface biotinylation on living schistosomes (Braschi and Wilson, 2006). Secondly, these are the only two antigens found to date that are selectively recognized by sera from humans identified as putatively resistant to schistosomiasis compared with chronically infected (Cardoso et al., 2006; Tran et al., 2006) and both produce a strong protective immune response upon immunization with recombinant protein (Tran et al., 2006; Cardoso et al., 2008). In unpublished results, we have shown that SmTSP-2 and Sm29 are recognized by antibodies in schistosome-infected rat sera at significantly higher titer than equivalent infected mouse sera. Furthermore, Sm29 is released from living adult worms by treatment with phosphatidylinositol phospholipase C (Dr. Alan R. Wilson, personal communication). Our results add further evidence in support of SmTSP-2 and Sm29 as promising, host-exposed targets of a schistosomiasis vaccine.

Three of the scFvs that we found to recognize exposed epitopes on living schistosomes, Teg5, 20 and 37, clearly recognize apical-enriched antigens by ELISA but did not recognize discrete apical antigen species on Western blots. It may be that the epitopes recognized by these scFvs are derived from antigens present in the tegumental extracts at a level below detection, or are conformationally sensitive epitopes that are denatured by SDS-PAGE. Alternatively, the antigens recognized by these scFvs may be non-proteinaceous in nature and not migrate as a discrete species on these gels. It is clear that adult schistosomes have non-protein components exposed at the host/parasite interface (Skelly and Wilson, 2006). Cercariae are known to be coated with a glycocalyx largely composed of carbohydrate (Samuelson and Caulfield, 1985; Caulfield et al., 1987; Khoo et al., 1995) and the exposed surface of living adults is also known to contain substantial carbohydrates (Klabunde et al., 2000). A valuable feature of the schistosome surface binding scFvs is that these binding proteins are reagents that will permit the purification and characterization of these antigens that comprise the critical host/parasite interface on adult schistosomes.

The role of antibodies in protective immunity to schistosomiasis in the rat model has been clearly demonstrated although the mechanism by which effector function is exerted remains uncertain (reviewed by Capron and Capron, 1986; Khalife et al., 2000). Recognition of worm surface antigens seems likely to participate in antibody effector action, and evidence has accumulated in support of this view (Dissous et al., 1982; Barker et al., 1985). To date, the specific antigen targets of protective antibodies targeting the exposed surface on living worms have not been identified. Recent studies have found a strong correlation of antibody recognition of two schistosome surface antigens, SmTSP-2 and Sm29, with putative resistance in schistosome-infected people in Brazil (Cardoso et al., 2006; Tran et al., 2006). It is probably not a coincidence that these are the same two antigens that we identified in this study as the host-exposed targets of antibodies from schistosome-resistant rats. The availability of scFvs recognizing these antigens will permit identification of the exposed epitopes and the design of peptide immunogens.

The staining of mammalian stage schistosomes by immune sera, or by the rat scFvs selected for worm surface binding, has a patched appearance that is often arrayed in a belt-like pattern in the younger worms. The source or cause of the patchy appearance is unknown at present, although it closely resembles the pattern of newly deposited material formed by the eruption of cytons during the initial formation of the tegument during cercarial transformation (Skelly and Shoemaker, 2001). It seems possible that the patches of staining observed in this study somehow result from the deposition of the outer tegumental membrane that “erupts” from the

underlying cytons. It has been postulated that the outer tegument of schistosomes is formed from multilamellar bodies which initially, upon deposition, appear as membrane stacks (Hockley and McLaren, 1973). Perhaps this recently deposited material is more available to antibody binding than older tegument or, alternatively, the deposited materials may be enriched in antigens recognized by serum antibodies from infected animals.

A provocative result of this study was the observation that the surface of living schistosomula and lung schistosomules stained well with *S. mansoni*-infected rat sera and some of the rat scFvs, while the surface of adult worms at 3 weeks or older did not stain with these agents unless the worms were fixed in formaldehyde. We do not know which tegument characteristics change during worm maturation that allow living adult worms to become refractory to antibody staining. The loss of staining is not due to the loss of antigens at the surface because the worms are readily stained by the antibodies following formaldehyde fixation. Possible explanations for the loss of antibody staining include the secretion of proteases, increased turnover of the tegument and some form of antigen masking that is obviated by formalin treatment. Whatever the mechanism, it seems likely that it plays a role in schistosome immune evasion. In this context, it is interesting to note that the point in schistosome maturation at which they become refractory to antibody staining (~7 days) is the same time at which schistosomes become refractory to killing by immune rat serum in passive transfer experiments (Mangold, 1981). The worm surface binding scFvs identified in this study should be useful reagents to identify and characterize the tegumental properties that develop after the lung stage and that lead to the inability of antibodies to remain associated with the surface of live adults.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Schistosoma mansoni-infected rats produce a higher titer of antibodies recognizing apical membrane proteins than mice. A) Dilution ELISA comparing 5 week *S. mansoni*-infected rat sera (squares) to similarly infected mouse sera (circles) for antibody recognition of *S. mansoni* apical membrane antigens (filled) with non-apical membrane antigens (open). Antigens were coated at 3 ug/ml protein. Both sera were diluted to 1:100 in the first well, 1:1,000 in the second well and then two-fold serial dilutions. B) Western blot comparing 5 week *S. mansoni*-infected rat sera (1:2,000) with similarly infected mouse sera (1:2,000) for antibody recognition of *S. mansoni* apical (A) membrane antigens and non-apical (NA) membrane antigens.

Fig. 2.

Immunofluorescence on recently transformed, living, *Schistosoma mansoni* schistosomes stained by sera from rats twice infected with *S. mansoni*. The focal plane of the photographs is at the worm surface. Living *S. mansoni* schistosomula were cultured for 5 days following cercarial transformation and stained with: A) non-immune control rat sera (1:100); or B.) 2× *S. mansoni*-infected rat sera (1:100). C) Living *S. mansoni* lung stage schistosomules isolated from mice 5 days following cercarial infection. Equivalent lung worms incubated with non-immune rat sera displayed no detectable fluorescence, similar to A. In all cases, sera was incubated with schistosomes and washed, then bound antibodies were labeled with FITC/anti-rat-IgG and detected by fluorescence microscopy.

Fig. 3.

Immunofluorescence on *Schistosoma mansoni* schistosomes, formaldehyde-fixed during perfusion from mice 3 weeks p.i., stained by sera from *S. mansoni*-infected rats. *Schistosoma mansoni* juvenile adults were recovered from infected mice 3 weeks after cercarial infection by liver perfusion using a formaldehyde solution. Sera, 1:100, from uninfected control rats (A) or 2× *S. mansoni*-infected rats (B–D) were incubated with the fixed schistosomes and bound antibodies detected with FITC/anti-rat-IgG and detected by fluorescence microscopy. Whole worm images are shown in A and B. The same worm is photographed in B–D. The areas of the worm shown in C and D are boxed in B.

Fig. 4.

Immunofluorescence on *Schistosoma mansoni* adult schistosomes, formaldehyde-fixed following recovery from mice 7 weeks p.i., stained by sera from rats 2× infected with *S. mansoni*. *Schistosoma mansoni* adults were recovered from infected mice 7 weeks after cercarial infection, and then fixed in a formaldehyde solution. Serum, 1:100, from uninfected control rats (A) or 2× infected rats (B) was incubated with the fixed schistosomes and bound antibodies detected with FITC/anti-rat-IgG and detected by fluorescence microscopy. Whole worm images (a) or higher magnification images (b) of the surface of the same worms are shown.

Fig. 5.

ELISA to compare rat Teg single-chain antibody Fv (scFv) recognition of apical versus non-apical extracts of adult schistosomes. ELISA plates were coated with 10 $\mu\text{g}/\text{ml}$ of *Schistosoma mansoni* apical extract (circles) or non-apical extract (squares). Three times serial dilutions of the indicated rat Teg scFv or the anti-Sm23 scFv (S1; Sepulveda, 2008) were incubated in the well with concentration range shown. Bound scFv was detected by absorbance at 450 nm and displayed as relative absorbance to compare recognition of the two antigen extracts.

Fig. 6.

Western blot of *Schistosoma mansoni* apical membrane antigens probed with the rat Teg single-chain antibody Fv (scFvs). Apical membrane extracts (50 μ g/lane) of adult *S. mansoni* were resolved by SDS-PAGE and transferred to filters for Western blotting. Filter strips were incubated with 1 μ g/ml of purified Teg scFvs as indicated, or a negative control scFv (Neg). Bound scFv was detected with a horseradish peroxidase (HRP) anti-epitope (E)-tag monoclonal antibody recognizing an epitopic tag on each scFv. The position of the band specifically recognized by Teg4 is indicated by an arrow. Molecular marker migration positions are shown.

A

B **Fig. 7.**

Immunofluorescence on living *Schistosoma mansoni* schistosomula stained by rat Teg single-chain antibody Fv (scFvs). Living *S. mansoni* schistosomula were maintained in culture following cercarial transformation and then incubated with 1 µg/ml of soluble scFv for 1 h. After washing, bound scFv was labeled with FITC/anti-epitope (E)-tag monoclonal antibody and detected by fluorescence microscopy. A) Five day cultured schistosomula were incubated with the indicated Teg or negative control scFv (Neg). Images were taken with the focal plane at the center of the schistosomula. B) Ten day cultured schistosomula were incubated with the indicated scFv. Images were taken with focal plane at the surface of the schistosomula.

Fig. 8.

Immunofluorescence on formaldehyde-fixed *Schistosoma mansoni* adults stained by selected rat Teg single-chain antibody Fv (scFvs). *Schistosoma mansoni* adults were obtained by liver perfusion and then fixed in formaldehyde. Fixed adult worms were then incubated with soluble scFvs at 1 μg/ml for 1 h. After washing, bound scFv was labeled with FITC/anti-epitope (E)-tag monoclonal antibody and detected by fluorescence microscopy. A) Fixed worms stained with the indicated Teg or negative control scFv (Neg) were imaged with the focal plane at the side edge of the worm. B) Fixed worms stained with the indicated scFv were imaged with the focal plane at the top surface of the worm.