Proteomic Identification of IPSE/alpha-1 as a Major Hepatotoxin Secreted by *Schistosoma mansoni* Eggs

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Abstract

**Background:** Eggs deposited in the liver of the mammalian host by the blood fluke parasite, *Schistosoma mansoni*, normally drive a T-helper-2 (Th2)-mediated granulomatous response in immune-competent mice. By contrast, in mice deprived of T-cells and incapable of producing granulomata, egg-secreted proteins (ESP) induce acute hepatic injury and death. Previous work has shown that one such ESP, the T2 ribonuclease known as omega-1, is hepatotoxic *in vivo* that specific antisera to omega-1 prevent hepatocyte damage.

**Methodology/Principal Findings:** Using an in vitro culture system employing mouse primary hepatocytes and alanine transaminase (ALT) activity as a marker of hepatic toxicity, we demonstrated that *S. mansoni* eggs, egg-secreted proteins (ESP), soluble-egg antigen (SEA), and omega-1 are directly hepatotoxic and in a dose-dependent manner. Depletion of omega-1 using a monoclonal antibody abolished the toxicity of pure omega-1 and diminished the toxicity in ESP and SEA by 47 and 33%, respectively. Anion exchange chromatography of ESP yielded one predominant hepatotoxic fraction. Proteomics of that fraction identified the presence of IPSE/alpha-1 (IL-4 inducing principle from *S. mansoni* eggs), a known activator of basophils and inducer of Th2-type responses. Pure recombinant IPSE/alpha-1 also displayed a dose-dependent hepatotoxicity *in vitro*. Monoclonal antibody depletion of IPSE/alpha-1 abolished the latter’s toxicity and diminished the total toxicity of ESP and SEA by 32 and 35%, respectively. Combined depletion of omega-1 and IPSE/alpha-1 diminished hepatotoxicity of ESP and SEA by 60 and 58% respectively.

**Conclusions:** We identified IPSE/alpha-1 as a novel hepatotoxin and conclude that both IPSE/alpha-1 and omega-1 account for the majority of the hepatotoxicity secreted by *S. mansoni* eggs.

Introduction

Schistosomiasis is a chronic parasitic disease that affects more than 200 million people worldwide [1]. The central pathological characteristic during chronic infection is a granulomatous reaction around trapped parasite eggs in the host’s liver, bladder, or intestine [2]. Granulomatous inflammation in the liver may result in fibrosis, scarring, portal hypertension, and in the worst cases hemorrhaging and death [3].

*Schistosoma mansoni* infection in mice is the most common experimental model employed. Approximately five-to-six weeks post-infection, parasite eggs deposited by adult worms induce a T-helper-2 (Th2)-type-polarized immune response [4]. A number of the immunodominant molecules in eggs have been described [5,6,7,8], in addition to the two molecules central to this report (see below). The ability of *S. mansoni* eggs to induce Th2-type differentiation during infection is underscored by the observation that eggs alone, or soluble egg antigen (SEA) released by the eggs through pores in the shell, are sufficient to drive Th2 polarization in naive uninfected mice [9,10,11].

Research from the late 1960s and 1970s has documented that mice lacking T-cells due to genetic loss or surgical removal of the thymus [12,13,14], or administration of specific immunosuppressors [15,16,17], do not develop a typical granulomatous response to trapped parasite eggs. In these T-cell depleted mice, infection was associated with extensive hepatic parenchymal damage suggestive of a cytotoxic egg product(s) diffusing into hepatic tissue [18]. Histopathology of livers from schistosome-infected immunocompromised mice displayed microvesicular hepatocyte steatosis [18,19,20], nuclear degeneration, and hepatocyte apoptosis [21]. Coincident with hepatocyte injury, there is an increase in liver cell transaminases in the plasma [19]. Immunosuppressed mice also have higher mortality once egg deposition in the liver begins [17,19,19,19,22]. In immunologically intact mice, circumoval granulomata, and antibody responses to released *S. mansoni* egg components, likely protect against hepatocyte damage. Also, hepatotoxicity is prevented in infected T cell-deprived mice by transfer of serum from intact mice immunized with *S. mansoni* eggs or egg homogenate, whilst antiserum against other lifecycle stages do not prolong survival [19]. Egg-induced hepatotoxicity appears to be...
Author Summary

The flatworm disease, schistosomiasis, is a major public health problem in sub-Saharan Africa, South America and East Asia. A hallmark of infection with Schistosoma mansoni is the immune response to parasite eggs trapped in the liver and other organs. This response involves an infiltration of cells that surround the parasite egg forming a "granuloma." In mice deprived of T-cells, this granulomatous response is lacking, and toxic products released by eggs quickly cause liver damage and death. Thus the granuloma protect the host from toxic egg products. Only one hepatotoxic molecule, omega-1, has been described to date. We set out to identify other S. mansoni egg hepatotoxins using liver cells grown in culture. We first showed that live eggs, their secretions, and pure omega-1 are toxic. Using a physical separation technique to prepare fractions from whole egg secretions, we identified the presence of IPSE/alpha-1, a protein that is known to strongly influence the immune system. We showed that IPSE/alpha-1 is also hepatotoxic, and that toxicity of both omega-1 and IPSE/alpha-1 can be prevented by first mixing the proteins with specific neutralizing antibodies. Both proteins constitute the majority of hepatotoxicity released by eggs.

identified IPSE/alpha-1 as an abundant protein in egg secretions [5,36,37,30]. IPSE/alpha-1 binds immunoglobulin and activates naïve basophils, leading to histamine release and facilitating the production of Th2-type cytokines [32]. In vitro, IPSE/alpha-1 induces interleukin (IL)-4 secretion from murine basophils in an IgE-dependent but antigen-independent manner [39]. Most recently, IPSE/alpha-1 has been shown to contain a functional C-terminal, monopartite, nuclear localization sequence that binds DNA such that it may alter gene expression in the host cell [40].

We employed a primary hepatocyte in vitro culture system to identify and measure direct toxicity of S. mansoni eggs and their derived fractions, including pure proteins. Hepatotoxicity of omega-1 was confirmed, and IPSE/alpha-1 was identified as a novel hepatotoxin. Both proteins together account for more than half of the egg-derived toxicity measured.

Materials and Methods

Ethics statement

These studies were performed in accordance with the recommendations by the University of California San Francisco Institutional Animal Care and Use Committee. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of California San Francisco (Permit Number: AN000237-03). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. The protocol followed these guidelines in the study: All U.S. Federal Policy and Guidelines governing the use of laboratory animals, Public Health Service Policy on Humane Care and Use of Laboratory Animals, Guide for the Care and Use of Laboratory Animals, National Academy Press, USDA Animal Welfare Act and Regulations, and NIH Office of Laboratory Animal Welfare Guidelines.

In vitro maintenance of S. mansoni and collection of Egg-Secreted Proteins (ESP)

S. mansoni eggs were isolated from the livers of female golden hamsters six weeks following infection with 500 cercariae, as previously described [41]. Approximately 0.5 million eggs were washed twice in serum-free RPMI-1640 supplemented with 100 mg/ml streptomycin. Eggs were then resuspended in 2 ml RPMI-1640, and 500 μl aliquots were placed in 12-well culture plates (Costar). Cultures were checked daily by microscopy to ensure sterility. Medium was harvested after 72 h, and centrifuged for 10 min at 200 ×g to remove eggs. ESP, usually containing approximately 0.5 mg/ml protein by Bradford assay [42], was stored at −80°C. After collection of ESP, egg viability was confirmed by hatching of miracidia; normally >85% of the eggs hatched. Hatching of eggs during the collection period was <1%. SEA was prepared, as described previously [43].

Purified natural omega-1 [30] and recombinant (r)IPSE/alpha-1 proteins [32], and anti-omega-1 (140-3E11) and anti-IPSE/alpha-1 (74-1G2) monoclonal antibodies [32] were kindly supplied by Drs. Gabrielle Schrann and Helmut Haas of the Research Center Borstel, Germany. Experiments to deplete ESP and SEA (each 10 μg/ml) of omega-1 and IPSE/alpha-1 involved incubation for 1 h with 5 μg/ml of the respective monoclonal antibodies bound to Protein G Sepharose (GE healthcare Biosciences Pittsburgh, PA). Protein G Sepharose was then removed by centrifugation for 30 min at 100 ×g.

Fractionation of ESP by anion exchange chromatography

ESP (2 mg) were added to 2 ml 30 mM Tris-HCl, pH 8.0, and centrifuged at 5000 ×g for 10 min at 4°C. The supernatant was loaded onto an Hr 5/5 Mono Q column (GE Healthcare), and
equilibrated with the same buffer and elute by a 0 to 1 M linear NaCl gradient in 6 column volumes of Tris-NaCl buffer. Flow-through and eluted fractions (1 ml) were stored at −80°C prior to testing for toxicity with cultured primary hepatocytes (applied volume 100 μl of each fraction to 2 × 10⁶ hepatocytes/0.5 ml).

**Primary hepatocyte preparation, culture and exposure to egg-derived material**

Hepatocytes were isolated from C57/BL6 mice by *in situ* perfusion of liver with collagenase, as described previously [44]. The portal vein was severed to permit outflow followed by cannulation of the inferior vena cava with a 22-gauge catheter. The liver was then flushed with a calcium-chelating buffer (liver perfusion medium) for 3 to 5 min, followed by perfusion with collagenase (liver digest medium) for an additional 6–8 min. At the end of the digestion, the liver was removed to a sterile dish and minced thoroughly with a scissors. This crude liver cell isolate was suspended in 25 ml of Dulbecco's modified Eagle's medium/Ham's F-12, filtered through sterile gauze, centrifuged at 70 × g for 2 min, and resuspended in Dulbecco's modified Eagle's medium/Ham's F-12. After an additional round of centrifugation and resuspension, hepatocytes were isolated by centrifugation using a 50% Percoll gradient. Hepatocytes were cultured at a density of 2 × 10⁷/0.5 ml per well in a 12-well culture plate (Costar), previously coated with a 5 mm layer of matrigel (BD Bioscience) [45], which is a tumor biomatrix prepared from the Engelbroth-Holm-Swarm mouse sarcoma [46]. Hepatocytes were allowed to attach for 1 hour at 37°C. Culture plates were gently swirled and the medium containing unattached cells and debris was aspirated. Cultures were then incubated for 72 h in a final volume of 0.5 ml RPMI medium containing 50, 100, or 200 eggs, unfraccionated ESP (10 μg/ml) or 100 μl of chromatography fractions. Supernatants were collected and stored at −20°C and analyzed for alanine transaminase (ALT), a serum marker of hepatotoxicity [47]) using a Beckman Chemical Analyzer in the Clinical Chemistry Laboratory of the San Francisco Veterans Affairs Medical Center (VAMC).

**Proteomic Analysis of ESP**

ESP was fractionated by SDS-PAGE, then silver stained [48,49], and 40 evenly spaced protein bands were sliced out of the gel (Fig S1). The gel slices were then diced into small cubes, reduced and alkylated with diithiothreitol and iodoacetamide, and in-gel digested with trypsin [50,51]. The resulting peptides were extracted and analyzed by on-line liquid chromatography/mass spectrometry, using an Eksigent nanoflow pump and a Famos autosampler, which were coupled to quadrupole-orthogonal-acceleration-time-of-flight hybrid mass spectrometer (QStar XL, Applied Biosystems). Peptides were fractionated on a reversed-phase column (C18, 0.75 × 150 mm), and a “5–50% B gradient-in-gradient” was developed in 35 min at a 350-nl/min flow-rate. Solvent A was 0.1% formic acid in water and solvent B was 0% formic acid in acetonitrile. Data were acquired in information-dependent acquisition mode: 1 sec MS surveys were followed by 3 sec CID experiments on computer-selected multiply charged precursor ions. Peak lists were generated using Analyst 2.0 software (Applied Biosystems) with the Mascot script 1.6b20 (Matrix Science). Database searches were performed using ProteinProspector v. 5.1.7 [http://prospector2.ucsf.edu] [52]. Searches were performed first on the SwissProt databank (December 16, 2008, 405,506 entries) to evaluate sample purity, followed by searching in the *S. mansoni* database SchistoDB v. 4.0 (www.schistodb.net; 13,174 entries downloaded July 2009). Batch-Tag settings were selected for samples prepared with trypsin allowing a maximum of one missed cleavage and no non-specific cleavages. Peptide modifications searched for included carboxamide (Cys) as the only fixed modification, and up to two variable modifications from among the following: oxidation (Met), acetyl (N-term), oxidized acetyl (N-term), pyroglutamate (Gln) and Met-loss (N-term). The mass accuracy considered was 200 ppm, and 300 ppm for the precursor and fragment ions, respectively. The following acceptance scores for database matches were required: a minimum protein score of 22, a minimum peptide score of 15, and a maximum expectation value of 0.02 required for both peptide and protein identification. These criteria resulted in an approximate 2% false determination rate. Protein identifications are reported with a minimum of two peptide matches per protein. For the analysis of ESP anion exchange fraction #11, the maximum expectation value was changed to 0.05, and no decoy proteins were identified using these acceptance criteria.

**Results**

5. *S. mansoni* eggs and their secretions are hepatotoxic *in vitro*

To measure hepatotoxicity caused by parasite eggs ESP, and their chromatographic fractions, we employed an *in vitro* system involving murine primary hepatocytes cultured on matrigel. ALT was employed as a hepatotoxicity biomarker. With parasite eggs, measurements were taken 24, 48 and 72 h. No alteration in ALT levels was seen at 24 or 48 h (not shown); however, by 72 h, ALT had increased markedly and in a dose-dependent manner (Figure 1A). Dose-dependent hepatocellular injury elicited by ESP was also measured after 72 h (Figure 1B).

To aid identification of those ESP constituents responsible for hepatotoxicity, ESP was fractionated by Mono Q anion exchange chromatography. The flow-through (component not bound to the column), and each of the eluted fractions (100 μL), was co-incubated with cultured hepatocytes. At 72 h, fraction #11 induced an approximate two-fold greater release of ALT relative to the other eluted fractions and flow-through, such that it was the equivalent of 10 μg/ml unfraccionated ESP (Figure 2).

**Proteomic analysis of ESP and hepatotoxic fraction #11: identification of IPSE/alpha-1**

SDS-PAGE and trypic digestion followed by mass spectrometry of ESP and hepatotoxic fraction #11 identified 99 and nine proteins, respectively (Tables 1 and 2). Previously, total proteomic analysis of ESP identified 188 proteins [36], and many are common between this and the present dataset (Table 1). Among the nine proteins identified in fraction #11 were metabolic enzymes involved in glucose metabolism, glycogen storage, in addition to chaperones. IPSE/alpha-1 was also identified; and, given its potent immunomodulatory properties [32,34], was of immediate interest in discovering of whether or not it was hepatotoxic.

**In vitro confirmation that Omega-1 is hepatotoxic**

Omega-1 is an egg-secreted glycoprotein with RNase activity and in vivo-demonstrated hepatotoxicity [18,28]. To measure direct toxicity to primary cultured hepatocytes, purified native omega-1 was co-incubated with primary hepatocytes. At 72 h, a dose-dependent release of ALT was measured (Figure 3A). Likewise, *Aspergillus oryzae* T2 RNase (25 μg/ml) Invitrogen, # 18031-013 Carlsbad, CA) was toxic. Importantly, pre-incubation of pure omega-1 with a monoclonal anti-omega-1 antibody bound
to Protein G Sepharose abolished cytotoxicity (Figure 3B). Depleting ESP and SEA with the same antibody decreased toxicity by 47 and 33%, respectively. All reductions in hepatoxicity were statistically significant (Figure 3B).

IPSE/alpha-1 is also a hepatotoxin in vitro

IPSE/alpha-1 was an abundant protein in the hepatotoxic fraction #11 from anion exchange chromatography (Table 2). Recombinant IPSE/alpha-1 was added to hepatocyte cultures, and a dose-dependent
Table 1. Proteins identified in ESP by geLC-MS/MS and searching in SchistoDB.

| Protein Name | Accession # | Peptides | % Cov | Score | Expect | MW (kDa) | Gel Band |
|--------------|-------------|----------|-------|-------|--------|----------|----------|
| phosphoenolpyruvate carboxykinase | Smp_00580 | 34 | 46 | 4.76 | 2.50E-08 | 70.4 | 8, 13, 14, 19, 20, 30 ++ |
| macroglobulin/complement | Smp_089670 | 30 | 12 | 4.57 | 7.50E-08 | 222.2 | 34–36 – |
| enolase (2-phosphoglycerate dehydratase) | Smp_024110 | 22 | 42 | 4.96 | 1.40E-08 | 47.0 | 8, 9, 12, 24, 25 ++ |
| fructose 1,6 bisphosphate aldolase | Smp_042160.2 | 20 | 53 | 4.75 | 3.40E-08 | 39.6 | 20–22 ++ |
| beta tubulin | Smp_035760 | 17 | 37 | 5.9 | 2.60E-10 | 49.8 | 12–14, 19, 20 ++ |
| glutathione S-transferase 26 kDa (GST 26) (GST class-mu) | Smp_102070 | 20 | 68 | 4.62 | 6.00E-08 | 25.3 | 15–18 ++ |
| phosphoglycerate mutase | Smp_096760 | 17 | 56 | 5.19 | 5.30E-09 | 28.4 | 18 ++ |
| thioredoxin peroxidase (Prx 1) | Smp_059480 | 17 | 51 | 5.11 | 7.60E-09 | 21.1 | 12–14, 20, 21 ++ |
| glutathione S-transferase 28 kDa (GST 28) (GST class-mu) | Smp_054160 | 19 | 57 | 4.46 | 1.20E-07 | 23.8 | 16–18 ++ |
| malate dehydrogenase | Smp_035270.2 | 18 | 34 | 5.98 | 1.90E-10 | 36.2 | 10, 11, 14–16, 20, 21 ++ |
| triosephosphate isomerase | Smp_003990 | 16 | 43 | 4.81 | 2.70E-08 | 28.1 | 16–18 + |
| purine nucleoside phosphorylase | Smp_090520 | 15 | 31 | 4.76 | 3.30E-08 | 45.2 | 16–18 ++ |
| family C56 non-peptidase homologue (C56 family) | Smp_082030 | 10 | 69 | 6.02 | 1.50E-10 | 19.1 | 12, 13 ++ |
| alpha tubulin | Smp_090120.1 | 9 | 24 | 5.52 | 1.30E-09 | 50.0 | 9, 12–15, 26 ++ |
| alpha-galactosidase/alpha-n-acetylgalactosaminidase | Smp_179260 | 12 | 10 | 3.57 | 1.10E-06 | 108.5 | 19, 20, 25, 26 + |
| interleukin-4-inducing protein precursor (IPSE/alpha-1) | Smp_112110 | 9 | 49 | 4.83 | 2.50E-08 | 15.4 | 13, 14, 19–21, 24 ++ |
| hepatotoxic ribonuclease omega-1 precursor | Smp_193860 | 13 | 63 | 3.31 | 1.60E-06 | 15.1 | 12, 18, 19 ++ |
| aminopeptidase PILS (M01 family) | Smp_174530 | 14 | 13 | 2.9 | 1.40E-05 | 110.8 | 29, 31, 34, 35 – |
| heat shock protein 70 | Smp_106930.1 | 11 | 19 | 4.72 | 1.50E-08 | 69.0 | 10, 16, 17, 24, 32, 39 ++ |
| thioredoxin glutathione reductase | Smp_048430 | 9 | 16 | 4.3 | 2.40E-07 | 64.8 | 8, 27, 28–31 ++ |
| peroxiredoxin, Prx-2 | Smp_158110 | 12 | 30 | 3.71 | 3.60E-07 | 21.7 | 8, 12, 13 ++ |
| peroxiredoxins, prx-1, prx-2, prx-3 | Smp_062900 | 5 | 14 | 4.5 | 1.00E-07 | 21.7 | 12–14 – |
| proteasome subunit alpha 2 (T01 family) | Smp_179630 | 4 | 20 | 4.08 | 5.90E-07 | 19.5 | 18, 25, 26 + |
| ATP:guanidino kinase (Smc74), putative | Smp_150240 | 10 | 18 | 3.21 | 5.10E-07 | 31.1 | 9, 12–15, 26 ++ |
| annexin | Smp_096890 | 10 | 45 | 3.53 | 4.40E-06 | 25.8 | 16 – |
| expressed protein | Smp_152710.2 | 8 | 34 | 2.9 | 5.90E-07 | 22.0 | 9, 13, 14–16 ++ |
| actin | Smp_046950 | 7 | 17 | 4.87 | 1.70E-08 | 41.7 | 8, 10, 11, 13, 19 ++ |
| serpin | Smp_090080 | 13 | 25 | 2.27 | 2.10E-05 | 46.0 | 20, 25–30 + |
| ferritin light chain | Smp_087760 | 8 | 25 | 3.43 | 5.90E-07 | 20.2 | 11, 14 – |
| nucleoside diphosphate kinase | Smp_092750 | 11 | 52 | 3.22 | 2.60E-07 | 16.9 | 11–13 + |
| phosphoglycerate kinase | Smp_187370 | 7 | 33 | 5.8 | 3.90E-10 | 18.5 | 11, 12, 19, 20, 25 ++ |
| expressed protein | Smp_150240 | 10 | 18 | 3.21 | 5.10E-07 | 31.1 | 9, 15, 21, 24, 25, 32, 33 + |
Table 1. Cont.

| Protein Name | Accession # | Peptides | % Cov* | Score | Expect | MW (kDa) | Gel Band |
|--------------|-------------|----------|--------|-------|--------|----------|----------|
| heat shock protein (Major egg antigen, P40) | Smp_049250 | 7 | 21 | 2.38 | 1.30E-04 | 40.2 | 14, 22 | ++ |
| superoxide dismutase (Cu-Zn) | Smp_176200 | 3 | 44 | 5.32 | 3.10E-09 | 15.9 | 11 | ++ |
| dihydrolipoamide dehydrogenase | Smp_046740 | 6 | 14 | 2.49 | 2.80E-05 | 52.9 | 10, 11, 25, 26 | ++ |
| proteasome subunit alpha 6 (T01 family) | Smp_130110 | 4 | 5 | 3.64 | 3.90E-06 | 76.9 | 10, 17 | – |
| thioredoxin | Smp_008070 | 4 | 40 | 4.05 | 2.50E-07 | 11.9 | 6–8 | – |
| expressed protein | Smp_011030 | 3 | 16 | 4.32 | 2.10E-07 | 20.1 | 6, 7, 14 | ++ |
| expressed protein | Smp_088720 | 5 | 20 | 3.09 | 8.40E-07 | 27.4 | 16, 18, 19 | – |
| transaldolase | Smp_070600 | 7 | 18 | 1.92 | 4.70E-05 | 37.8 | 18-20, 22 | + |
| calcium-binding protein | Smp_096390 | 3 | 21 | 4.48 | 1.10E-07 | 16.7 | 9, 10 | 2 |
| calpain (C02 family) | Smp_157500 | 3 | 2 | 4.3 | 1.00E-07 | 172.9 | 12, 13 | ++ |
| 22.6kDa tegument-associated antigen | Smp_086470 | 3 | 12 | 3.77 | 2.00E-08 | 21.6 | 4, 8, 9 | ++ |
| phosphomannomutase | Smp_087860 | 3 | 14 | 4.01 | 8.10E-07 | 28.1 | 16, 18 | + |
| glucose-6-phosphate isomerase | Smp_22400 | 4 | 6 | 3.37 | 2.10E-07 | 61.1 | 26 | + |
| 14-3-3 protein, putative | Smp_009760 | 8 | 30 | 2.06 | 1.20E-05 | 32.0 | 30, 31 | – |
| 200-kDa GP1-anchored surface glycoprotein | Smp_017730 | 3 | 2 | 3.6 | 1.70E-06 | 36.0 | 8, 14 | – |
| uridine phosphorylase | Smp_082420 | 2 | 12 | 5.15 | 6.20E-09 | 32.8 | 10, 18 | 2 |
| cyclophilin | Smp_040130 | 5 | 22 | 3.54 | 1.90E-07 | 17.7 | 8, 11–13 | ++ |
| ferritin light chain | Smp_047650 | 5 | 16 | 3.1 | 6.90E-09 | 19.7 | 36 | + |
| alpha-glucosidase | Smp_133210 | 7 | 8 | 1.72 | 3.10E-05 | 102.5 | 32–34 | – |
| expressed protein | Smp_187410 | 2 | 8 | 5.42 | 1.00E-10 | 32.0 | 30, 31 | 2 |
| glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | Smp_056970.1 | 4 | 12 | 2.78 | 3.10E-06 | 36.4 | 11, 20, 21 | ++ |
| glycogen phosphorylase | Smp_143840 | 8 | 11 | 1.75 | 1.50E-04 | 80.0 | 8, 9, 31, 33 | ++ |
| proteasome subunit alpha 3 (T01 family) | Smp_092280 | 3 | 13 | 3.46 | 7.20E-08 | 28.1 | 17, 18 | 2 |
| ribose-5-phosphate isomerase, putative | Smp_092220 | 3 | 3 | 2.9 | 4.90E-06 | 159.4 | 18 | 2 |
| venom allergen-like (VAL) 5 protein | Smp_120670 | 2 | 11 | 3.87 | 8.70E-07 | 16.4 | 16 | 2 |
| expressed protein | Smp_170410 | 6 | 19 | 2.19 | 2.90E-06 | 29.3 | 25, 26, 29 | – |
| leucine aminopeptidase (M17 family) | Smp_030000 | 6 | 11 | 2.28 | 1.30E-05 | 56.7 | 26–28 | – |
| methylthioadenosine phosphorylase | Smp_028190 | 3 | 11 | 3.55 | 1.50E-07 | 34.6 | 12, 13 | – |
| superoxide dismutase [Mn] | Smp_056440 | 3 | 11 | 2.68 | 1.20E-05 | 24.3 | 15 | ++ |
| L-lactate dehydrogenase, putative | Smp_089370 | 4 | 16 | 3.22 | 4.20E-07 | 30.0 | 8, 14 | + |
| NAD dependent epimerase/dehydratase | Smp_089370 | 4 | 16 | 2.19 | 7.10E-06 | 29.8 | 5, 9, 10 | – |
| PwLAP aminopeptidase (M17 family) | Smp_083870 | 7 | 13 | 1.49 | 2.90E-05 | 59.7 | 27, 28 | – |
| annexin | Smp_045560 | 5 | 18 | 2.07 | 2.30E-05 | 36.9 | 19, 20 | + |
| SmVal26 | Smp_154260 | 2 | 15 | 2.52 | 1.40E-04 | 20.5 | 10, 11 | – |
| proteasome catalytic subunit 2 (T01 family) | Smp_074500.2 | 2 | 11 | 3.23 | 1.80E-06 | 22.8 | 14 | – |
| ribosomal protein related | Smp_160460 | 2 | 9 | 1.81 | 3.00E-04 | 53.8 | 8, 19, 26 | ++ |
| dynein light chain | Smp_056780 | 2 | 27 | 2.81 | 1.40E-06 | 10.8 | 8, 9 | – |
| cytochrome c | Smp_033400 | 2 | 18 | 2.99 | 1.80E-06 | 12.0 | 9 | – |
| SmVal27 | Smp_154290 | 3 | 20 | 1.92 | 3.10E-05 | 20.7 | 12 | – |
| calmodulin | Smp_026560.1 | 1 | 10 | 3.75 | 1.30E-06 | 17.2 | 12 | + |
| titin, putative | Smp_126240 | 3 | 1 | 1.47 | 4.20E-05 | 67.4 | 35, 36 | – |
| peptidylglycine mono-oxygenase | Smp_145300 | 3 | 7 | 1.46 | 2.10E-04 | 26.5 | 25, 29 | ++ |
| SmVal23 | Smp_160250 | 1 | 6 | 3.36 | 6.60E-06 | 22.8 | 12 | – |
| inosine triphosphate pyrophosphatase | Smp_063120.1 | 2 | 10 | 1.77 | 6.40E-05 | 21.2 | 13 | – |
toxicity was measured at 72 h ALT levels that was significantly elevated relative to negative controls (Figure 4A). Similar to that found for omega-1, specific neutralization of rIPSE/alpha-1 with an anti-rIPSE/alpha-1 monoclonal antibody abolished activity and decreased the cytotoxicity of ESP and SEA by 32 and 35%, respectively (Figure 4B). All reductions in hepatoxicity were statistically significant.

Table 1. Cont.

| Protein Name                              | Accession # | Peptides | % Cov | Score   | Expect | MW (kDa) | Gel Band |
|-------------------------------------------|-------------|----------|-------|---------|--------|----------|----------|
| Sodium/potassium-transporting ATPase subunit beta | Smp_033550   | 2        | 9     | 1.73    | 1.40E-05 | 32.1      | 20       | –        |
| proteasome subunit alpha 7 (T01 family)   | Smp_076230   | 2        | 13    | 2.33    | 1.20E-05 | 29.7      | 18, 19   | –        |
| low-density lipoprotein receptor (LDL)    | Smp_159420   | 4        | 4     | 1.17    | 3.50E-04 | 102.7     | 35       | –        |
| arginase                                  | Smp_059980   | 2        | 7     | 2.45    | 1.40E-06 | 39.9      | 11, 20   | +        |
| heme binding protein                      | Smp_016730   | 3        | 22    | 1.55    | 2.10E-05 | 20.6      | 14       | +        |
| dipeptidyl-peptidase III (M49 family)     | Smp_019010   | 2        | 3     | 1.81    | 7.90E-05 | 76.2      | 31, 32   | +        |
| expressed protein                         | Smp_153230   | 2        | 2     | 1.71    | 9.90E-06 | 146.3     | 36       | –        |
| ATP synthase alpha subunit mitochondrial  | Smp_002880.1 | 4        | 9     | 1.55    | 1.70E-05 | 59.6      | 26       | –        |
| adenylate kinase                          | Smp_071390   | 2        | 10    | 1.52    | 1.30E-04 | 22.3      | 14       | +        |

Note: cutoff above 70% (++), below (+) and (–) not found.

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Table 2. Proteins identified in hepatotoxic fraction #11 by LC/MS/MS analysis after SDS-PAGE fractionation and in-gel digestion, or after in-solution digestion.

| Gel-band | Rank | Protein Name                              | Acc #       | Num Unique | % Cov | Best Disc Score | Best Expect Val | Protein MW |
|----------|------|-------------------------------------------|-------------|-----------|------|-----------------|-----------------|------------|
| 1        | [1]  | glycogen phosphorylase                    | Smp_143840  | 32        | 50.7 | 5.36            | 2.60E-09        | 80035      |
| 1        | [2]  | glycogen phosphorylase                    | Smp_143850  | 7         | 51.1 | 4.32            | 2.10E-07        | 16835      |
| 2        | [1]  | heat shock protein 70                     | Smp_182190.2| 31        | 41.3 | 5.61            | 8.90E-10        | 69830      |
| 3        | [1]  | aldehyde dehydrogenase                    | Smp_050390  | 5         | 11.6 | 4.33            | 2.10E-07        | 53763      |
| 3        | [2]  | expressed protein                         | Smp_170410  | 2         | 4.2  | 2.7             | 1.70E-05        | 29253      |
| 4        | [1]  | heat shock protein (Major egg antigen (P40)) | Smp_049250 | 10        | 25.1 | 4.76            | 3.30E-08        | 40192      |
| 5        | [1]  | heat shock protein 70                     | Smp_182190.2| 39        | 52   | 5.48            | 1.50E-09        | 69830      |
| 6        | [1]  | aldehyde dehydrogenase                    | Smp_050390  | 11        | 28.9 | 4.9             | 1.90E-08        | 53763      |
| 6        | [2]  | utp-glucose-1-phosphate uridylytransferase 2 | Smp_133600 | 4         | 12.8 | 4.92            | 1.70E-08        | 52729      |
| 6        | [3]  | expressed protein                         | Smp_170410  | 3         | 9.2  | 3.57            | 5.30E-06        | 29253      |
| 6        | [4]  | heat shock protein 70                     | Smp_106930  | 2         | 3.3  | 0.99            | 2.30E-04        | 69004      |
| 7        | [1]  | heat shock protein (Major egg antigen (P40)) | Smp_049250 | 2         | 6.9  | 2.86            | 1.10E-04        | 40192      |
| 8        | [1]  | heat shock protein (Major egg antigen (P40)) | Smp_049250 | 4         | 10.8 | 2.99            | 6.20E-05        | 40192      |
| 8        | [2]  | fructose-1,6-bisphosphatase-related       | Smp_097370  | 3         | 6.1  | 0.64            | 0.01            | 37472      |
| 9        | [1]  | interleukin-4-inducing protein precursor (IPSE/ALPHA-1) | Smp_112110 | 3         | 25.4 | 4.82            | 2.60E-08        | 15358      |
| 9        | [2]  | heat shock protein                        | Smp_049250  | 2         | 4.7  | 2.75            | 1.70E-04        | 40192      |
| in solution | [1] | heat shock protein 70                     | Smp_106930  | 21        | 33.2 | 5.7             | 5.90E-10        | 69004      |
| in solution | [2] | heat shock protein, (Major egg antigen (P40)) | Smp_049250 | 8         | 17.7 | 4.15            | 4.50E-07        | 40192      |
| in solution | [3] | glycogen phosphorylase                    | Smp_143840  | 5         | 6.6  | 3.12            | 3.50E-05        | 80035      |
| in solution | [4] | aldehyde dehydrogenase                    | Smp_050390  | 5         | 11.6 | 2.22            | 4.30E-05        | 53763      |
| in solution | [6] | macroglobulin/complement                  | Smp_089670  | 2         | 0.9  | 0.72            | 0.0054          | 222171     |

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Omega-1 and IPSE/alpha-1 are major hepatotoxins in ESP and SEA

To measure the combined contributions of omega-1 and IPSE/alpha-1 to the hepatotoxicity of ESP and SEA in vitro, both egg-derived preparations were depleted of both omega-1 and IPSE/alpha-1 with specific monoclonal antibodies prior to incubation with hepatocytes. The combination of both antibodies diminished hepatotoxicity of ESP and SEA by 60 and 58%, respectively (Figure 5).

Discussion

The pathogenesis of hepatic schistosomiasis is due to the host's granulomatous response to eggs deposited in the liver [2]. The initial cellular granuloma is characterized by the presence of activated macrophages, lymphocytes, and eosinophils, as reviewed in both Agnew and Pearce [23,53]. Over time, granulomata become fibrotic, and their accumulation in perportal areas, as is the case in chronic S.mansoni infection, can lead to portal hypertension, hemorrhaging, and death [3]. Ironically, in the

Figure 3. Omega (ω)-1 exhibits dose-dependent hepatotoxicity that is neutralized by a specific monoclonal antibody. (A) Hepatocyte cultures (0.5 ml in Dulbecco's modified Eagle's medium were co-incubated with various amounts of purified native omega-1, and egg-derived material (10 μg/ml). After 72 h, ALT, a biomarker for hepatotoxicity, was measured. Polymyxin B (80 μg/ml) was included in co-incubations with omega-1 to neutralize any potential LPS. Control cultures contained polymyxin B alone. Aspergillus oryzae T2 RNase (25 U/ml) was also used as a comparison in light of omega-1's described T2 RNase activity. A combination of 5 mM D-galactosamine hydrochloride (D-gal) and 1 μg/ml rTNF-α (D-gal/TNF-α) was employed as a known hepatotoxic control and negative control cultures used PBS. (B) Pre-incubation of a specific monoclonal antibody (5 μg/ml) with omega-1 abolished the latter's toxicity and respectively decreased cytotoxicity of ESP and SEA by 47 and 33%. Data are presented as the means ± SD from two independent experiments each performed in duplicate. *P<0.02, **P<0.001 and ***P<0.0001 using a one-sided paired Student's t-test.

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Figure 4. IPSE exhibits dose-dependent heptotoxicity that is neutralized by specific monoclonal antibody. (A) Hepatocytes were co-incubated with various amounts of rIPSE. After 72 h, the presence of ALT in the medium was measured. As positive controls, cells were co-incubated with 10 μg/ml each of ESP or SEA. A combination of 5 mM D-galactosamine hydrochloride (D-gal) and 1 μg/ml rTNF-α (D-gal/TNF-α) was employed as a known hepatotoxic control and negative control cultures used PBS. (B) Pre-incubation of a specific monoclonal antibody (5 μg/ml) with rIPSE abolished the latter’s toxicity and respectively decreased cytotoxicity of ESP and SEA by 32 and 35%. Data are displayed as the mean ± S.D. from two experiments each performed in duplicate. *P<0.05, **P<0.001 and ***P<0.0001 using a one-sided paired Student’s t-test. The hatched line represents the control baseline.

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absence of a granulomatous response, experimental hepatic schistosomiasis in mice leads to a more acute and lethal disease [17,18,19,20,22,54]. The understanding from such observations is that schistosome eggs release hepatotoxins, toxins that are normally prevented from diffusing by circumoval granulomata. To date, the only hepatotoxic molecule identified in S. mansoni eggs is omega-1 [18], which is RNaseT2 [28], that also induces a Th2 response [30]. We established an in vitro primary hepatocyte culture system using ALT as the metric for cell injury to identify egg components with direct hepatotoxicity. We first confirmed the toxicity of S. mansoni eggs and their derivatives, ESP and SEA, and then showed that pure native omega-1 is hepatotoxic in vitro, consistent with previous in vivo observations. Based on the present system, omega-1 was a major toxin released by S. mansoni eggs, as depletion of ESP or SEA with a specific monoclonal antibody decreased ALT levels by 47 and 33%, respectively.

To search for additional hepatotoxins, we combined anion exchange chromatography of ESP with proteomics. A single hepatotoxic fraction (#11) was identified which contained a short list of nine proteins. IPSE/alpha-1 stood out as a molecule of interest given its potent immunomodulatory properties [39,53]. Subsequent characterization of pure rIPSE/alpha-1 demonstrated that the molecule is indeed directly hepatotoxic. The finding was confirmed using a specific monoclonal antibody that essentially neutralized IPSE/alpha-1 toxicity while decreasing the cell injury produced by both ESP and SEA by approximately one-third. Further depletion of ESP and SEA with a combination of monoclonal antibodies targeting both omega-1 and IPSE/alpha-1 indicated that approximately 60% of the toxicity of the egg-derived material is due to these two proteins. This leaves room for additional hepatotoxins to be identified, perhaps by different chemical and physical separation approaches. We also note that although both omega-1 and IPSE/alpha-1 were identified in the total ESP proteome, only IPSE/alpha-1 was subsequently found in the single hepatotoxic fraction #11. This suggests that omega-1 was below the mass spectrometry detection limits used to identify proteins.

Recently, IPSE/alpha-1 was reported to be internalized by Chinese hamster ovary cells (CHO) and primary monocyte-derived dendritic cells, but not by peripheral blood basophils [40]; and in each case without apparent toxicity. This suggests that host cell-specific factors determine how cells interact with and respond to IPSE/alpha-1. Such factors might explain why IPSE/alpha-1 is directly toxic to hepatocytes. Studies to understand the mechanism of hepatotoxicity induced by IPSE/alpha-1, and other hepatotoxins such as omega-1, can now be undertaken with the present in vitro system. Ribonuclease activity is often associated with cytotoxicity, and Steinfelder et al noted that omega-1 was initially characterized as a hepatotoxic agent from S. mansoni [28]. Nevertheless, the Th2-promoting activity of omega-1 cannot be explained by a cytotoxic effect, as the molecule failed to induce a detectable reduction in dendritic cell viability. The exact mechanism(s) by which the ribonuclease activity of omega-1 may promote Th2 responses is currently under investigation [31].

The results presented here underscore the paradox of the granulomatous response in hepatic schistosomiasis. Though detrimental to the host in the longer term due to its contribution to disease sequelae such as portal hypertension, it nevertheless protects against more acute hepatocyte injury resulting from toxins released by the schistosome egg.

**Supporting Information**

**Figure S1** SDS-PAGE preparation of ESP. ESP (20 μg) was loaded into SDS-PAGE, the gel was silver stained, then sliced into 40 bands as indicated for in gel trypsin digestion and peptide sequencing by LC-MS/MS. The picture shows Fluorescence image for SyproRuby stained gel.

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**Author Contributions**

Conceived and designed the experiments: M-HA K-CL CRC JHM. Performed the experiments: M-HA K-CL CRC. Analyzed the data: M-HA K-CL. Contributed reagents/materials/analysis tools: M-HA K-CL CRC. Wrote the paper: M-HA CRC JHM.

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