ANALYSIS OF THE MOUSE IMMUNOME REACTIVE AGAINST
SCHISTOSOMA MANSONI EGG ANTIGENS

An Undergraduate Research Scholars Thesis

by

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>1</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>3</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>4</td>
</tr>
<tr>
<td>NOMENCLATURE</td>
<td>5</td>
</tr>
<tr>
<td><strong>CHAPTER</strong></td>
<td></td>
</tr>
<tr>
<td><strong>I</strong> INTRODUCTION</td>
<td>6</td>
</tr>
<tr>
<td>Overview of Schistosomiasis</td>
<td>6</td>
</tr>
<tr>
<td>Life Cycle of <em>S. mansoni</em></td>
<td>8</td>
</tr>
<tr>
<td>Symptoms and Diagnosis of Schistosomiasis</td>
<td>11</td>
</tr>
<tr>
<td>Treatment of Schistosomiasan</td>
<td>11</td>
</tr>
<tr>
<td>Mouse Model of Schistosomiasan</td>
<td>12</td>
</tr>
<tr>
<td><strong>II</strong> MATERIALS AND METHODS</td>
<td>13</td>
</tr>
<tr>
<td>Overview of Procedure</td>
<td>13</td>
</tr>
<tr>
<td>Protein Extraction from Liver Tissue of Mice</td>
<td>13</td>
</tr>
<tr>
<td>Rehydration of IPG Strips 4-10</td>
<td>14</td>
</tr>
<tr>
<td>Isoelectric Focusing of IPG Strips 4-10</td>
<td>14</td>
</tr>
<tr>
<td>Equilibration of IPG Strips 4-10</td>
<td>15</td>
</tr>
<tr>
<td>Two-Dimensional Electrophoresis</td>
<td>15</td>
</tr>
<tr>
<td>Western Blot</td>
<td>16</td>
</tr>
<tr>
<td>Protein Analysis</td>
<td>17</td>
</tr>
<tr>
<td><strong>III</strong> RESULTS</td>
<td>18</td>
</tr>
<tr>
<td>Results of Two-Dimensional Electrophoresis</td>
<td>18</td>
</tr>
<tr>
<td>Results of Western Blot</td>
<td>19</td>
</tr>
<tr>
<td>Results of Protein Analysis</td>
<td>20</td>
</tr>
<tr>
<td><strong>IV</strong> DISCUSSION</td>
<td>21</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>24</td>
</tr>
</tbody>
</table>
ABSTRACT

Analysis of the Mouse Immunome Reactive against Schistosoma mansoni Egg Antigens. (May 2015)

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Department of Biology

Schistosomiasis is a waterborne parasitic infection affecting more than two hundred million people in the tropical and subtropical regions of the world. Currently, the disease is treated with the drug Praziquantel, but reinfection is common. If an anti-pathology vaccine were created, the rate of infection and reinfection of schistosomiasis would be significantly reduced. Humans with asymptomatic intestinal schistosomiasis and mice with moderate splenomegaly syndrome (MSS) share certain cross-reactive idiotypes (CRI), which are absent in the anti-egg antigen specific antibody responses of symptomatic hepatosplenic patients and mice with hypersplenomegaly syndrome (HSS). We hypothesize that CRI unique to MSS down-regulate the progress of the disease, while CRI unique to HSS may be pro-inflammatory. Therefore, identifying the proteins unique to each disease state could be used to produce a vaccine. CBA/J male mice with twenty-week infections develop the full chronic disease as seen in humans. Predictably 10 - 20% of infected mice will develop severe HSS. In order to identify all soluble proteins from schistosome egg antigens (SEA), proteins were separated based on size, pH, and pI by two-dimensional electrophoresis. Then unique antigen reactivity of the CRI was detected by Western blotting. To identify the antigens, the stains were compared to an SEA Proteomic map generated in The Center for Biodiscovery and Proteomics, University of Wellington, New Zealand, where protein
analysis was completed via Matrix-Assisted Laser Desorption/Ionization – Time of Flight (MADLI-TOF) mass spectrometry. Results indicate that, while an estimate of thirty antigens are represented in both disease states, there are a few differences. Three antigens have been identified as unique to MSS: Calreticulin precursor, Calreticulin, and SJCHGCO1881 protein. Two antigens unique to HSS are Enolase (2phosphoglycerate dehydratase) and one unidentified protein. Future research would investigate the effectiveness of these antigens as anti-pathology vaccines, perhaps by using those only unique to MSS to promote down-regulation.
DEDICATION

This work is dedicated to those who have lost loved ones to schistosomiasis.
ACKNOWLEDGEMENTS

Many thanks go to Dr. Barbara Doughty for mentoring me in her laboratory and to all who have supported my research and education.
## NOMENCLATURE

<table>
<thead>
<tr>
<th>Abbreviation</th>
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</tr>
</thead>
<tbody>
<tr>
<td>2DE</td>
<td>two-dimensional electrophoresis</td>
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<tr>
<td>CRI</td>
<td>cross-reactive idiotypes</td>
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<tr>
<td>gi</td>
<td>genInfo identifier</td>
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<tr>
<td>HSS</td>
<td>hypersplenomegaly syndrome</td>
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<td>Id</td>
<td>idiotype</td>
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<td>IgG</td>
<td>immunoglobulin</td>
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<td>IL-10</td>
<td>interleukin-10</td>
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<tr>
<td>IPG</td>
<td>immobilized pH gradient</td>
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<tr>
<td>MQ</td>
<td>Milli-Q water</td>
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<tr>
<td>MSS</td>
<td>moderate splenomegaly syndrome</td>
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<tr>
<td>PBST</td>
<td>phosphate buffered saline/tween</td>
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<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>SEA</td>
<td>soluble egg antigen</td>
</tr>
<tr>
<td>TNF-a</td>
<td>tumor necrosis factor alpha</td>
</tr>
</tbody>
</table>
CHAPTER I
INTRODUCTION

Overview of Schistosomiasis

More than 200 million people worldwide suffer from the disease schistosomiasis, which is caused by infection from the trematode fluke known as a schistosome (“Schistosomiasis,” 2012). Three species of the fluke cause schistosomiasis: Schistosoma (S.) mansoni, S. hematobium and S. japonicum (Coligan, 1991). The disease is concentrated near regions with bodies of freshwater. For example, cases of S. mansoni are typically reported in sub-Saharan Africa and South America. There is also a low risk for contracting infection via S. mansoni in the Caribbean. S. hematobium is more common in North Africa, Egypt and throughout sub-Saharan Africa, and in the Middle East. S. japonicum causes most infections in Southeast Asia, Indonesia, and China. The people in these areas are most susceptible to the disease because of their poor or nonexistent sanitation systems (“Schistosomiasis,” 2012).

FIG. 1: Distribution of Schistosomiasis, Worldwide, 2009 (World Health Organization)
This research will focus on *S. mansoni*, which has a complex life cycle involving a snail intermediate and a human host. In humans, the adult male and female worms live in copula in the mesenteric veins of the small intestine, continuously releasing eggs. Some of the eggs break through the host’s intestinal wall and enter the intestinal lumen to be excreted with feces, while other eggs are trapped in the liver and elicit a range of immune responses (“Schistosomiasis FAQs,” 2012).

There are two main disease states of schistosomiasis. In most patients, the disease becomes chronic without major pathology (Mbow et al., 2012). This is known as “asymptomatic schistosomiasis,” which results in moderate hepatic fibrosis. It is possible to live for many years while suffering from this disease state of schistosomiasis. However, in some patients (~10%), the infection leads to “symptomatic schistosomiasis” as a result of eggs entrapped in the precapillary sinusoids of the liver, eliciting severe hepatic granuloma formation and fibrosis that most often results in death (“Schistosomiasis FAQs,” 2012). The granuloma is composed of primarily macrophages, T-lymphocytes, and fibroblasts, and it forms to protect the body from the soluble egg antigens (SEAs) that the egg releases, which are toxic to the liver (“General Pathology Associated with Schistosome Infections,” 2010). From this information, it has been found that those suffering from asymptomatic schistosomiasis do not produce the same anti-SEA antibodies as those suffering from symptomatic schistosomiasis, discussed in depth later. Those suffering from symptomatic schistosomiasis react to the parasite with a protective inflammatory response, whereas those suffering from asymptomatic schistosomiasis produce a down-regulatory response. The down-regulatory response results in a slower fibrosis of vital organs, resulting in a longer lifespan. The goal of this research is to identify any discrepancies in antibodies produced in each disease state. By doing so, it may be possible to produce a vaccine that promotes the
down-regulatory response in the infected person, rather than promoting the lethal inflammatory response.

Similar percentages of these disease states have been reported in a CBA/J mouse model of schistosomiasis. Therefore, the asymptomatic form of schistosomiasis, or “moderate splenomegaly syndrome” (MSS), and the symptomatic form, or “hepatic splenomegaly syndrome” (HSS), are comparable to the human disease states and serve as a realistic model for laboratory experiments.

**Life Cycle of *S. mansoni***

![Life cycle of *S. mansoni* ("Schistosomiasis: Biology," 2012)](image)

FIG. 2: Life cycle of *S. mansoni* ("Schistosomiasis: Biology," 2012)
The lifecycle of \textit{S. mansoni} begins when \textit{S. mansoni} eggs hatch to release miricidia, the term for the ciliated larvae of parasitic flukes, into a water source. The miricidia swim and search for an intermediate host, typically a snail ("Schistosomiasis: Disease," 2012). The species of snail \textit{Biomphalaria glabrata} commonly hosts \textit{S. mansoni} ("Life Cycle of \textit{S. mansoni} - Host Snails of Human Schistosomes," 2010). Once the miricidia have penetrated the snail, a series of asexual developments begin. Namely, two generations of sporocysts develop: a mother sporocyst and a daughter sporocyst. The daughter sporocyst produces cercariae, the term for the free-swimming infectious larvae of parasitic flukes ("Life Cycle - Host Snails," 2010). The cercariae have characteristic forked tails, allowing them to survive in water for about forty-eight hours while they search for a vertebrate host ("Schistosomiasis: Disease," 2012).

![Diagram of cercariae anatomy](image)

**FIG. 3:** Anatomy of the cercariae of \textit{S. mansoni} ("Life Cycle - The Schistosome Cercaria," 2010)
Once the vertebrate host is located, the cercaria attaches to and penetrates the host’s skin by secreting proteolytic enzymes from glands in its head (“Life Cycle - The Schistosome Cercaria,” 2010). It takes the cercaria roughly thirty minutes to penetrate the epidermis, but it can take up to a day for it to completely penetrate the basal lamina, and an additional ten hours to completely penetrate the dermis. (“Life Cycle - Skin Penetration,” 2010). After penetrating all of the dermal layers, the parasite enters the bloodstream.

![Image: Cercaria Penetration of the Epidermis](Image)

FIG. 4: Cercaria Penetration of the Epidermis (“Life Cycle of *S. mansoni* - Skin Penetration & Infection,” 2010)

Immediately after dermal penetration, the cercaria loses its tail to enter its next stage of life as a schistosomulum (“Life Cycle - The Schistosome Cercaria,” 2010). As a schistosomulum, the parasite begins to circulate in the body through the veins, passing through the pulmonary artery to the lungs before reaching the liver via the hepatic portal vein and entering its adult stage. While in the lungs, the schistosome undergoes an intermediate growth stage by consuming plasma, and it only fully matures in the liver. Here, the mature male schistosome mates with a mature female, and the schistosomes lay eggs for approximately a month at a time. (“Life Cycle – Schistosomula,” 2010). At this point, the eggs may be excreted in feces, starting the life cycle
over again, or they will become lodged in the liver and spleen, causing the granuloma formation and fibrosis characteristically symptomatic of the disease.

**Symptoms and Diagnosis of Schistosomiasis**

Humans do not typically show symptoms of schistosomiasis until the schistosomes burrow into the blood vessels and mature into adults. At this point, a skin rash may develop and persistently worsen. Between one to two months of infection, the human host may exhibit flu-like symptoms including “fever, chills, cough, and muscle aches” ("Schistosomiasis: Disease," 2012). There is also a small chance that schistosome eggs may travel to the brain or spinal cord. This causes seizures, paralysis, and spinal cord inflammation. ("Schistosomiasis: Disease," 2012). A blood test is necessary to diagnose schistosomiasis. Stool samples can also be collected to examine for eggs; however, because eggs are produced cyclically, there is a chance that eggs will not be found in the feces. The most accurate way to diagnose schistosomiasis is with the blood test. ("Schistosomiasis: Disease," 2012)

**Treatment of Schistosomiasis**

Typically, administration of the drug Praziquantel adequately treats schistosomiasis by killing the parasite. However, the only way to truly prevent schistosomiasis is to properly clean and maintain the population’s water supply as well as eliminate the intermediate host snail (USAID).
Mouse Model of Schistosomiasis

The development of HSS in CBA/J male mice with twenty-week infections correlates with elevations in tumor necrosis factor alpha (TNF-a) liver mRNA and collagen deposition and with lower levels of interleukin-10 (IL-10), an anti-inflammatory cytokine (Sadler et al., 2003). In addition, SEA-specific antibodies present in the sera of MSS mice display a distinct set of SEA specific antibody specificities known as idiotypes (Id) compared to those from sera of infected mice with HSS (Ludolf et al., 2014). The Id preparations from the sera of MSS mice (MSS Id) stimulate T cell proliferation and the production of distinctive cytokine patterns by spleen cells from infected mice while Id prepared from sera of HSS animals (HSS Id) is not stimulatory (Mbow et al., 2012). These differences parallel those observed in human patients with asymptomatic and symptomatic forms of the disease. The most exciting aspect of these parallels is the observation that humans with asymptomatic schistosomiasis and mice with MSS share certain idiotypes, as demonstrated by the cross-reactivity of rabbit antisera specific for asymptomatic patients’ Id with MSS Id. In contrast, these cross-reactive idiotypes (CRI) are absent in the anti-egg antigen specific antibody responses of both mice with HSS and symptomatic schistosomiasis patients. In the CBA/J chronic infection model, CRI is present in the sera of mice that develop MSS by six weeks after infection whereas mice that develop HSS chronic pathology or die before twenty weeks of infection never produce CRI. In summary, MSS results in immunosuppressive CRI that prevent severe fibrosis of the liver and spleen, and these CRI are absent in HSS. Identifying the CRI would allow them to be used in vaccinations to promote the down-regulatory response in those suffering from schistosomiasis.
CHAPTER II
MATERIALS AND METHODS

Overview of Procedure

There are two main steps involved in the protein analysis procedure: two-dimensional electrophoresis (2DE) and western blot. 2DE separates the SEA based on pH, isoelectric point (pI), and size. Of four gels, one gel is stained as a reference and the other three are transferred to nitrocellulose for Western blot analysis. Staining the gel allows all the separated antigens to stain, while a Western blot allows only the antigens that reacted to the specific antibodies to stain.

The following procedures have been adapted from Current Protocols in Immunology.

Protein Extraction of soluble egg antigens

Frozen S. mansoni eggs were obtained from BRMI (Biomedical Research Institute) in Rockville, Maryland and stored at -80°C until use. Eggs were washed five times in sterile phosphate buffered saline, then resuspended in Lysis Buffer (LB) pH 8.4 (30 mM TrisHCL pH 8.8, 2M thiourea, 7M urea, 4% (w/v) CHAPS) and homogenized in a motor driven pestle for 20 minutes on ice. The ratio of eggs to buffer is 50,000 eggs to 1 ml of LB. Eggshells and cellular debris were removed with ultracentrifugation at 50,000 XG at 4°C for 60 minutes. The supernatant was collected, and stored at -80°C until use. Protein determinations were performed prior to performing IEF (Nezlin).
**Rehydration of IPG Strips 4-10**

For this procedure a solution of 40 microliters of SEA (80 – 100 µg), 150 microliters of Rehydration Buffer (2M thiourea, 7M urea, 2% IPG buffer 3-10, 2% DTT, 4% (w/v) CHAPS), and 5 microliters of Ampholytes 3-10 was used per IPG strip. The volume of the solution adds up to 195 microliters per strip. The strips were inserted into the rehydration tray positive end first. Then each solution was spread through its individual lane of the rehydration tray along the length of the strip, and care was taken to avoid creation of air bubbles in the lane to guarantee even hydration. The tray set overnight for processing (Nezlin).

**Isoelectric Focusing of IPG Strips 4-10**

The strips were inserted positive side towards the anode into the IPG cassette. 150 microliters of MQ water were added to two paper wicks, which touch both ends of the gel strips to ensure current flow. The anodic and cathodic electrodes were then attached to the paper wicks. The strips were isoelectric focused at 1000 V for one hour. Isoelectric focusing sorts the proteins based on their pl (Nezlin).

![Diagram](image)

FIG. 5: Proteins are sorted by pl independent of size via isoelectric focusing. (“2-D Electrophoresis for Proteomics”)
Equilibration of IPG Strips 4-10

Performing the equilibration procedure reduces and alkylates the proteins in the strips. By reducing, the double bonds in the protein are broken, and the globular protein becomes a linear protein. Alkylation treats the double bonds to ensure they will not reform later in the procedure. For this procedure, the strips were incubated in 5-15 mL of the Reducing Solution (5 mL of equilibration buffer, 0.1 g of 1% dithiothreitol for two strips) for fifteen minutes at room temperature, and then they were incubated in 5-15 mL of the Alkylating Solution (5 mL of equilibration buffer, 0.125 g of 2.5% iodoacetamide for two strips) for fifteen minutes at room temperature. The strips were then immediately set up to perform the two dimensional electrophoresis as detailed below (Nezlin).

Two-Dimensional Electrophoresis

For this procedure, NuPAGE Novex 4-12% Bis-Tris ZOOM Gels were used in addition to an IPG Well of 1.0 mm.
Before beginning, the MOPS NuPage buffer was prepared with 40 mL MOPS and 760 mL MQ water. After preparing it, the comb was removed from the cassette, and the cassette was rinsed in MQ water. The equilibrated strip was then rinsed and trimmed, and it was inserted into the cassette such that the positive end faced towards the marker well. The MOPS NuPage buffer was poured over the strip to cover it. 5 mL of molecular weight marker was added to its respective well. Agarose sealing solution (100 mL Laemmli SDS electrophoresis buffer, 0.5g agarose, 200 microliters1% Bromophenol blue stock solution) was added to cover the strip. The agarose solution stabilizes the strip so the proteins run into the polyacrylamide gel in a uniform fashion. After appropriately positioning the cassette, MOPS NuPAGE buffer was poured into the front,
center, and back electrophoresis chambers. Then 500 microliters of 2-Mercaptoethanol was added to the center chamber to act as an antioxidant in the procedure. The 2DE was performed at 200 V for one hour, and then the gel was removed from the cast and set in fixer overnight. The next day, the gel was washed for ten minutes three times with MQ water. It was then stained with colloidal staining solution (170 g (NH4)2SO4, 340 mL CH3OH, 30 mL H3PO4) and set for an hour. After that, 100 microliters of 0.06% Coomassie G-250 (0.06 g Coomassie G-250 dissolved in 100 mL MQ water) were added and the gel was set overnight to stain (Nezlin).

**Western Blot**

To properly perform the Immunoblot Western Blot technique, first nitrocellulose was moistened with distilled water that had been autoclaved to destroy indigenous phosphatases. Then the membranes were blocked for thirty minutes with the Blocking Solution, comprised of PBST (800 mL distilled water, 8g NaCL, 0.2g KCl, and 5% Skim Milk. Skim milk is the blocking agent in the solution because it binds to any particles without proteins. The membranes were then washed with autoclaved water for five minutes before incubating in the primary antibodies (normal, MSS, and HSS) for one hour. The membranes were washed again for five minutes in autoclaved water before incubating in the secondary antibody (anti-mouse IgG) diluted 1:1,000 in PBST for one hour. Then the membranes were washed with PBST three times and with autoclaved water two times. Finally, they were incubated in Chromagen with rotation for one hour in order to cause any particles reacting with the IgG to turn black. The membranes were then washed one final time in autoclaved water and air-dried (Nezlin).
Protein Analysis

In order to identify the resulting stained proteins from this experiment, the antigens must be compared to an SEA Proteomic map. The map used for this analysis was generated in The Center for Biodiscovery and Proteomics, University of Wellington, New Zealand. There, protein analysis was completed via Matrix-Assisted Laser Desorption/Ionization – Time of Flight (MALDI-TOF) mass spectrometry. The MALDI-TOF procedure results in peptide mass fingerprinting that allows for identification of each particular protein. Once the proteins were recognized, they were identified on the National Center for Biotechnology Information (NCBI) protein database.

FIG. 6: Protein Map of Schistosoma mansoni egg antigen (SEA): 2DE gel 4-12% Bis Tris, pl 3-10. Courtesy of The Center for Biodiscovery and Proteomics, University of Wellington, New Zealand.
CHAPTER III

RESULTS

The 2DE and Western blot procedures were repeated four separate times. The best of the results are included in this document for discussion.

Results of Two-Dimensional Electrophoresis

Illustrated below are the results of the stained 2DE of SEA pI 3-10.

FIG. 7: SEA 2D gel pI 3-10, Coomassie blue stain, 4-12% Bis-Tris
Results of Western Blot Analysis

The other three gels from the 2DE procedure were transferred to nitrocellulose for Western blot. Below are the results of the western blot analysis, with the gels incubated in the normal antibody, the MSS antibody, and the HSS antibody, respectively, in order to isolate the antigens only reacting to the specific types of antibodies.

FIG. 8: Western blot of antibody-specific antigens: normal, MSS, and HSS, respectively.
Results of Protein Analysis

By comparing the western blots of the different antibodies, it was possible to determine which SEA are unique to each type of antibody. As illustrated above, proteins 1, 2, and 3 are unique to MSS. Via the NCBI database, they have been identified as Calreticulin precursor, Calreticulin, and SJCHGCO1881 protein, respectively. Antigens 4 and 5 are unique to HSS. Antigen 5 has been identified as Enolase (2-phosphoglycerate dehydratase), but the identity of antigen 4 is unknown. The genInfo identifier (gi) number of Calreticulin precursor and Calreticulin is 1345835. The SJCHGCO1881 protein has a gi number of 56752775. Enolase’s gi number is 3023710.
CHAPTER IV

DISCUSSION

The purpose of this research is to search for a more effective anti-pathology vaccine for schistosomiasis. Using the identified proteins from the MSS disease state in a vaccination should, in theory, promote down-regulation of the disease.

To understand why this could occur, the documented characteristics of each protein should be examined. Using the UniProt database, this was possible. According to UniProt, Calreticulin and its precursor function in calcium binding that promotes healthy endoplasmic reticulum functioning (“Q06814 - CALR_SCHMA [Database record]”). The other protein of the MSS disease state, SJCHGCO1881, functions as a Phosphatase 2A inhibitor (“Q5DHV7 - Q5DHV7_SCHJA [Database record]”). Only one protein of the HSS disease state, Enolase, is documented, and it functions as a catalyst for the creation of phosphoenolpyruvate and water from 2-phospho-D-glycerate during glycolysis and carbohydrate digestion (“Q27877 - ENO_SCHMA” [Database record]). Future research would involve the effects of these proteins on the parasite’s functioning.

Alternate interpretations of this data must be considered. Another possible outcome indicated by this data is that there could be an isotype difference between the MSS and HSS disease states, which could cause the different immune responses of down-regulation or inflammation, respectively. Another alternate interpretation to consider is that within the estimated thirty identical proteins found in both disease states, there could be an isotype to the same antigen recognized in both disease states that promotes a down-regulatory or inflammatory response. In order to confirm if unique antigens are responsible for the different immune responses, immunoprecipitation must be performed to isolate and concentrate the proteins in question.
followed by further proteomic analysis. After that, trials to challenge CBJ/A mice with the antigens could begin to investigate whether the mice are protected against the inflammatory response after vaccination.

Throughout the literature, a limited number of researchers have investigated the possibility of an anti-pathology vaccination for schistosomiasis. A study from 2002 investigated the possibility of using carbohydrate antigens in anti-pathology vaccines. The N-glycans investigated expressed LDN, LDNF, and polymeric Lewis x (Le\(^x\)) \([\text{Galb1–4(Fuca1–3)}\text{GlcNAc}]n\text{-R}\) epitopes. The researchers found that \(S.\) \textit{mansonii}-infected patients primarily produced antibodies to LDN, LDNF, and Le\(^x\). However, the strongest immune responses resulted from exposure to LDN and LDNF (Nyame et al., 2003). Future studies could involve identifying these antigens in MSS and HSS and investigating the efficacy of the down-regulation effect of any N-glycans unique to MSS. Some researchers have considered the possibility of combining antigens in order to enhance effectiveness of antigen-based vaccinations. Researchers at Alexandria University in Alexandria, Egypt have investigated the combined effect of antigens Sm14 and Sm29 in mice infected with \(S.\) \textit{mansonii}. The effect proved positive, with a “40.3\%, 68.2\%, and 57.9\% reduction in adult worm burden, liver egg burden and intestinal eggs, respectively” (Ewaisha et al., 2014). Future tests could involve preparing vaccinations with combinations of the identified MSS and HSS antigens and other identified anti-pathogenic antigens, such as those mentioned above, to increase efficacy of a possible vaccine. In a more recent study, published in August 2014, Dr. Barbara C. Figueiredo et al. tested the potential of \(S.\) \textit{mansonii} syntenin (SmSynt) as an antigen in an anti-pathology vaccine in hopes that it would hinder the growth and development of the parasite. This protein was investigated because it is especially present in the worm’s intravascular stage of life (the stage in which it infects humans), and it is involved in a variety of
essential functions related to protein scaffold support within the worm. It is also thought to be involved in the blood-feeding of the parasite. During the study, the research team immunized C57BL/6 mice with three doses of the vaccine formulated as 25 mg of histidine-tagged rSmSynt or as 25 mg of a PBS control. They found that the vaccinated group showed an increase in antibodies produced, reduced liver granuloma, and reduced adult worm burden as compared to the control. However, given that the reduction in adult worm burden was proportional to the reduction in female worm burden, the researchers believe that the vaccination did not affect the female’s ability to produce and release eggs. Because of the Th-1 immune response resulting from vaccination with SmSynt, the researchers suggest that the partial protection provided by the antigen could be combined with another antigen to create a more successful vaccine (Figueiredo et al., 2014). Combining SmSynt with an MSS antibody could potentially increase down-regulation of the disease while simultaneously inhibiting cellular functions within the worm.
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