Identifying key antigens that drive antibody-mediated regulation of chronic schistosomiasis

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Abstract

Chronic schistosomiasis is a spectral illness where patients present with either moderate intestinal (INT) schistosomiasis or severe hepatosplenic schistosomiasis (HS). The different clinical manifestations are associated with the immune response in the patient against schistosome eggs that end up lodged in the liver and other organs. Inbred male CBA/J mice infected with *Schistosoma mansoni* present with two distinct syndromes, moderate splenomegaly syndrome (MSS) and hypersplenomegaly syndrome (HSS); the pathologies of which parallel human INT and HS forms, respectively. Furthermore, antibody with a cross-reactive idiotype (CRI) is produced in MSS mice and INT patients (Montesano, Freeman et al. 1990^a) that is absent in HSS mice and HS patients and is believed to play a protective role against severe pathology.

Our primary aim was to determine the specific antigens in soluble egg antigen (SEA) that react to protective CRI+ anti-SEA antibodies using the CBA/J mouse model of disease. Using one-dimensional Western blotting, we have shown that the specificities of anti-SEA antibodies present in MSS and HSS forms of schistosomiasis are different. Using two-dimensional differential in-gel electrophoresis, we isolated six spots that were of significant interest as disease-specific candidates and a further six candidates that were infection-specific. Finally, we characterized these antigens using mass spectrometry, where we managed to identify several of these isolated antigens. Our infection-specific antigens can be useful in developing diagnostic tools to identify individuals infected with

schistosomiasis, and individuals who are at risk of developing severe disease. Additionally, we found evidence for antigens that are specifically reactive to CRI+ antibodies and these can be used as candidates for the development of a vaccine that can drive the production of these protective antibodies. We also found evidence of CRI- antibody-specific antigens, and further analysis of these antigens may help increase our understanding of severe disease induction in chronic patients. Overall, this study identified several unique candidate antigens that merit further research in developing diagnostic and therapeutic tools, and added to our understanding of helminth interactions in human biology.

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List of Abbreviations

BSA: bovine serum albumin

CRI: cross-reactive idiotyp

DIGE: differential in-gel electrophoresis

ELISA: enzyme-linked immunosorbent assay

HS: hepatosplenic

HSS: hypersplenomegaly syndrome

HSS-Id: hypersplenomegaly syndrome idiotyp

IEF: isoelectric focussing

INT: intestinal

MALDI-TOF: matrix-assisted laser desorption/ionization time-of-flight

MSS: moderate splenomegaly syndrome

MSS-Id: moderate splenomegaly syndrome idiotyp

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate buffered saline

SEA: soluble egg antigen

Chapter 1: Introduction

1.1 Epidemiology

Schistosomiasis (also known as bilharzias) is a tropical parasitic disease caused by worms of the genus *Schistosoma*. The main species associated with human disease are *S. mansoni*, *S. haematobium*, and *S. japonicum*.(Gryseels, Polman et al. 2006)

Schistosomiasis is one of the most widespread human parasitic infections and is second only to malaria in terms of socioeconomic and public health importance in tropical and subtropical regions. It is endemic in 74 countries of the world and is the most prevalent water-borne disease in low to middle income countries.(Doumenge, Mott et al. 1987)

The parasite is estimated to infect more than two hundred million people from rural and agricultural areas in endemic countries, with up to six hundred million more people exposed to infection due to substandard living and sanitary conditions.(Iarotski and Davis 1981) In these low to middle income countries fourteen thousand deaths were caused directly by schistosomiasis in 2001, two thousand of which were from the Sub-Saharan Africa region. Furthermore, 1.53 million disability adjusted life years due to significant disease associated mortality were estimated for low to middle income countries in 2001, 1.18 million of which were from the Sub-Saharan Africa region.(Lopez, Mathers et al. 2006)

The causative parasite within the scope of this study is *Schistosoma mansoni* which is acquired from freshwater snails of the genus *Biomphalaria*.(Doumenge, Mott et al. 1987)

S. mansoni has a large geographical distribution, the majority of which is found in Sub-Saharan Africa and northeast Brazil (Figure 1.1). *S. mansoni* is also found in Suriname, Venezuela, the Caribbean, lower and middle Egypt and the Arabian peninsula.(Gryseels, Polman et al. 2006) The prevalence of *S. mansoni* infection in most endemic countries is generally greatest in the 10-24 year-old age group. It is estimated that 5-25% of the infected population excrete the eggs that contaminate the environment, and most of these individuals are 10-14 years of age.(Doumenge, Mott et al. 1987)

Figure 1.1: Global distribution of schistosomiasis.(Gryseels, Polman et al. 2006)

S.mansoni (green) is mainly found in Sub-Saharan Africa and northeast Brazil. *S. mansoni* is also found in Suriname, Venezuela, the Caribbean, lower and middle Egypt and the Arabian peninsula.

1.2 Lifecycle of *S. mansoni*

Schistosomes have a complex lifecycle, with humans being one of the main definitive hosts and fresh water snails the intermediate host (Figure 1.2). Infected individuals excrete *S. mansoni* eggs in the faeces and, upon contact with fresh water, the eggs hatch to free the miracidium which must find and penetrate the snail host. The miracidium transforms into highly replicating sporocysts which migrate from the site of penetration to the hepatopancreas of the snail. Here, the daughter sporocysts differentiate into thousands of cercariae. Cercariae are released from the snail into the environment following a circadian rhythm.(Rowan 1958) These highly motile, free-swimming, infective cercariae reside in fresh water areas in search of the definitive host.

Upon contact, the cercariae actively burrow through the skin of a person and enter the bloodstream becoming schistosomula. Schistosomula are capable of evading the person's immune system (Dissous, Torpier et al. 1990) and undergo further maturation in the lungs before migration to the liver. In the liver sinusoids, the schistosomula mature to adults, and males and females begin pairing (Figure 1.3). Once the pairs are fully mature, they migrate to the mesenteric veins of the host, where egg laying commences about five to six weeks after initial infection.(Warren, Domingo et al. 1967) Hundreds of eggs per day are released by the schistosome pairs, many of which are excreted with the faeces into the environment thus completing the lifecycle. The rest of the eggs fail to be excreted and are instead swept into the portal vein where they become lodged in the liver

or other organs where the eggs induce granulomatous lesions.(Gryseels, Polman et al. 2006)

Figure 1.2: Lifecycle of *Schistosoma mansoni* (Source: CDC)

Figure 1.3: Two images of male and female worm pairs (Source: CDC)

1.3 Acute phase schistosomiasis

After infection, the first stage of schistosomiasis is dermatitis due to the cercarial penetration of the patient's skin. At the site of cercarial penetration, a rash, otherwise known as swimmer's itch, can appear that persists for days. This dermatitis is reported more frequently in tourists and migrants (Visser, Polderman et al. 1995) however the dermatitis may be more common in endemic countries than reports indicate.(Appleton 1984) The acute phase of schistosomiasis, also known as Katayama fever, develops two to ten weeks after infection with the parasite, especially in patients who are exposed to the parasite for the first time, such as tourists and migrants.(Visser, Polderman et al. 1995) In endemic areas, the acute phase may pass unnoticed as infection occurs early in the patient's life and diagnosis may not be suspected.(Lambertucci 1993)

The acute phase in humans coincides with the schistosomula maturation, migration, and early egg-laying of the schistosome worm pairs.(Visser, Polderman et al. 1995) Deposition of schistosome eggs in the liver from the portal vein occurs five to six weeks after infection. An inflammatory response against these eggs occurs, resulting in granulomas surrounding them. The granuloma is a local inflammatory response composed of lymphocytes, eosinophils, and macrophages, which take up egg antigen. Six to nine weeks after egg deposition, the eosinophil-rich granulomas reach their peak size. The granuloma size diminishes thereafter, leading into the chronic phase.(Warren, Domingo et al. 1967)

The anatomic features of the acute disease include disseminated granulomas that reside in the liver, lung, pancreas and lymph nodes.(Gryseels, Polman et al. 2006; Caldas, Campi-Azevedo et al. 2008) This is usually associated with fever ($<39^{\circ}\text{C}$) and respiratory complaints, followed predominately by gastrointestinal symptoms due to passage of eggs from the bloodstream into the gut. Early inflammatory reaction to eggs trapped in the patient's liver is the main cause of hepatomegaly in children and adolescents infected with schistosomiasis.(Gryseels, Polman et al. 2006) Furthermore, these groups tend to have the highest faecal egg counts.(Kardorff, Stelma et al. 1996)

The symptoms of acute schistosomiasis vary according to the individual. For example, fever may or may not be present during infection yet fever may be so severe that patients require immediate hospitalisation. This can be accompanied by lung and abdominal symptoms and dyspnea.(Bottieau, Clerinx et al. 2006) Despite the inconsistency, morbidity can be correlated to patient egg output/burden irrespective of the patient's age or amount of water contact during the acute phase.(Rabello, Garcia et al. 1996)

1.4 Chronic phase schistosomiasis

Schistosome worm pairs may persist in the patient for over five to eight years while hundreds of eggs per day are released by the worm pairs in the mesenteric venules.(Hotez, Brindley et al. 2008) In humans, chronic schistosomiasis is a spectral

illness where patients present with one of two clinical forms. These are moderate, intestinal schistosomiasis (INT) or hepatosplenic schistosomiasis (HS). The differential clinical manifestations are associated with the immune response in the host against schistosome eggs that end up lodged in the liver and other organs. Generally, eggs secrete proteolytic enzymes that provoke eosinophilic and granulomatous inflammation around the eggs. Therefore, the severity of symptoms depends not only on the infection intensity but also the patient's own immune responses in resolving this granulomatous inflammation.(Gryseels, Polman et al. 2006)

1.4.1 Intestinal schistosomiasis

Most chronically infected patients (~90%) develop INT schistosomiasis.(Nash, Cheever et al. 1982) Common symptoms are abdominal pain and discomfort, loss of appetite, and diarrhoea.(Gryseels, Polman et al. 2006) This is associated with the presence of the adult schistosome worms in the mesenteric vasculature and the migration of eggs through the intestinal wall which cause lesions to occur in the bowel.(Cheever, Kamel et al. 1978)

Six to nine weeks after egg deposition, the eosinophil-rich granulomas reach their peak size. The granuloma size diminishes thereafter.(Warren, Domingo et al. 1967) Thus, response to the eggs that became lodged in the liver is regulated and inflammation surrounding the eggs is limited, minimising damage that occurs in the liver. However, chronic schistosome infection can be linked to more insidious persistent health conditions

such as anaemia, growth retardation, malnutrition, fatigue and poor cognitive development.(Hotez, Brindley et al. 2008) Although these conditions characterise more subtle morbidities among patients, they still result in significant negative effects for economic production and quality-adjusted life years.(Lopez, Mathers et al. 2006)

1.4.2 Hepatosplenic schistosomiasis

After years of infection, patients with diffuse periportal thickening may develop severe periportal thickening, over two thirds of which also develop splenomegaly.(Kardorff, Stelma et al. 1996; Abath, Morais et al. 2006) These symptoms constitute HS schistosomiasis which is more commonly found in adolescents and young adults.(Kardorff, Stelma et al. 1996; de Jesus, Miranda et al. 2000) Up to ten percent of infected individuals progress to this most severe disease form.(Nash, Cheever et al. 1982) The development of HS schistosomiasis is not solely due to infection intensity as the clinical signs, such as abdominal pain, weakness, diarrhoea and inability to work, are still present despite low infection intensity.(Tanabe, Gonçalves et al. 1997)

HS schistosomiasis morbidity is caused by a dysregulated immune reaction resulting in liver fibrosis and severe complications due to the fibrosis.(Warren 1975; Dunne and Pearce 1999) During the acute phase, granulomatous inflammation to eggs that were deposited in the peripheral portal veins occurs. The egg granulomas are composed of lymphocytes, eosinophils and macrophages. Six to nine weeks after egg deposition, the

granulomas reach their peak size towards the end of the acute phase.(Boros and Warren 1970) However, leading into the chronic stage of infection, the immune response is not properly down-regulated at this point.(Warren 1975) After 10-12 weeks the inflammation surrounding the eggs subsides and is replaced by irreversible Symmer's pipestem fibrosis.(Cheever 1968) This fibrosis leads to portal hypertension, splenomegaly, venous collateral circulation, portocaval shunting and gastrointestinal and oesophageal varices. Bleeding from varices is the main complication that commonly leads to death.(Cheever, Hoffmann et al. 2000; Gryseels, Polman et al. 2006)

It is unclear why certain individuals develop this most severe form of disease. Certain factors have been proposed as contributing conditions to its development such as increased worm burden (Cheever 1968) however it is not intensity alone that is responsible.(Mohamed-Ali, Elwali et al. 1999) For example, polymorphisms within the anti-fibrinogenic IFN- γ receptors have been linked to severe schistosomiasis.(Dessein, Hillaire et al. 1999) Furthermore, an IL-13 decoy receptor system, which sequesters IL-13, has been shown to modulate the egg-induced inflammatory response and prolong patient survival depending on its functionality within the infected individual.(Mentink-Kane, Cheever et al. 2003) These findings suggest that an individual's genetic predisposition to developing HS schistosomiasis can also play a key role in the causative mechanisms that lead to severe disease.

1.5 Immunology of Schistosomiasis

1.5.1 Cellular response

Early in acute phase schistosomiasis, lymphocytes react strongly to soluble egg antigen (SEA) indicating an initial, unregulated inflammatory response during initial infection.(Gazzinelli, Montesano et al. 1987) CD4⁺ T cells are also activated at this time and the frequency of these cells is increased.(Martins-Filho, Cunha-Melo et al. 1999) It has been shown that these T cells are required for the granulomatous response during infection.(Hernandez, Wang et al. 1997) In the peripheral blood mononuclear cells (PBMC) there is production of pro-inflammatory cytokines TNF- α , IL-1, IL-6 (de Jesus, Silva et al. 2002) and IFN- γ (Montenegro, Miranda et al. 1999). The majority of SEA-stimulated cultures taken from acutely infected individuals also show high levels of IL-5, which is important in tissue eosinophilia.(Malaquias, Falcao et al. 1997) Thus the cytokine profile of these activated T cells is mixed T helper 1 (Th1) and T helper 2 (Th2).(de Jesus, Silva et al. 2002)

Th2-associated cytokines are highly induced after the onset of egg-laying and deposition (Grzych, Pearce et al. 1991) and these cytokines play a role in patient survival during this disease phase. For example, a failure to mount an initial IL-4 response to SEA exposure results in a severe form of acute schistosomiasis from a lack of Th2 immune modulation, resulting in cachexia and death likely due to excessive reactive oxygen and nitrogen intermediate production.(Brunet, Finkelman et al. 1997) This is supported by treatment with a peroxynitrite scavenger which ameliorates severe disease.(La Flamme, Patton et

al. 2001) Additionally, neutralisation of IL-4 decreases the proliferation response of PBMC upon exposure to SEA suggesting that it plays a role in the general early acute schistosomiasis response.(Malaquias, Falcao et al. 1997) Furthermore, towards the end of the acute phase of schistosomiasis infection, a modified Th2 response develops which is responsible for the down-regulation of granulomatous inflammation leading into chronic schistosomiasis. A Th2 cytokine, IL-6, has been shown to mediate the anti-inflammatory response by down-regulating Th1 responses.(La Flamme, MacDonald et al. 2000) Other anti-inflammatory cytokines, such as IL-10, are produced at this time and are believed to play a role in this down-regulation.(Montenegro, Miranda et al. 1999; Sadler, Rutitzky et al. 2003) The importance of the Th2-associated response is further supported by the fact that a failure to initiate such a response can be fatal.(MacDonald, Patton et al. 2002)

During the chronic phase of schistosomiasis, differences arise in cell populations between patients that have either intestinal (INT) or hepatosplenic (HS) schistosomiasis. PBMC taken from INT patients still retain the proliferation responsiveness to SEA and soluble worm antigen preparation (SWAP) that is present in acute disease; however, this responsiveness is not apparent in HS individuals.(Gazzinelli, Montesano et al. 1987) Furthermore, HS patients have a lower percentage of CD4⁺ T-cells in PBMC and splenic populations, resulting in a lower CD4⁺ T-cell ratio that correlates with their unresponsiveness to parasite antigen.(Colley, Katz et al. 1983)

A balance between Th1/Th2 cytokine responses also play an important role in modulating pathology during the chronic phase of schistosomiasis.(Cheever, Hoffmann et al. 2000) For example, IL-13 is a key pro-fibrotic Th2 cytokine implicated in the progression towards HS schistosomiasis during the chronic phase. It has been shown that severe schistosomiasis does not develop when IL-13 has been rendered ineffective in IL-4R α knockout mice (Jankovic, Kullberg et al. 1999), neutralised by an inhibitor (Chiaramonte, Donaldson et al. 1999), sequestered by the IL-13 decoy receptor system,(Mentink-Kane, Cheever et al. 2003) or removed in IL-13 deficient mice.(Fallon, Richardson et al. 2000) Furthermore, switching the Th2 response in chronic schistosomiasis to a Th1 response using IL-4/IL-10-deficient and IL-4/IL-13-deficient mice resulted in less fibrosis in these mice although the mice died due to the lack of IL-4.(Hesse, Modolell et al. 2001)

Additionally, Th2 cytokines have shown to be valuable in controlling morbidity in chronic schistosomiasis. For example, IL-4 is beneficial as IL-4-deficient and IL-4/13-deficient mice experience increased hepatocyte damage and mortality after infection.(Fallon, Richardson et al. 2000) It has also been shown that HS schistosomiasis in humans is linked with low levels of IL-5 and high levels of IFN- γ , TNF and TNF receptor.(Mwatha, Kimani et al. 1998) Another Th2 cytokine, IL-10, is produced in large amounts early in schistosomiasis disease progression (Montenegro, Miranda et al. 1999) and is also known to play roles in disease regulation and morbidity control during the chronic phase.(Araujo, Jesus et al. 1996) It was demonstrated that neutralization of IL-10 increased the proliferative response of PMBC obtained from INT patients, but had no effect on PBMC from HS or acute patients, indicating that IL-10 modulates INT patient

cell proliferation in the presence of parasite proteins. This is reinforced by the addition of recombinant IL-10 which restored the regulated cell response characteristic of INT patients.(Malaquias, Falcao et al. 1997) Furthermore, neutralisation of IL-10 on SEA stimulated PBMC taken from INT, but not acute or HS, patients result in an increase in granuloma formation.(Falcão, Malaquias et al. 1998) Finally, Th1-polarized IL-10/IL-4-deficient mice, and Th2-polarized IL-10/IL-12-deficient mice both develop severe, yet distinct, forms of schistosomiasis, associated with excessive Th1 or Th2 responses reinforcing the role that IL-10 likely plays in regulating the cytokine production during infection.(Hoffmann, Cheever et al. 2000) This shows that a balance between the Th1 and Th2 responses is critical for a regulated immune response and the contributions of both Th1 and Th2 cytokines during the chronic phase is very complex.

1.5.2 Humoural response

During the acute stages of infection with *S. mansoni* the initial granulomatous response is not B-cell dependant.(Hernandez, Wang et al. 1997) However, B-cells play a role in regulating the initial granulomatous response as it was shown that tissue pathology increased in B-cell-deficient mice.(Jankovic, Cheever et al. 1998) Also, in these B-cell deficient mice, the down-regulation of the granulomatous response leading into the chronic phase did not occur. This indicated that the down-regulation of the CD4+ T-cell response at this point is also B-cell dependant.(Jankovic, Cheever et al. 1998)

Soon after the initial stages of infection, the Th1-dominant profile is followed by a Th2-dominated one.(Pearce, Caspar et al. 1991) While Th1 profiles help cell-mediated immune responses, Th2 profiles can provide help for the humoral response. For example, a lack of IL-6, a Th2 cytokine, leads to an early defective development of B-cells.(La Flamme and Pearce 1999) Using B-cell deficient mice, it was shown that B-cells do not alter the T-cell proliferative responses at this time, nor do they affect the Th1/Th2 balance.(Jankovic, Cheever et al. 1998) Conversely, other studies using B-cell deficient mice showed that B-cells do promote a Th2 response and are therefore critical in driving the Th1/Th2 switch at this point.(Hernandez, Wang et al. 1997)

During the chronic phase of schistosomiasis, there is evidence that there is humoral regulation of the cellular response. It has been proposed that this regulation can be due to idiotypic/anti-idiotypic interactions regulating cellular immunity; interactions prevalent in endemic populations.(Lima, Gazzinelli et al. 1986) For example, T-cells in the PBMC from active and former patients with schistosomiasis can respond well to anti-SEA antibodies. These anti-SEA antibodies express idiotypes that stimulate specific anti-idiotypic T-cell proliferation (Lima, Gazzinelli et al. 1986), and the anti-idiotypic T-cells respond to these anti-SEA idiotypes directly.(Parra, Lima et al. 1988) Additionally, anti-SEA antibodies from chronically infected patients stimulate the PBMC of some former and active schistosomiasis patients, and a patient's response to anti-SEA antibodies is independent of the patient's PBMC response to SEA itself.(Lima, Gazzinelli et al. 1986) Moreover, levels of IgG4 antibody specific to SEA are highest in groups that present with the initial fibrosis, which suggests that a specific humoral response is responsible for the

development of fibrotic schistosomiasis.(Silveira, Bethony et al. 2002) Studies in the humoral responses present in the CBA/J mouse model of schistosomiasis are described later, showing how differential antibody responses to SEA can regulate chronic pathology and drive the outcome of disease.

Specific humoral interactions can also play a role in schistosomiasis sensitisation and may explain why symptoms of acute schistosomiasis are rarely seen in endemic populations. For example, it was shown that children born of infected mothers experienced first contact with schistosomiasis *in utero* through cell-mediated sensitisation from anti-idiotypic antibodies provided by the mother, or by circulating antigen itself. The newborns demonstrated the same IgG anti-schistosome antibody activity as their mother.(Correa-Oliveira, Oliveira et al. 1987; Novatosilva, Gazzinelli et al. 1992) This differed from naive individuals from non-endemic areas, who received their first contact later in life. Furthermore, the production of anti-idiotypic T lymphocytes can begin *in utero* due to idiotypic sensitisation from maternal anti-schistosome antibodies that express the relevant idiotypes.(Eloi-Santos, Novato-Silva et al. 1989)

Antibody specificities have been proposed as diagnostic tools to determine whether a patient has acute or chronic schistosomiasis. Individuals with acute or chronic schistosomiasis do not differ significantly in the amount of IgG and IgM reactive to SWAP and SEA (Rabello, Garcia et al. 1996), although IgA levels are significantly higher in individuals with acute, compared to chronic, schistosomiasis.(Alves-Brito,

Simpson et al. 1992) Reactivity to SWAP has also been shown to increase with the age of the patient, and anti-SWAP antibodies from patients with INT and HS schistosomiasis show a high degree of heterogeneity. However there are subtle differences. Generally, INT patients recognise a 31kDa antigen while HS patients recognise 14kDa and 66kDa antigens. This antigenic recognition has been postulated to be a valuable diagnostic tool.(Correa-Oliveira, Oliveira et al. 1987) Nonetheless, to date no specific differences in antigenic recognition by INT and HS schistosomiasis patients have been identified.

1.6 Chemotherapies and Diagnostics

Praziquantel kills both adult worms and mature eggs and is the drug used for mass chemotherapy programmes for schistosomiasis.(Colley and Secor 2004) The control strategy consists of annual administration of praziquantel to at-risk populations within endemic communities. Review of past programmes has deemed them to be cost-effective in terms of investment versus increased productivity of the treated individual.(Croce, Porazzi et al. 2010) However, praziquantel is the only drug used to treat schistosomiasis and its implementation is not without limitations. For example, there is evidence that resistance to this drug is developing in countries that have been implementing chemotherapy control strategies for some time.(Ismail, Botros et al. 1999) Furthermore, maintaining such programmes is costly and will have to be implemented in the long term.(Siddiqui, Ahmad et al. 2008) Though cost-effective, many affected areas cannot

support such programs autonomously and generally require outside financial assistance.(Croce, Porazzi et al. 2010)

Praziquantel-based chemotherapeutic treatments are widely used but there is no vaccine that is routinely used to prevent schistosomiasis.(Bergquist, Utzinger et al. 2008) A vaccine that is long lasting and potent can contribute greatly to disease control and provide an alternative to chemotherapy treatment programs. It is argued that such a vaccine can be found because mice can be immunized against schistosomiasis by using irradiated cercariae. Also, endemic populations seem to develop some natural immunity.(Richter, Reynolds et al. 1993; Siddiqui, Ahmad et al. 2008) An individual person's immune response plays a role in drug effectiveness as well. For example, it has been shown that complement, linked to Th2 response development, affects the ability of praziquantel to clear schistosome infection.(La Flamme, MacDonald et al. 2003) Thus, it has been suggested that current functional vaccines can be used in conjunction with, rather than alternatively to, existing chemotherapy programs. By using vaccines that moderate the immune response in individuals exclusively treated with praziquantel, it can extend the time between treatments and improve the efficiency of such programs.(Bergquist, Leonardo et al. 2005)

For decades, there has been a need for improved diagnostic markers for schistosomiasis. Mass praziquantel therapy programmes require accurate, sensitive and accessible diagnostic tools in order to differentiate individuals that are sub-clinically infected with

disease, to those that are succumbing to severe disease.(Colley and Secor 2004; Hotez, Brindley et al. 2008) It is also critical to track the effectiveness of such programs and validate control strategies from area to area.(Kabatereine, Kemijumbia et al. 2004) Various diagnostics do exist for schistosomiasis infection but their effectiveness is limited. For example, egg detection via microscopy is specific, however is not very sensitive due to the varying amounts of detectable eggs excreted in host stool. This is especially problematic in chemotherapeutically-treated endemic communities with low intensity infections. It is also ineffective in identifying infection early in the disease course.(Zhang, Luo et al. 2009) Eosinophil counting has been used to help diagnose disease but is not a standalone diagnostic as it is not specific for schistosome infection.(Bottieau, Clerinx et al. 2006) Antigen-based diagnostic tools exist, but fail to reliably distinguish between current and past infection, rendering them unsuitable for measuring treatment success. They also fail to pick up infection in asymptomatic, acute infections. Additionally, they are mainly used clinically in non-endemic areas as there is less risk of co-infection with other helminths, which can cause cross-reactivity.(Chand, Chiodini et al. 2010)

Diagnostic tools to distinguish intestinal patients and patients with or at risk of developing hepatosplenic schistosomiasis may also be critical in focussing control strategies. Sonography can diagnose enlarged livers and spleens during schistosomiasis infection (Dittrich, Milde et al. 1982; Kabatereine, Kemijumbia et al. 2004) and ultrasonography is used to determine the extent of hepatic fibrosis in infected individuals. This technique can be used to diagnose the degree of morbidity and also judge

effectiveness of disease control programs over time.(Homeida, Ahmed et al. 1988; de Jesus, Miranda et al. 2000) Other methods, such as differences in antigenic recognition, have been postulated as diagnostic tools for disease form in individuals infected with schistosomiasis (Correa-Oliveira, Oliveira et al. 1987) but suitable candidate proteins for such tools are still being sought.

1.7 Mouse Model of Schistosomiasis

1.7.1 CBA/J mouse pathology

Inbred male CBA/J mice infected with chronic schistosomiasis present with two distinct syndromes, moderate splenomegaly syndrome (MSS) and hypersplenomegaly syndrome (HSS); the pathologies of which parallel human intestinal (INT) and hepatosplenic (HS) forms, respectively. Because this mouse model consistently develops differential manifestations of disease, it is a valuable tool for studying the molecular pathogenesis of schistosomiasis. Liver and spleen enlargements during the chronic stage distinguish both forms, with these organs significantly enlarged in HSS mice compared to MSS mice (Figure 1.4).

MSS occurs in 80% of infected male CBA/J mice. Although these mice do have granulomatous reactions to eggs in the liver, the amount of fibrosis that follows is significantly less than what is found in HSS. Thus, these mice are relatively

asymptomatic and suffer only from characteristic moderate splenomegaly.(Henderson, Nix et al. 1993)

HSS is analogous to the severe HS form in humans and 20% of infected male CBA/J mice spontaneously develop this form. Similar to HS schistosomiasis in humans, HSS is associated with the immune response that is initiated in response to eggs lodged in the liver. As is believed to occur in humans, the immune response is dysregulated resulting in extreme splenomegaly, profound anaemia, cachexia, ascites and thymic atrophy. Finally, there is extensive hepatic fibrosis and granulomatous inflammation like in human HS schistosomiasis.(Henderson, Nix et al. 1993)

Figure 1.4: Infected CBA/J mice with HSS develop severe splenomegaly compared to MSS and normal mice. (Henderson, Nix et al. 1993)

1.7.2 Cellular response in CBA/J mice

A difference in inflammatory cytokines (especially TNF- α , IL-4, IL-5 and GM-CSF) is believed to be responsible for the differential development of granulomatous inflammation surrounding eggs in the livers of infected MSS and HSS mice. HSS hepatic cells produce up to three fold more TNF- α than MSS mice suggesting that a failure to down-regulate TNF- α production in the liver contributes to fibrotic HSS pathology.(Adewusi, Nix et al. 1996) However it has also been found that spleen cell TNF- α is correlated to less severe, MSS pathology (Cetre-Sossah, Montesano et al. 2007) suggesting that the pathological role of TNF- α in schistosomiasis is complex. IL-4, IL-5 and GM-CSF intracellular expression in splenic CD4+ T-cells correlates to more severe pathology (Cetre-Sossah, Montesano et al. 2007) which is expected of Th2 cytokines implicated in granuloma formation and fibrosis. Additionally, T-cell specificity is believed to have a role in the cellular response of infected individuals as it has been found that V β TCR usage, which is a major determinant of T-cell antigen specificity, is significantly different between MSS and HSS T-cell repertoires.(Secor and Freeman 2001)

1.7.3 Humoural response in CBA/J mice

The most striking immunological feature of chronic schistosomiasis in CBA/J mice is the production of distinct anti-soluble egg antigen (SEA) antibodies that are exclusively produced in chronic MSS mice and acutely infected mice that eventually develop MSS,

but not in HSS mice.(Henderson, Nix et al. 1993; Montesano, Colley et al. 2002) Anti-SEA antibodies from MSS mice and INT patients both produce a cross reactive idiotype (CRI) which is not present in the HSS/HS form.(Montesano, Freeman et al. 1990^a) These CRI+ anti-SEA antibodies have been shown to modulate hepatic cellular responses which may result in less severe chronic phase pathology. For example, anti-SEA antibodies from MSS mice have been shown to stimulate IFN- γ production in splenic cells.(Montesano, Colley et al. 1999^a) These differ from anti-SEA antibodies from acutely infected mice, which stimulate IL-4 and IL-10 production, and anti-SEA antibodies from HSS mice which do not stimulate cytokine production at all.(Montesano, Freeman et al. 1997) Furthermore, the CRI+ MSS idiotypic antibodies are produced five to six weeks into infection, indicating that the immune response is fixed by then and that the clinical outcome of disease can be predicted during the acute phase of infection in mice.(Montesano, Colley et al. 2002)

Finally, it has been shown that mice from uninfected mothers which received neonatal injections of CRI+ anti-SEA antibodies had less severe pathology and increased survival eight weeks after infection with schistosomiasis, compared to mice that were injected with CRI- anti-SEA antibodies.(Montesano, Colley et al. 1999^a) Thus, a balance between specific antibody responses and their relationship to T-cell stimulation is important to the progression of disease in the acute and chronic phase.

1.8 Aims & Objectives

The current hypothesis is that CBA/J mice with moderate splenomegaly syndrome (MSS) and hypersplenomegaly syndrome (HSS) share anti-soluble egg antigen (SEA) idiotypic repertoires to humans with intestinal and hepatosplenic forms, respectively (Montesano, Freeman et al. 1990^a), and that the presence of anti-SEA antibodies bearing cross-reactive idiotypes (CRI) play a role in the regulation of the immune reaction against schistosome eggs that are present in the liver of infected patients.(Montesano, Colley et al. 2002)

CRI+ anti-SEA antibodies have a number of important characteristics that suggest their protective role. For example, neonatal injection containing CRI+ anti-SEA antibodies have shown to alter the development of pathology in mice after subsequent schistosome infection.(Montesano, Colley et al. 1999^a; Montesano, Colley et al. 1999^b) Furthermore, CRI+ anti-SEA antibodies have been directly correlated to the production of the Th2 cytokines implicated in MSS infection, such as IL-4 and IL-10, and have found to stimulate differential T cell proliferation and cytokine production.(Montesano, Freeman et al. 1997; Secor and Freeman 2001)

The aim is to determine what specific antigens in SEA react to CRI+ anti-SEA antibodies using the CBA/J mouse model of disease. By elucidating these antigens, they may be useful in developing differential diagnostic markers to determine an individual's immune response to schistosome infection. These antigens may also be useful in determining a vaccine based on driving the production of a regulated, protective immune response. An

effective vaccine lies in its ability to reduce morbidity by decreasing infection, schistosome fecundity or, in this case, pathology.(Siddiqui, Ahmad et al. 2008) Also, these antigens may be helpful in further understanding the immunological mechanism behind CRI+ anti-SEA antibody induction and help control severe pathology in afflicted individuals.

Chapter 2: General Methods

2.1 Mouse and soluble egg antigen samples

Individual mouse sera samples were provided by E. Secor and contained sera from infected male CBA/J mice with moderate splenomegaly syndrome (MSS), infected mice with hypersplenomegaly syndrome (HSS) or normal, uninfected mice. The individual MSS samples were designated as 1C, 2C, 3C, 7C, 8C and 9C. The HSS samples were designated 5C, 6C, 10C, 12C and 17C. The uninfected samples were designated 1N, 2N, 3N, 4N and 5N. These sera samples contained anti-soluble egg antigen (SEA) antibodies that were used for our experiments. As shown in Table 2.1, it can be noted that spleen/body weight ratio is the main indicator of differentiating MSS or HSS disease for mice with chronic disease

Pooled sera samples were also provided by E. Secor. The samples contained pooled antibodies from a group (~50 individuals) of CBA/J mice which presented with either MSS or HSS chronic disease and were suspended in PBS (145mM NaCl, 10mM NaH₂PO₄). These pooled MSS or HSS antibodies were affinity-purified on an SEA-bound sepharose bead column. As such, these antibodies were SEA-specific and are referred to as pooled MSS-idiotypic (MSS-Id) and HSS-idiotypic (HSS-Id). Anti-SEA concentrations for individual sera and pooled Id samples are listed in Table 2.1 and were determined from experiments we carried out in Chapter 3.5.

SEA used for Western blotting was provided E. Secor, and is of the same SEA used to affinity-purify the MSS- and HSS-Id. This ensured that all anti-SEA IgG present in the pooled Id samples would react to the SEA. This SEA was suspended in PBS (145mM NaCl, 10mM NaH₂PO₄). SEA samples that were suspended in lysis buffer (30mM TrisHCl, 2M Thiourea, 7M Urea, 4% (w/v) CHAPS) were provided by B. Manivannan (Uradey) and were preferably used for mass spectrometry. However, the PBS suspended SEA samples were also used, provided they were precipitated to remove the contaminating PBS buffer which interfered with isoelectric focussing when used in amounts required for mass spectrometry.

MSS mouse sera sample data			
Mouse number	Sample date	SBW ratio	[anti-SEA IgG](mg/mL)
1C	17/12/2003	0.42	6.15E-02
2C	10/12/2003	0.54	1.46E-01
3C	17/12/2003	0.54	6.21E-03
7C	17/12/2003	0.45	3.89E-02
8C	17/12/2003	0.68	1.72E-02
9C	17/12/2003	0.8	3.23E-01

HSS mouse sera sample data			
Mouse number	Sample date	SBW ratio	[anti-SEA IgG](mg/mL)
5C	17/12/2003	1.71	3.33E-02
6C	17/12/2003	2.16	4.05E-02
10C	17/12/2003	2.29	2.02E-02
12C	17/12/2003	3.27	6.89E-02
17C	24/12/2003	2.14	6.34E-03

Uninfected mouse sera sample data		
Mouse number	Sample date	SBW ratio
1N	2/01/2004	0.24
2N	2/01/2004	0.43
3N	2/01/2004	0.25
4N	2/01/2004	0.23
5N	2/01/2004	0.21

Pooled sera sample data	
Type	[anti-SEA IgG](mg/mL)
MSS-Id	6.53E-01
HSS-Id	2.41E-02

Table 2.1: Data tables of pooled and individual sera samples used in all experiments

The above tables indicate sera sample collection date and spleen/body weight (SBW) ratio of the individual mouse, distinguishing MSS or HSS disease state. For MSS mouse sera, HSS mouse sera and pooled sera samples, anti-SEA IgG concentrations were elucidated in Chapter 3.5.

2.2 Enzyme-linked immunosorbent assay

The concentration of anti-SEA antibodies in each sera and Id sample was determined by antigen-specific enzyme-linked immunosorbent assay (ELISA). Absorbance values of antibodies at various dilutions were related to the linear range of the moderate splenomegaly syndrome idiotypic (MSS-Id) dose response curve standard.

BD Falcon® Microtest™ 96-well ELISA plates were coated with 50µL Coating buffer (pH ~ 9.0 0.1M sodium carbonate / sodium bicarbonate buffer) containing 0.5µg soluble egg antigen per well. The plates were incubated overnight at 4°C. The next day, the antigen was removed and the plates washed 5 times in Wash buffer (0.05% Tween-20 in PBS). To block, 200µL ELISA Blocking buffer (5% fetal calf serum in PBS) was added to each well and the plates were incubated for 1 hour at 37°C.

The ELISA Blocking buffer was removed and the plates washed 6 times in Wash buffer. To all wells, 100µL ELISA Blocking buffer was added. To the first column, samples containing primary anti-SEA antibody were added at 1:105 dilution and five-fold serial dilutions were carried out. The plates were incubated for 2 hours at 37°C. After incubation, the plates were washed 6 times in wash buffer to remove the primary antibody solution. 100µL secondary antibody solution containing horseradish peroxidase-labelled anti-mouse IgG diluted 1:5000 in blocking buffer was added. The plates were incubated for 1 hour at room temperature.

After incubation, the plates were washed 8 times in Wash buffer to remove the secondary antibody solution. 100µL freshly prepared 1:1 solution of BD OptEIA™ TMB Substrate Reagent Substrate A (containing hydrogen peroxide, warmed to room temperature) and Substrate B (containing TMB, warmed to room temperature) was added to each well. The reaction was left to develop for 1 minute, and stopped with the addition of 100µL Stop solution (0.18M H₂SO₄) to each well. The plates were read at 405nm, using a Molecular Devices tunable microplate reader, immediately after completion of the development reactions.

2.3 Bradford assay

Enzyme-linked immunosorbent assay (ELISA) comparing moderate splenomegaly syndrome idiomotype (MSS-Id) and hypersplenomegaly syndrome idiomotype (HSS-Id) levels were hard to determine as the linear range of the dose response curves did not overlap. A Bradford assay was used to determine total protein concentrations of the HSS-Id and MSS-Id samples as the samples were immunity column purified for schistosome egg antigen (SEA). This gave the protein concentrations of the purified pooled sera, which could be compared to anti-SEA IgG concentrations obtained in the ELISA.

BD Falcon® Microtest™ 96-well flat bottom tissue culture plates were used. To generate the standard solutions, phosphate buffered saline (PBS) was added to bovine

serum albumin (BSA) at a $\frac{3}{4}$ dilution starting at 2mg/ml, for a total of 12 standards. In each well, 1 μ L standard solution was combined with 19 μ L MilliQ water and 250 μ L Bradford's reagent (0.01% (w/v) Coomassie Blue Dye, 50mL, 5% (95%) ethanol, 10% (85% w/v) phosphoric acid, filtered before use). To determine the concentration of the MSS-Id pooled sera sample in duplicate wells 2 μ L MSS-Id sample was combined with 18 μ L PBS and 250 μ L Bradford's reagent. To determine the concentration of the more diluted HSS-Id pooled sera sample in duplicate wells 18 μ L HSS-Id sample was combined with 2 μ L PBS and 250 μ L Bradford's reagent. Blank wells contained 20 μ L PBS with 250 μ L Bradford's reagent. Wells were mixed by light tapping on the side of the plate and left for ten minutes. The plates were read at 570nm, using a Molecular Devices tunable microplate reader, immediately after completion of the development reactions.

2.4 One-dimensional polyacrylamide gel electrophoresis

One-dimensional polyacrylamide gel electrophoresis (PAGE), in conjunction with Western blotting, was used to compare the ability for moderate splenomegaly syndrome (MSS) and hypersplenomegaly syndrome (HSS) mouse sera to recognize soluble egg antigen (SEA) to see if there are any notable differences in specificities between clinical groups.

SDS-PAGE gels (10% separating gel and 4% stacking gel, 1% sodium dodecyl sulphate) were prepared and placed in a BioRad electrophoresis tank. Load samples were prepared by combining 5 μ L SEA (1.1mg/mL) with 6 μ L Running buffer (Tris 25mM, Glycine 192mM, SDS 0.1% (w/v)) containing Coomassie blue and MilliQ water to make up to 15 μ L. The central chamber was filled with Running buffer and the 15 μ L samples were loaded to appropriated lanes. 2 μ L protein ladder was also added. The gels were run at room temperature at 200V for 55 minutes, or until the dye front ran off the bottom of the gel. The apparatus was disconnected and the gel immediately underwent Western transfer.

2.5 Two-dimensional polyacrylamide gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (PAGE), as part of differential in-gel electrophoresis (DIGE), was used to compare the ability for moderate splenomegaly syndrome (MSS) and hypersplenomegaly syndrome (HSS) mouse sera to react to soluble egg antigen (SEA) to see if there are any notable differences in reactivity between clinical groups. Two-dimensional PAGE, in conjunction with protein precipitation, was used to obtain gel spots for matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF).

For first dimension separation of protein based on isoelectric point, the protein sample was made up to a volume of 125 μ L with Rehydration buffer (2M Thiourea, 7M Urea, 2% IPG buffer 3-10, 2% DTT, 4% (w/v) CHAPS, with bromophenol blue). Adequate amounts of protein and Rehydration buffer were prepared for every IPG strip (Immoboline™ Drystrip pH 3-10 non-linear, 7cm) to be separated via isoelectric focussing (IEF).

The protein sample was vortexed and spun down and incubated at room temperature for 15 minutes. After incubation the sample was loaded onto the lane of a rehydration tray about the length of an IPG strip. A new IPG was laid on the sample, gel-side down and allowed to rehydrate in the protein sample for 30 minutes. It was important to check that there were no bubbles under the strip. To prevent dehydration of the IPG strips during the rest of the incubation, the strip was covered with 3mL Covering Oil which was loaded into the lane of the rehydration tray containing the strip. The rehydration lid was replaced and the strip was left to incubate overnight at room temperature.

The next day, the rehydrated strip underwent IEF on the Ettan™ IPGphor II™ Isoelectric Focusing System. To set up the system, 110mL Covering Oil was poured onto the ceramic focussing tray. Each lane can be used once for each IPG strip. The IPG strip was placed on the focussing tray, gel-side up and the acidic end towards the anode. Damp paper wicks were placed on each gel end of the IPG strip and the apparatus electrodes make contact with these to facilitate the flow of current through the IPG gel

strip. The focussing parameters used for the system to focus the 3-10 IPG strips were as follows: Phase S1, Step, 300V for 30 minutes; Phase S2, Gradient, 1000V for 30 minutes; Phase S3, Gradient, 5000V for 1 hour 25 minutes; Phase S4, Step, 5000V for 25 minutes. When prompted, the number of strips to be focussed was adjusted accordingly, and the temperature was set at 20°C. Once focussing began, the current started at 30µA to 50µA and gradually rose to 50µA before S2 phase began (depending on the sample volume used).

At the conclusion of the IEF, to prevent diffusion of the protein in the gel, the strip was removed immediately from the system and either the strip underwent gel electrophoresis (next step of Two-Dimensional Electrophoresis) or frozen at -80°C until further use. The start and end current and voltage were recorded and, if possible, the LOGfile for the IEF was saved for future reference.

For second dimension separation based on size, immediately following the conclusion of IEF or from the -80°C frozen state, each strip was equilibrated in 2.5mL fresh 1% DTT Equilibration buffer (50mM Tris, 6M Urea, 30% Glycerol, 2% SDS, fresh 1% Dithiothreitol, with bromophenol blue) for 10 minutes at room temperature. This was followed by equilibration in 2.5mL fresh 2.5% IAA Equilibration buffer (50mM Tris, 6M Urea, 30% Glycerol, 2% SDS, fresh 2.5% Iodoacetamide, with bromophenol blue) for 10 minutes at room temperature.

During equilibration, the PAGE system was set up. For each strip to be run, a Precast gel (Invitrogen Novex Mini Cell NuPAGE® 4-12% Bis-Tris ZOOM™ Gel, 1.0mm x IPG well) was set up in the PAGE apparatus, making sure to remove excess gel from the strip well and checking for quality of the gel, e.g. correct gel casting. The IPG strip was cut to size and fitted into the single well of the precast gel, with the acidic end toward the marker well and gel-side toward the central chamber. MOPS NuPage working buffer (40 mL Stock MOPS, 760 mL MilliQ water) was placed in the central chamber first to ensure the chamber is sealed, followed by the outer chamber (800mL needed). 500µL antioxidant (Invitrogen NuPAGE® antioxidant) was added to the central portion of the tank and the gels were run at 200V for 55 minutes. At the conclusion of the PAGE separation the gel was removed immediately from the system, to prevent diffusion of the protein in the gel, and either underwent Western transfer (see methods on Western Blot) or gel staining.

For gel staining immediately following two-dimensional PAGE, the gel was removed from the system and placed in Fixer (50% ethanol, 3% phosphoric acid) from 30 minutes to overnight. After the fixation, the gel was washed in MilliQ water three times at 30 minutes each or left overnight. After washing, the gel was placed in Colloidal staining solution (17% ammonium sulphate, 34% methanol, 3% phosphoric acid) from 30 minutes to overnight. After priming, a few grains of Coomassie G-250 were placed in the Colloidal staining solution and the gel was left overnight for staining. After staining, the gel was washed in MilliQ water as needed before being scanned using a Molecular Dynamics Personal Densitometer SI.

2.6 Western blot

One-dimensional or two-dimensional polyacrylamide gel electrophoresis (PAGE) was carried out as required prior to Western blot. Filter paper (Whatman® 3mm chromatography paper) sheets were cut with the gel (two sheets per gel) such that it had a slightly larger area than the gel. The filter paper, along with fibre gel pads (two pads per gel) were soaked in Western Transfer buffer (25 nM Tris, 192 mM Glycine, 20% Methanol v/v) in the arrangement tray until needed.

PVDF membranes (Amersham Biosciences Hybond-LFP PVDF transfer membranes) were cut to the size of the gel (one membrane per gel), taking care to avoid the membrane being of a larger area than the gel (which may affect the flow of current during the transfer). The membranes were soaked in 100% methanol for 20 seconds, soaked in MilliQ water for 20 seconds then left to equilibrate in Western Transfer buffer for 10 minutes. Gels were removed from their casts and stacking gel and excess separating gel were removed with a spatula. The prepared gel was left to equilibrate in Western Transfer buffer for 8 minutes.

The sandwich for the gel was opened and the black side of the holder was laid down. Stacked on top was the fibre gel pad, followed by a piece of filter paper. The gel was placed on top of the filter paper. The membrane was placed on top of the gel, followed by a second sheet of filter paper and fibre gel pad. To avoid bubbles in between the layers which could affect transfer of protein, bubbles were rolled away with a roller after

applying each layer. The sandwich was closed and locked in the gel holder, ensuring that the black side of the sandwich faced the black cathode side of the holder, and placed in a BioRad Trans-blot tank. An ice slab was placed into the Trans-blot tank. The Trans-blot tank was placed in a styrofoam box filled with ice. The Trans-blot tank was filled with ice cold Western Transfer buffer and taken to the 4°C cold room. The Trans-blot tank was connected to a power pack and run on ice for 4 hours at 50V (a dual gel setup will result in a current of 0.18A).

The membranes were removed and fluorophore-stained as required. Alternatively, the PVDF membrane was stained with Coomassie blue R-250 stain for confirmation of transfer of proteins of interest, if it was not going to be Western blotted. For Western blotting with antibodies, the membranes were transferred into clean labelled clip-lock containers, placing the membrane with the side that was touching the gel face up and rinsed with T-TBS (Tris 20mM, NaCl 136mM, Tween-20 5% (v/v)) for 3 minutes. The T-TBS rinse was discarded and ice cold Blocking buffer (5% skim milk in T-TBS) was added. The membrane was placed on a rocker in a 4°C cold room overnight.

The following day the Blocking buffer was removed and fresh Blocking buffer containing primary antibody to the desired dilution was added. The membrane was placed on the rocker at room temperature for 1 hour. The membrane was rinsed with T-TBS for 5 minutes. Fresh T-TBS containing secondary antibody to the desired dilution was added and the membrane was placed on the rocker for 1 hour. In the case of the

Goat anti-mouse IgG Cy5 fluorophore, 4µl fluorophore in 1680µl T-TBS gave a 1:600 dilution and the container was wrapped in tinfoil to keep the fluorophore in the dark. The membrane was washed three times with T-TBS for 5 minutes then rinsed in MilliQ water three times for 5 minutes. Immediately after washing, the membranes were scanned using the Fujifilm FLA-5100 scanner. The membranes were then left to dry overnight at room temperature in the dark and scanned again when dry.

2.6.1 Western blot optimisation

The optimal transfer time was based on the highest amount of protein that could be transferred from the gel to the PVDF membrane. A balance between the voltage used during transfer and the total transfer running time were compared for effective transfer of proteins. One-dimensional PAGE gels were loaded with sample and subsequently Western transferred to PVDF membrane. As shown below, the Western Transfer run for 20 hours at 20V in 4°C resulted in the transfer of lower molecular weight molecules but the higher molecular weight molecules remained within the gel (Figure 2.1). Slightly increasing the transfer times did little to facilitate further transfer of higher molecular weight proteins. Increasing the voltage and lowering the transfer time resulted in an extensive transfer of protein across the entire molecular weight range. As shown in Figure 2.2, the Western Transfer run for 4 hours at 50V in 4°C resulted in the transfer of both lower and higher molecular weight molecules with no, or very little, protein

remaining in the gel. This transfer time was deemed the optimum transfer time and was used for the main experiments.

Figure 2.1: A Western Transfer run for 20 hours at 20V in 4°C showed high molecular weight molecules remain within the gel.

Scan of a gel after Western transfer. All lanes contained 1 μ l sera and were subsequently Western transferred. Lane 1 is 1 μ l mouse sera, Lane 2 is 1 μ l tuatara sera, Lane 4 is 1 μ l mouse sera, Lane 5 is 1 μ l tuatara sera, Lane 7 is 1 μ l mouse sera, Lane 8 is tuatara sera, * denotes Precision Plus Dual Colour Protein Standard ladder.

Figure 2.2: A Western Transfer run for 4 hours at 50V in 4°C showed no high molecular weight molecules remain within the gel.

Scan of a gel after Western transfer. All lanes contained mouse sera and were subsequently Western Transferred. Lanes 2-8 (continuous) is 10 μ l mouse sera, * denotes Precision Plus Dual Colour Protein Standard ladder.

The optimal dilution of the Goat anti-mouse IgG Cy5 fluorophore was based on the highest dilution that did not result in a loss of signal. One-dimensional PAGE gels were loaded with 1 µl mouse sera and subsequently Western transferred to PVDF membrane. Differing dilutions of Goat anti-mouse IgG labelled with Cy5 dye were blotted against the transferred mouse antibody present in the sera. As shown in Figure 2.3, a 1:600 dilution (1 µl at 0.7 mg/ml of fluorophore per 420 µl blocking buffer) is the highest dilution that does not result in a loss of fluorophore signal.

The specificity of the Goat anti-mouse IgG Cy5 fluorophore was checked to ensure that it did indeed bind to mouse IgG as asserted. One-dimensional PAGE gels were loaded with sample and subsequently Western transferred to PVDF membrane. 1 µL rabbit sera (which would contain rabbit antibody) was run on a gel and transferred to PVDF membrane, where it was blotted against the anti-mouse fluorophore. 1 µL mouse sera (which would contain mouse antibody) was also run on the same gel and transferred to PVDF membrane, where it was blotted against the anti-mouse fluorophore. As shown in Figure 2.4, the anti-mouse fluorophore only bound to molecules present in the mouse sera showing evidence for species specificity.

SEA was run on a same gel and transferred to PVDF membrane, where it was blotted against the anti-mouse fluorophore. 1 µl mouse sera, 1 µl mouse IgG1 and 1 µl mouse IgG2a were also Western blotted from the same gel. The visible bands indicating binding of fluorophore to mouse sera protein lined up with the mouse IgG1 and mouse IgG2a

bands, verifying that the fluorophore bound antibody present in the mouse sera sample. No binding in the SEA indicates that the fluorophore does not bind to SEA, nor does it contain mouse antibody (Figure 2.5).

Figure 2.3: 1:600 is the highest dilution that does not result in a loss of signal.
All lanes contained 1 μ l mouse sera vs. Goat anti-mouse IgG Cy5 fluorophore. Lane 1 is 1:100 dilution, Lane 2 is 1:200 dilution, Lane 3 is 1:400 dilution, Lane 4 is 1:600 dilution, Lane 5 is 1:800 dilution, * denotes Precision Plus Dual Colour Protein Standard ladder.

Figure 2.4: The Goat anti-mouse IgG Cy5 fluorophore did not bind to Rabbit sera.
All lanes contained 1µl sera vs. Goat anti-mouse IgG Cy5 fluorophore. Lane 1 is 1µl rabbit sera, Lane 2 is 1µl mouse sera, * denotes Precision Plus Dual Colour Protein Standard ladder.

Figure 2.5: The Goat anti-mouse IgG Cy5 fluorophore did not bind to SEA.

All lanes contained protein vs. Goat anti-mouse IgG Cy5 fluorophore. Lane 1 is 5µL SEA, Lane 2 is 1µl mouse sera, Lane 3 is 1µl mouse IgG1, Lane 4 is 1µl mouse IgG2a, * denotes Precision Plus Dual Colour Protein Standard ladder.

2.7 Differential in-gel electrophoresis

To better determine antibody-reactive proteins in the SEA preparations Differential in-gel electrophoresis (DIGE) was performed to compare antibody binding specificities between all moderate splenomegaly syndrome (MSS) and hypersplenomegaly syndrome (HSS) sera and Id. It combines two-dimensional polyacrylamide gel electrophoresis (PAGE) and Western blotting.

From stock solution, 1 μ L fresh Cy3 fluorophore (400 μ M in DMF) was added to every 50 μ g soluble egg antigen (SEA) to be labelled with Cy3. The solution was centrifuged and spun down briefly then left on ice in the dark for 30 minutes. To stop the conjugation reaction for minimal labelling, equivalent 10mM lysine (to CyDye) was added. The solution was mixed by pipetting, spun down and left on ice for a further ten minutes. The Cy3 SEA must be kept in the dark and can be stored for at least 3 months at -70 °C. Two-dimensional PAGE was performed as normal, using 9.5 μ g Cy3 SEA per gel. Western blotting was also performed. The membranes were blotted with either equal amounts of HSS or MSS mouse sera (pooled and individual), or sera containing equivalent of 0.48 μ g anti-SEA IgG. This was done for one hour at room temperature in 2400 μ L Blocking buffer. The secondary antibody used was Cy5 conjugated Goat anti-mouse IgG in a 1:600 dilution. This was done for one hour at room temperature in T-TBS. After blotting, the membranes were scanned. The membranes were dried overnight in the dark and scanned again when dry.

Generally, dry scans have higher signal and less noise. In some cases, as shown in Figure 2.6, when working with the pooled Id samples the dry membrane took on a grainy appearance. Despite the appearance of the dry scan compared to the wet scan, the values remained consistent. Since dry scans were generally of better quality, dry scan values were used across all membranes for consistency when comparing absolute binding values. To compare differences in antibody reactivity between the clinical forms of schistosomiasis, DIGE images were compiled using Fluorochrome Separation (Multicolor Version 2.2, Copyright ©1999 Molecular Dynamics). ImageQuant (Version

5.2, Copyright ©1999, Molecular Dynamics) was used to assign reactive spots and quantify the volume of antibody binding (arbitrary units) at that spot. The data was used to determine whether SEA preferentially bound antibodies from HSS or MSS sera. The volume threshold set for absolute values to prevent false positives was set at 16,500 to ensure that faint spots would be found without false positives (Figure 2.7).

Figure 2.6: Dry scans were used for ImageQuant analysis

Panels show a membrane blotted with pooled HSS-Id and scanned before and after drying the membrane. Major spots were unaffected by the grainy appearance. For consistency, dry scan values were used for all membranes.

Figure 2.7: The reactive spot 66 is an example of faint, yet positive, binding

The spot numbered 66 (taken from the membrane corresponding to mouse 9C) was given an absolute binding value of 16,583 and is close to the minimum value of 16,500 for a positive site of binding.

2.8 Protein precipitation

To prepare the soluble egg antigen (SEA) sample for matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), at least 60µg protein had to be run on two-dimensional polyacrylamide gel electrophoresis (PAGE). MALDI-TOF experiments used SEA samples that were suspended in lysis buffer (30mM TrisHCl, 2M Thiourea, 7M Urea, 4% (w/v) CHAPS) or SEA samples suspended in PBS (145mM NaCl, 10mM NaH₂PO₄). During two-dimensional PAGE, PBS buffer interferes with isoelectric focussing (IEF) as the contaminating salts disrupt the current. Figure 2.8 shows that protein precipitation was successfully used to concentrate protein samples and remove PBS buffer so that they can run in IEF. Strip washing was initially attempted as described (Heppelmann, Benson et al. 2007) however this was met with limited success. The following method is based on product guidelines for the Calbiochem® ProteoExtract™ Protein Precipitation Kit.

The protein sample (<200µl) was placed in a microcentrifuge tube with a conical bottom. Four volume equivalents of -20°C cold Precipitation Agent (Calbiochem® ProteoExtract™ reagent catalogue number 539180) were added to the sample and vortexed briefly. The sample was spun briefly and incubated overnight at -20°C. The following day the sample was spun for 10 minutes at 13,400 rpm at room temperature. The supernatant was aspirated completely and disposed of (noting the position of the protein pellet). The pellet was washed to remove residual sample buffer. 500µl -20°C Wash solution (Calbiochem® ProteoExtract™ reagent catalogue number 539180) was

added to the sample pellet and vortexed briefly. The sample was spun for 10 minutes at 13,400 rpm at room temperature (in the same orientation as before to minimise disruption of the sample pellet). The supernatant was aspirated completely and disposed of. Again, 500µl -20°C Wash solution was added to the sample pellet and vortexed briefly. The sample was spun for 10 minutes at 13,400 rpm at room temperature. The supernatant was aspirated completely and disposed of.

The sample pellet was dried to remove excess Wash solution. The microcentrifuge with sample was opened and incubated for 1 hour at room temperature within the fume hood. Care was taken not to over-dry the pellet as this would hinder resuspension of the sample pellet in the new buffer solution. The protein pellet was resuspended in the desired buffer solution, vortexed and spun briefly. The sample was left overnight at 4°C before further use to promote full reconstitution of the sample pellet into the new buffer.

Figure 2.8: Scans showing how IEF is affected before and after protein precipitation on the PBS suspended SEA

The left image shows the effect of PBS during IEF without protein precipitation. Note that the first dimension focussing did not resolve well. The right image shows the same IEF conditions except that the SEA underwent protein precipitation first to remove the PBS buffer.

2.9 Matrix-assisted laser desorption/ionization time-of-flight

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) was undertaken to determine proteins present in the SEA sample. Focus was placed on reactive spots first, followed by non-reactive spots. Protein spots to be identified using MALDI-TOF were first removed from the gel containing the sample of interest. To do this, the gel was arranged on a clean glass plate. A printout of the gel scan, with protein spots to be picked appropriately numbered, was used as a reference for picking the spots of interest. A spot picker pipette with sponge barrier tips picked spots from the gel (while making sure to keep the gel wet with MilliQ water) and the gel spots containing sample were dispensed into PP-Microplate 96-well V- shaped (Greiner Bio-one) plate wells containing 50 μ L MilliQ water. The gel spot number and well it was placed into was recorded for future

reference. The spots were washed twice with MilliQ water by aspirating the wells and replacing with fresh MilliQ water. The gel spots were incubated at 4 °C overnight in Valve 2 solution (20 mM Ambic) to remove any stain on them if desired, otherwise peptide extraction commenced immediately.

The gel plugs were placed in the Ettan Digestor and underwent automatic peptide extraction. The parameters for the extraction were as follows: Wash 4 x 30 minutes in 100µL Valve 1 solution (50mM Ambic, 50% Methanol); Digest 300 minutes in 10µL Valve 2 solution containing 0.05µg trypsin; Transfer 3 x 20 minutes in 35µL Valve 3 solution (0.1% Trifluoroacetic acid, 50% Acetonitrile). Once the autodigestor run was complete, the extracted proteins were spotted on a 100-well digestor plate in 1µL matrix (alpha-cyano-4-hydroxycinnamic acid in 1:1 ACN and MilliQ water with 0.25% TFA acid). Calibration spots were also placed between wells, containing Calmix 2 protein mixture.

Spectra were obtained from the spots using a time-of-flight mass spectrometer (Voyager-DE™ PRO BioSpectrometry™ Workstation). The parameters for spectra collection were as follows: Instrument mode reflector positive; Accelerating voltage 20,000V, grid voltage 75%, guide wire voltage 0.01%; Delay time 180ms; 120 shots/spectrum, mass range 800-3000 daltons, low mass gate 800 daltons. The obtained spectra were analysed using Data Explorer (Version 4.0.0.0 Copyright ©1997-2000, Applied Biosystems). First, external calibration was carried out, followed by internal trypsin peak calibration.

The spectra were de-isotoped and peak lists were obtained. The *Schistosoma spp.* database available on MASCOT (provided by the Centre for Biodiscovery) was searched with the following parameters for all peak lists; protein modification carbamidoethyl, partial modification oxidation M; tolerance at 50ppm, MH+ and one missed trypsin cleavage allowance.

Chapter 3: Determining the best strategy to compare antigenic recognition by MSS and HSS sera

3.1 Introduction

Infection of male CBA/J mice with *S. mansoni* leads to spontaneous development of moderate splenomegaly syndrome (MSS) in ~80% of mice or hypersplenomegaly syndrome (HSS) in ~20% of mice.(Henderson, Nix et al. 1993) These two disease forms are similar to the intestinal (INT) and hepatosplenic (HS) forms in human schistosomiasis and thus the mouse model is a valuable tool for studying the molecular pathogenesis of schistosomiasis. A distinguishing feature between MSS and HSS is the size of the spleen and liver which are significantly enlarged in HSS mice compared to MSS mice (Figure 1.4/Figure 3.1). One difference between MSS/INT and HSS/HS which has been found is the production of a protective cross-reactive idiotype (CRI) in MSS mice and INT patients (Montesano, Freeman et al. 1990^a) that is absent in HSS mice and HS patients.

Because the idiotypes of the CRI+ antibodies found in MSS mice are distinctly different to the CRI- antibodies found in HSS mice, we anticipate that there will be detectable differences between the specificity of the antibodies to soluble egg antigen (SEA) in MSS compared to HSS mice. SEA contains a large mixture of different proteins and the ability

to distinguish reactive proteins is important in determining MSS/HSS-specificities. If, as we believe, distinct differences exist in the antigen specificity, disease form-specific antigen/antibody interactions could serve as indicators of chronic disease, and the uniquely recognized antigens could be developed for diagnostic tools that take advantage of differences in antigenic recognition in individuals infected with schistosomiasis.(Correa-Oliveira, Oliveira et al. 1987)

The experiments in this chapter aimed to determine whether the antibodies in MSS or HSS mouse sera were not only infection specific, but also if the antibody profiles were specific to either form. To this end, we tested whether differences in SEA specificity could be detected in mice with MSS or HSS and in pooled, purified anti-SEA antibody preparations. Additionally, the anti-SEA antibody titres of infected mice were determined to test whether antibody titre correlated to spleen/body weight ratios, a measure of disease severity, of infected mice. Furthermore, elucidating the anti-SEA antibody titres across all mice was important to determine if the low level of reactivity in some samples was due solely to a reduced level of total anti-SEA antibody. Ultimately, this knowledge would allow the normalization of antibody titres for future DIGE experiments and allow a more precise analysis of the differential recognition of specific antigens by individual mice.

Figure 3.1: Graph of spleen / body weight ratios between normal, MSS and HSS CBA/J mice

3.2 One-dimensional gel and Western Blot analysis for individual mouse sera

The ability for MSS and HSS mouse sera to recognize soluble egg antigen (SEA) was compared to see if there are any notable differences in specificities between clinical groups. SEA was separated by one-dimensional polyacrylamide gel electrophoresis (PAGE) and anti-SEA responses were assessed by Western blot analysis to determine if any differences in the recognition of specific antigens could be seen. Serum from one MSS mouse (3C) and one HSS mouse (12C) were incubated with the SEA-transferred PVDF membrane and subsequently detected with a Cy5-labelled goat anti-mouse IgG

antibody. From Figure 3.2 it is clear that the anti-SEA IgG profiles of the mice are different.

Using the migration of protein ladder, the size of each protein band was estimated (Appendix B). Both mice recognized antigens at 200, 150, 71, and 35kDa (Table 3.1). However, the HSS mouse serum reacted strongly to unique 76 and 43kDa bands, while the MSS mouse serum detected a unique band at 116kDa (Table 3.1). These results indicate that distinct differences in reactivity to SEA can be detected in individual mice with different disease forms by Western blotting.

Figure 3.2: Sera from infected MSS and HSS mice show distinct patterns of reactivity.

SEA (5.5 µg/lane) was run on a 10% polyacrylamide gel and transferred to a PVDF membrane before being incubated with sera from an MSS (12C; 1:600 dilution) or HSS mouse (2C; 1:600 dilution). Specific antibody binding was detected using Cy5-labelled goat anti-mouse IgG antibody (1:600 dilution). A protein ladder is labelled in the middle lane.

MSS (3C)	HSS (12C)
206	202
150	150
116	Not detected
Not detected	76
71	71
Not detected	43
35	35

Table 3.1: Estimated antigen sizes (in kDa) recognized by individual MSS or HSS mouse sera

3.3 Extended individual mouse anti-SEA reactivity

To understand if the differences between the reactivity of the individual MSS and HSS mice were specific to the disease form, similar experiments were performed to compare antibody binding specificities of three other MSS and two other HSS mice. Additionally, to confirm that the bands were infection-specific, we investigated whether anti-SEA antibodies were present in uninfected CBA/J mice. All schistosome-infected mice had antibodies that recognized SEA by Western blot while no uninfected mouse sera showed any reactivity confirming that the antibodies are infection-specific (Appendix C).

Although sera from all infected mice recognized proteins in SEA, there was variability between serum samples in the specificity and intensity of the recognition (Figure 3.3). The anti-SEA IgG profiles of the mice can be seen and the sizes of bound antigens were calculated (Table 3.2; Appendix B). Sera from all infected mice showed strong binding at 35kDa, and most sera recognized antigens at 205, 160, 72, 53 and 45kDa suggesting that these are common and not disease specific. The HSS mice showed a similar profile while

each MSS mouse had a slightly different pattern. Interestingly, MSS mice 7C and 9C bound a unique 55 kDa antigen that was not detected by 3C or by sera from either HSS mouse. In contrast, there was an HSS unique band at 74kDa in 5C and 17C, which was also present in 12C.

These data show that each mouse possesses a different pattern of antibody specificities to SEA. However, despite the variability in responses certain commonalities could be detected. In particular, most MSS mice recognized a band that migrated at 55 kDa and all HSS mice recognized a band at 74 kDa. Normal mice, as expected, did not contain anti-SEA antibodies and so did not show any positive binding in the WB membranes. Taken together these results suggest that disease form-specific differences in reactivity can be detected by Western blot.

Figure 3.3: Sera from infected MSS and HSS mice show patterns of reactivity unique to each individual mouse.

SEA (5.5µg/lane) was run on a 10% polyacrylamide gel and transferred to a PVDF membrane before being incubated with sera (1:600 dilution) from an MSS (3C, 7C and 9C), HSS (5C and 17C), or uninfected (5N, 3N, and 1N) mouse. Specific antibody binding was detected using Cy5-labelled goat anti-mouse IgG antibody. A protein ladder is labelled in the middle lane.

MSS			HSS	
3C	7C	9C	5C	17C
Not detected	205	205	205	205
Not detected	160	160	160	160
Not detected	Not detected	Not detected	74	74
72	72	Not detected	72	72
Not detected	55	55	Not detected	Not detected
Not detected	53	53	53	53
Not detected	45	45	45	45
35	35	35	35	35

Table 3.2: Estimated antigen sizes (in kDa) recognized by individual MSS (3C, 7C, and 9C) or HSS (5C and 17C) mouse sera

3.4 Two-dimensional gel and Western Blot analysis for pooled and individual mouse sera

Preliminary studies using MSS and HSS mice showed that there were notable differences in antibody binding between clinical groups; however, because SEA contains a large mixture of different proteins, identification of the distinct proteins that migrate at similar molecular weights would be difficult. In order to more accurately determine antibody-reactive proteins in the SEA preparations differential in-gel electrophoresis (DIGE), combining two-dimensional PAGE with Western blot analysis, was performed using pooled and individual sera samples. The pooled samples were provided by E. Secor and contained antibodies from infected MSS or HSS CBA/J mice that were affinity-purified on an SEA-bound sepharose bead column. Thus all antibodies in these pooled preparations were SEA-specific and are referred to as MSS-idiotypic (MSS-Id) and HSS-idiotypic (HSS-Id). Previous studies have shown that the majority of these antibodies are IgG isotype.(Lima, Gazzinelli et al. 1986)

SEA was fluorescently labelled with Cy3, separated by two-dimensional PAGE and transferred to a PVDF membrane. MSS-Id and HSS-Id anti-SEA IgG samples were diluted 1:600 and detected with the same goat anti-mouse IgG antibody as previously used. The data show a difference in the specificities (for both volume and number of spots) of MSS-Id and HSS-Id antibodies reactive to SEA (Figure 3.4a & Figure 3.4b). As discussed later, this difference can be attributed in part to a significant variation in the amount of primary antibody added to each membrane, with more MSS-Id added than

HSS-Id. In spite of the difference in the level of reactivity, many of the major spots appear to be recognized by both MSS-Id and HSS-Id preparations (Figure 3.4). As expected, normal mouse sera did not show reactivity to SEA (Appendix C). These experiments demonstrate the benefit of using two-dimensional PAGE to separate the different protein antigens in SEA.

Figure 3.4: SEA reactivity differs greatly between MSS-Id (top) and HSS-Id (bottom)
SEA (9.5µg/gel) was labelled with Cy3, separated by two-dimensional PAGE, and transferred to a PVDF membrane before being incubated with (a) MSS-Id sample diluted 1:600 or (b) HSS-Id sample diluted 1:600. Specific antibody binding was detected using Cy5-labelled goat anti-mouse IgG antibody.

Given the variability in anti-SEA responses detected with one-dimensional PAGE using individual mouse serum samples, we chose to more fully investigate these differences using DIGE as described earlier. Sera from four MSS and four HSS mice were used as primary antibodies and added in a 1:600 dilution as with the previous Western blots. The results from four mouse sera (two MSS and two HSS samples) are shown in Figure 3.5 and confirm that there are significant individual differences in the specificities of MSS and HSS mouse sera although some common antigens appear to be recognized by most if not all animals. Additionally it appears that, like the MSS-Id and HSS-Id preparations, some of the differences can be attributed to variation in the amount of primary antibody in the serum samples (Appendix C). In order to identify the true differences in antibody binding attributed to differences in specificity, the same amount of antibody must be added to each membrane. Thus, we elucidated the SEA-specific antibody concentrations of the MSS-Id, HSS-Id and the individual sera samples.

Figure 3.5: SEA reactivity differs greatly between MSS mouse 9C and HSS mouse 5C

SEA (9.5 µg/gel) was labelled with Cy3 (green), separated by two-dimensional PAGE, and transferred to a PVDF membrane before being incubated with serum from infected MSS or HSS mice (diluted 1:600). Specific antibody binding was detected using Cy5-labelled goat anti-mouse IgG antibody (red, diluted 1:600). The scanned membrane images show anti-SEA antibodies present and the proteins they react to. (a) MSS mouse 9C, (b) HSS mouse 5C.

3.5 Anti-Soluble Egg Antigens Antibody Titres Across Disease

Forms

To normalize the amount of antibody used for each membrane, the concentration of anti-SEA antibodies in each sample was determined by antigen-specific enzyme-linked immunosorbent assay (ELISA). Because all of the antibodies in the Id preparations were SEA-specific, the antibody concentration of these preparations could be verified by

protein assay and used to calculate the concentration of SEA-specific antibodies in the serum samples. Finally, by determining the absolute concentration of SEA-specific antibodies, we could assess whether there was a relationship between anti-SEA antibody concentration and chronic disease form in infected mice.

Initial ELISA data showed a vast difference in the anti-SEA IgG titres between the pooled HSS-Id and MSS-Id samples, more than the value that was initially given by E. Secor (Figure 3.5a). Because the samples had been affinity column-purified for SEA, a Bradford assay was used to determine the total protein concentration of the pooled sera samples. The true concentrations of the HSS-Id and MSS-Id were calculated at 0.653 mg/ml and 0.024 mg/ml, respectively (Appendix B). Spectral analysis by the nanodrop also confirmed these new concentrations (Appendix B). Retesting the MSS and HSS-Id with these concentrations showed that both preparations had a similar level of reactivity by ELISA (Figure 3.6b). The difference in antibody concentration is likely the reason for the relatively poor reactivity of the HSS-Id by Western blot.

Figure 3.6: The true concentrations of the HSS-Id and MSS-Id samples are reflected in the ELISA
 (a) The given concentrations of 2.09 mg/ml and 0.43 mg/ml for the MSS-Id and HSS-Id samples, respectively, did not fit with the ELISA data. (b) The true concentrations of 0.653 mg/ml and 0.024 mg/ml for the MSS-Id and HSS-Id samples, respectively, fit well with the ELISA data.

To determine if the overall level of antibody to SEA differed between MSS and HSS mice, we assessed the total anti-SEA IgG levels by antigen-specific ELISA. The linear range of the known MSS-Id dose response curve was used to determine the concentration of SEA-specific antibodies in the sera samples (Appendix B). Similar to the results with the two-dimensional PAGE Western blots, individual MSS (Figure 3.7a) and HSS (Figure 3.7b) mouse sera had very different levels of anti-SEA antibodies.

The spleen to body weight ratios of schistosome-infected CBA/J mice are a standard indicator of disease state (Figure 1.4). As previously reported (Henderson, Nix et al. 1993), HSS mice have the highest spleen to body weight ratios ($>>1$), and while MSS mice have lower spleen to body weight ratios (<1), this ratio was significantly higher than uninfected mice. Despite significant difference in spleen to body weight ratios between the groups, this difference did not correlate to variations in SEA-specific antibody concentrations in the infected individual mice (Figure 3.8). Additionally, although the

average of antibody concentration was higher in MSS mice (0.0989 mg/mL) compared to HSS mice (0.0338 mg/mL), this difference was not significant.

Figure 3.7: Anti-SEA IgG concentrations differ between and within MSS and HSS groups

MSS-Id and MSS sera (a) or HSS sera (b) samples containing primary anti-SEA antibody were added (0.957 μ L) at 1:105 dilution and five-fold serial dilutions were carried out. Relative antibody amounts at each dilution were compared to the known MSS-Id standard curve values (black). Specific antibody was detected using horseradish peroxidase-labelled anti-mouse IgG (diluted 1:5000). All sample dose response curves were generated from two sets of dilutions. The recorded values for the diluted sera samples lay in the linear range of the MSS-Id standard curve and were used to determine anti-SEA IgG concentration.

Figure 3.8: Serum anti-SEA IgG titres of infected mice cannot infer spleen/body weight ratio or MSS/HSS disease state of mice

Spleen/body weight ratios <1 indicate data taken from MSS mice. Spleen/body weight ratios >1 indicate data taken from HSS mice.

3.6 Discussion

We tested differences in SEA specificity across mice with either MSS or HSS and showed that CRI+ anti-SEA antibodies found in MSS mice possess distinctly different specificities to the CRI- antibodies found in HSS mice. These differences were visualised by one-dimensional PAGE Western blots (Figure 3.2 & Figure 3.3) that looked at the anti-SEA antibody reactivity found in MSS and HSS sera. Anti-SEA antibodies found in MSS and HSS mouse sera bound strongly to antigens at 35kDa. There was also common binding from most sera to antigens at 205, 160, 72, 53 and 45kDa indicating that these antigens are infection- but not disease form-specific. The one-dimensional Westerns

blots from HSS mice showed similar profiles while the MSS mice had more varied patterns which can be attributed to individual differences across mice. Despite this, disease-specific commonalities in reactivity to SEA were detected in mice with either MSS or HSS disease forms. Most MSS mice recognized a band that migrated at 55kDa which was not reactive in any HSS mice. Furthermore, all HSS mice recognised a band at 74kDa that was not reactive in any MSS mice. This differential specificity was shown previously in a study by Correa-Oliveira *et al* where sera from INT patients bearing CRI+ anti-SEA antibodies recognised a 31kDa antigen while sera from more HS patients bearing CRI- anti-SEA antibodies recognised 14kDa and 66kDa antigens.(Correa-Oliveira, Oliveira et al. 1987) Although this study did not determine the identity of these differentially recognized antigens, it supports the hypothesis that antigenic recognition is distinct between the two disease forms. Overall our preliminary studies support the use of the CBA/J mouse model for characterizing disease form-specific antigen recognition during chronic schistosomiasis by demonstrating the existence of specific antigens that have potential to be developed into diagnostic tools.(Correa-Oliveira, Oliveira et al. 1987)

The one-dimensional Western blot data demonstrate that there are differences in specificity between disease forms, however SEA contains a large mixture of different proteins and the ability to distinguish reactive proteins is important in determining more subtle MSS/HSS-specificities. DIGE, utilising two-dimensional PAGE and Western blot analysis using fluorescent Cy3 SEA, showed vast differences in reactivity between pooled MSS-Id and HSS-Id reactivity levels (Figure 3.4) however reactivity with major

spots was consistent between the samples. Significant differences in reactivity levels were also found with individual sera samples irrespective of disease form (Figure 3.5). We hypothesized that these differences were due to differing anti-SEA IgG titres between MSS and HSS samples and found that indeed the differences in reactivity by Western blot were likely due to variations in the levels of anti-SEA antibodies in the individual serum samples. These concentration variations made it difficult to assess subtle differences in specificity between groups as differences in reactivity to particular antigens could not be attributed to relative differences in specificity compared to all other SEA-specific antibodies, as opposed to absolute differences in antibody recognition. Despite this, DIGE was shown to be an effective method to separate the various proteins found in SEA, providing both a classification of all reactive and non-reactive proteins simultaneously and a means to isolate such proteins for further identification.

Once similar concentrations of antibodies were added, we found that MSS-Id and HSS-Id possess similar levels of anti-SEA IgG reactivity to SEA and allowed us to estimate the absolute amounts of anti-SEA antibodies in each serum sample. Additionally, because known and equal amounts of anti-SEA antibodies from these samples were then added to each membrane as primary antibody in later experiments, the absolute binding values could be directly compared across different membranes. Any antibody reactivity differences found between clinical forms could then be attributed to antibody specificity rather than unequal amounts of antibody used in each experiment. Finally, the spread of antibody titres across all infected mice, regardless of the manifested chronic disease, suggests that anti-SEA antibody titre cannot be an indicator of schistosomiasis disease

state (Figure 3.8). This finding is in agreement with human studies that have shown that anti-SEA IgG ELISA titres vary widely across individuals with specific clinical presentations of disease.(Lima, Gazzinelli et al. 1986)

Overall, our initial studies confirmed that there are distinct differences in antibody specificity between CRI+ and CRI- anti-SEA IgG and that such reactivity is infection-specific. It was also demonstrated that DIGE is a suitable method to separate and determine reactive proteins in SEA. Having determined the true concentrations of the MSS-Id and HSS-Id samples as well as the anti-SEA IgG concentrations in each individual sera sample, our next step was to fully characterize the antibody reactivity using pooled Id and individual CBA/J mouse sera samples using DIGE. Using DIGE we planned to see whether there were antigens recognized uniquely by each group, which would in turn highlight antigens that can be indicators of disease state for individuals infected with schistosomiasis.

Chapter 4: Determining antibody reactivity in MSS and HSS sera

4.1 Introduction

We have shown that antibodies against soluble egg antigen (SEA) produced in *S. mansoni*-infected CBA/J mice recognize a wide range of antigens. Additionally, we found that although individual mice differ in their anti-SEA antibody repertoire, certain commonalities in specificity can be found among mice which manifest with moderate splenomegaly syndrome (MSS) or hypersplenomegaly syndrome (HSS). Because the CBA/J mouse model is an established model for human schistosomiasis, we believe these antibodies reflect the human antibody response and represent the idiotypes characteristic of each form. In particular, a unique cross-reactive idiotypic (CRI) is exclusively found in chronic MSS mice and in acutely infected mice that eventually develop MSS, but this antibody is not in HSS mice (Henderson, Nix et al. 1993; Montesano, Colley et al. 2002). Similarly, human patients with moderate intestinal (INT) schistosomiasis produce CRI+ anti-SEA antibodies like MSS mice whereas human patients with severe hepatosplenic (HS) schistosomiasis produce CRI- anti-SEA antibodies like HSS mice do. This finding further supports the use of the CBA/J mouse model of schistosomiasis to study human disease and the CRI+ anti-SEA profiles found in each form.

We first looked at reactivity in pooled MSS idiotypic (MSS-Id) or HSS idiotypic (HSS-Id) samples that were affinity column-purified for SEA. Secondly, we investigated anti-SEA antibodies found in individual mice and isolated the antigens to which these antibodies reacted. By determining specific antibody/antigen interactions that characterise each form, knowledge of the antigen could lead to a diagnostic tool that distinguishes the two chronic forms of schistosomiasis. Furthermore, knowledge of the antigen may provide insight into the mechanism that determines which disease form manifests in humans and could lead to the development of vaccines to drive production of the protective, CRI+ anti-SEA antibodies.

4.2 Pooled sera reactivity results

To fully characterize the differences in the specific egg antigens to which MSS-derived and HSS-derived antibodies reacted, we used difference in gel electrophoresis (DIGE). In brief, SEA was fluorescently labelled with Cy3, separated by two-dimensional polyacrylamide gel electrophoresis (PAGE) and transferred to a PVDF membrane. Each membrane was reacted with the equivalent of 0.48µg anti-SEA antibodies from pooled MSS-Id or HSS-Id samples, and bound antibodies were detected with a Cy5-labelled goat anti-mouse IgG antibody. Compared to the preliminary results shown in Figure 3.4, Figure 4.1 shows that the reactivity of the HSS-Id increases to the level found with MSS-Id when similar concentrations are used. This result confirms that there is no reduced reactivity by the HSS-Id as suggested by the initial work in Chapter 3.

Figure 4.1: Antibody reactivity increases after equalising primary antibody amounts added to Western blot for low anti-SEA antibody titre pooled HSS-Id sample

(Before) HSS-Id reactivity when anti-SEA IgG was diluted 1:25000. (After) HSS-Id reactivity when anti-SEA IgG was diluted to 1:5000.

Membranes were classified according to the primary antibody used. For the two MSS-Id blotted membranes, they are called M1 and M2, and for the HSS-Id blotted membrane, it is classified H. For each membrane, spots designating reactive SEA were numbered. Figure 4.2 shows a representative membrane showing the numbering system used across all membranes. The membrane is for the highly reactive pooled MSS-Id sample, M2. The results from both MSS-Id experiments (M1 and M2) and one HSS-Id (H) experiment are shown in Figure 4.3 and confirm that many common antigens appear to be recognized by MSS-Id and HSS-Id antibodies. Furthermore, the vast difference in reactivity seen in Figure 3.4 is not present after normalising antibody levels applied during the Western blot. Given that reactivity for many SEA antigens are shared between MSS-Id and HSS-Id, it is likely that the disease form-specific differences are subtle. Moreover, from the Western blots shown in Figure 4.3, it is clear that many of the highly reactive antigens (i.e. red) in SEA are not the most abundant (i.e. green).

In order to look for subtle differences in antibody specificity that may be present between the clinical forms of schistosomiasis, ImageQuant was used to quantify the volume of antibody bound at each numbered reactive spots. Binding values (arbitrary units) were directly compared across all membranes and major sites of binding that preferentially bound anti-SEA IgG from either MSS-Id or HSS-Id are listed in Table 4.1 (see Appendix A for a full list). Of the 77 reactive spots we designated in SEA, 16 preferentially bound pooled MSS-Id and 11 bound pooled HSS-Id anti-SEA antibodies. These results provide evidence that there are antibody specificities present in one clinical form that are not found in the other.

Figure 4.2: Representative gel showing the numbering system for reactive proteins present in SEA
 SEA (9.5µg/gel) was labelled with Cy3 (green), separated by two-dimensional PAGE, and transferred to a PVDF membrane before being incubated with MSS-Id (1:5000). Specific antibody binding was detected using Cy5-labelled goat anti-mouse IgG antibody (red, diluted 1:600).

Figure 4.3: SEA reactivity shows many commonalities between MSS-Id and HSS-Id samples
 SEA (9.5µg/gel) was labelled with Cy3 (green), separated by two-dimensional PAGE, and transferred to a PVDF membrane before being incubated with MSS-Id or HSS-Id (diluted 1:5000). Specific antibody binding was detected using Cy5-labelled goat anti-mouse IgG antibody (red, diluted 1:600). (a) Pooled MSS-Id anti-SEA IgG sample, M1. (b) Pooled MSS-Id anti-SEA sample, second experiment, M2. (c) Pooled HSS-Id anti-SEA IgG sample, H.

Spot	Pooled Sera			Reactivity
	M1	M2	H	
6				MSS
9				MSS
29				MSS
32				MSS
33				MSS
42				MSS
43				MSS
56				MSS
64				MSS
65				MSS
66				MSS
72				MSS
73				MSS
75				MSS
76				MSS
77				MSS

Spot	Pooled Sera			Reactivity
	M1	M2	H	
3				HSS
4				HSS
5				HSS
11				HSS
12				HSS
22				HSS
36				HSS
48				HSS
59				HSS
60				HSS
67				HSS

Legend:

Spot Volume	MSS	HSS
< 16500		
< 50000		
< 150000		
< 300000		
< 500000		
> 500000		

Reactivity	MSS HSS	HSS MSS
< 1		
< 2		
< 3		
< 4		
< 5		
> 5		

Table 4.1: Spot reactivity shows evidence of antigens that react specifically to MSS-Id or HSS-Id

Spot refers to the assigned spot number given to a particular reactive protein found in SEA. M1 and M2 refer to membranes that were blotted with primary antibodies taken from the MSS-Id pooled sera. H refers to a membrane that was blotted with primary antibodies taken from HSS-Id pooled sera. Spot volume shows the absolute value (arbitrary units) for antibody bound to a particular protein and its corresponding colour. Reactivity relates to whether the protein preferentially binds MSS-Id or HSS-Id anti-SEA antibodies. Proteins listed were ones that bound specifically to one form and did not bind the other, or preferentially bound one form over the other if it bound both.

4.3 Individual sera reactivity results

To determine differences in individual mouse reactivity, SEA was labelled, separated by two-dimensional PAGE and transferred as stated previously. For each Western blot, the equivalent of 0.48 μ g anti-SEA antibodies from individual MSS and HSS mouse sera samples were added and detected with Cy5-labelled goat anti-mouse IgG antibody. We found an increase in reactivity (for both volume and number of spots) after increasing the antibody for samples with low anti-SEA IgG titre, represented by sera from HSS mouse 5C (Figure 4.4). This finding parallels the increased reactivity shown by HSS-Id, which also has a low anti-SEA IgG titre (Figure 4.1). Unfortunately, in some samples there was not enough mouse sera (e.g. MSS mouse 8C) to generate enough primary antibody for comparison with the other membranes, and these samples could not be included in the analyses.

Figure 4.4: Antibody reactivity increases after equalising primary antibody amounts added to Western blot for low antibody titre sera sample 5C

(Before) HSS mouse 5C sera reactivity when anti-SEA IgG was diluted 1:18000. (After) HSS mouse 5C sera reactivity when anti-SEA IgG was diluted 1:5000.

Membranes were classified according to the primary antibody used. For the five MSS sera blotted membranes, they are called 1C, 2C, 3C, 7C and 9C, and for the HSS sera blotted membrane, they are classified 5C, 6C, 10C, 12C and 17C. For each membrane, spots designating reactive SEA were numbered as shown in Figure 4.2. Figure 4.5 shows two MSS and two HSS representative membranes and their comparative reactivity to proteins found in SEA (see Appendix C for a complete list of membrane images). The results confirm that many common antigens appear to be recognized by MSS and HSS serum antibodies after antibody equalisation during Western blot, and reinforce the similar reactivity levels found in the pooled sera samples (Figure 4.3). These data also show that reactivity (for volume and number of spots) varies between individual mice as well as between clinical groups of mice.

In order to look for differences in antibody specificity that may be present between the clinical forms of schistosomiasis, ImageQuant was used to quantify the volume of antibody bound at each numbered spot as before. Binding values (arbitrary units) were directly compared across all membranes and major sites of binding that preferentially bind anti-SEA IgG from either MSS or HSS forms are listed in Table 4.2 (see Appendix A for a full list). Of the 77 reactive spots we designated in SEA, and across all individual mice samples, 10 preferentially bound MSS-Id and 5 preferentially bound HSS-Id anti-SEA antibodies. This finding supports previous evidence of antibodies present in one clinical form that are not found in the other.

Figure 4.5: SEA reactivity shows varied reactivity between individual MSS and HSS sera samples
SEA (9.5μg/gel) was labelled with Cy3 (green), separated by two-dimensional PAGE, and transferred to a PVDF membrane before being incubated with MSS-Id or HSS-Id (diluted 1:5000). Specific antibody binding was detected using Cy5-labelled goat anti-mouse IgG antibody (red, diluted 1:600). (a & b) MSS sera samples, 1C and 2C. (c & d) HSS sera samples, 5C and 6C.

Spot	Individual Mice Sera										Reactivity	
	1C	2C	3C	7C	9C	5C	6C	10C	12C	17C		
5												MSS
6												MSS
11												MSS
29												MSS
34												MSS
35												MSS
61												MSS
62												MSS
66												MSS
72												MSS

Spot	Individual Mice Sera										Reactivity	
	1C	2C	3C	7C	9C	5C	6C	10C	12C	17C		
16												HSS
36												HSS
53												HSS
60												HSS
73												HSS

Legend:

Spot Volume	MSS	HSS	Reactivity	<u>MSS</u> HSS	<u>HSS</u> MSS
< 16500			< 1		
< 50000			< 2		
< 150000			< 3		
< 300000			< 4		
< 500000			< 5		
> 500000			> 5		

Table 4.2: Spot reactivity shows evidence of antigens that react specifically to MSS-Id or HSS-Id

Spot refers to the assigned spot number given to a particular reactive protein found in SEA. 1C, 2C, 3C, 7C and 9C refer to membranes that were blotted with primary antibodies taken from the MSS mouse sera. 5C, 6C, 10C, 12C and 17C refer to membranes that were blotted with primary antibodies taken from HSS mouse sera. Spot volume shows the absolute value (arbitrary units) for antibody bound to a particular protein and its corresponding colour. Reactivity relates to whether the protein preferentially binds MSS-Id or HSS-Id anti-SEA antibodies. Proteins listed were ones that bound specifically to one form and did not bind the other, or preferentially bound one form over the other if it bound both.

4.4 Combined pooled and individual sera reactivity results

Reactive spots that were preferentially recognised by one disease form were compared from both the individual sera and pooled Id sample data. When the specific spots recognized by the pooled Ids were compared with those recognized by individual mice sera samples, we found that the preferentially reactive proteins in the pooled Id samples were also recognized by the individual mice. The pooled Id sample data with the individual sera sample data was combined. This created a more robust data set, as the individual mice sera sample data set is larger, using five MSS mice and five HSS membranes, compared to the two pooled MSS-Id and single pooled HSS-Id membranes. Furthermore, it was important to determine reactive proteins found in the individual mouse membranes were also present in the pooled sera sample membranes, to ensure that differences found between individual mice were due to differences in specificity as a result of clinical disease form as opposed to individual antibody specificity variations from one mouse to another. This analysis provides a validation of our data and suggests the selected spots are robust candidates recognized by CRI+ or CRI- antibodies.

Table 4.3 shows the comparison between the reactive spot data from the pooled and individual samples. There is evidence of antigens that react specifically to MSS or HSS anti-SEA IgG across all individual and pooled Id experiments. For MSS-Id, these are spots 6, 29, 66 and 72. For HSS-Id, these are spots 36 and 60. These reactive spots are shown on two membranes blotted with serum from MSS mouse 2C and HSS mouse 12C (Figure 4.6a & Figure 4.6b). Of all of the disease form-specific spots, the most consistent

spot is spot 36, which showed 100% reactivity to HSS derived anti-SEA IgG in both the current experiments and previous individual mouse experiments (Figure 4.7). Spot 36 did not show any reactivity to MSS derived antibodies in the current experiments nor the previous individual mouse experiments (Figure 4.8). There may be slight reactivity to spot 36 in the initial MSS-Id blotted membrane (Figure 3.4a), however given the high concentration of primary antibody used during the Western blot for that experiment, this reactivity is minimal. Some spots were consistently and highly reactive across both clinical groups; these are spots 1, 30, 31, 41, 70 and 71.

The isolation of the reactive antigens in SEA is important. For example, the MSS-specific spots can provide candidate antigens for developing a vaccine that drives the protective immune response. Also, the MSS- and HSS-specific spots provide candidate antigens that can be used in a diagnostic tool to determine clinical disease form in infected patients. Finally, the highly reactive spots, though not disease form-specific, may be important in providing insight into the immunological processes involved in the disease course and how these key antigens drive the immune response against schistosome eggs.

	Individual Mice Sera													Pooled Sera			
Spot	1C	2C	3C	7C	9C	5C	6C	10C	12C	17C	Reactivity			M1	M2	H	Spot
6												MSS					6
29												MSS					29
66												MSS					66
72												MSS					72

	Individual Mice Sera													Pooled Sera			
Spot	1C	2C	3C	7C	9C	5C	6C	10C	12C	17C	Reactivity			M1	M2	H	Spot
36												HSS					36
60												HSS					60

	Individual Mice Sera													Pooled Sera			
Spot	1C	2C	3C	7C	9C	5C	6C	10C	12C	17C	Reactivity			M1	M2	H	Spot
1												Both					1
30												Both					30
31												Both					31
41												Both					41
70												Both					70
71												Both					71

Legend:

Spot Volume	MSS		Reactivity	MSS		HSS	MSS
	MSS	HSS		HSS	MSS		
< 16500			< 1				
< 50000			< 2				
< 150000			< 3				
< 300000			< 4				
< 500000			< 5				
> 500000			> 5				

Table 4.3: Combined spot reactivity data shows evidence of antigens that react specifically to MSS-Id or HSS-Id in across individual and pooled sera samples

The top and middle panels show proteins present in SEA that were preferentially reacting to one form of anti-SEA IgG over the other on both the individual and pooled sera membranes. The top panel shows MSS-Id reactive spots. The middle panel shows HSS-Id reactive spots. The bottom panel show proteins present in SEA that reacted strongly to both forms of anti-SEA IgG on individual and pooled sera membranes.

Figure 4.6: Schematic of reactive spots from the combined spot data for an MSS and HSS membrane
 (a) Schematic using MSS mouse 2C membrane as template. (b) Schematic using HSS mouse 12C membrane as template. Green arrows indicate spots that correspond to specific MSS-Id reactive proteins. Orange arrows indicate spots that correspond to non-specific highly reactive proteins. Red arrows indicate spots that correspond to specific HSS-Id reactive proteins.

Figure 4.7: HSS-Id reactivity is universal to spot 36 across all HSS-Id blotted membranes

Top row from left; Pooled HSS-Id (H), 5C, 6C, 10C, 12C, 17C.

Figure 4.8: MSS-Id reactivity is absent to spot 36 across all MSS-Id blotted membranes

Top row from left; Pooled MSS-Id (M1), Pooled MSS-Id (M2), 1C, 2C, 3C, 7C, 9C.

4.5 Discussion

Previously we have shown that anti-SEA antibodies produced in CBA/J mice infected with *S. mansoni* express a wide range of antigen specificity. Furthermore, work by Montesano *et al* has shown that these antibodies reflect the human antibody response with respect to the presence or absence of the cross reactive idiotypes (CRI). CRI+ anti-SEA antibodies are exclusively produced in chronic MSS mice and acutely infected mice that eventually develop MSS, but not in HSS mice.(Henderson, Nix et al. 1993; Montesano, Colley et al. 2002) Our experiments in Chapter 3 showed that there are distinct patterns of specificity to antigens present in SEA depending on whether the serum came from MSS or HSS mice. To better identify the specific differences, DIGE was performed on pooled and individual sera samples to determine specific antibody/antigen interactions that characterise each form. We isolated these antigens and classified them according to their strong preference for MSS-Id anti-SEA, HSS-Id anti-SEA, or both. These were our antigens which would be useful candidates in developing

diagnostic tools, vaccines and/or increase understanding of how such antigens drive disease outcome in infected individuals.

The DIGE experiments used equal amounts of antibody during the Western blot step, giving us the ability to directly compare antibody reactivity to certain antigens found in SEA. This is an improvement on previous experiments which could compare differences in antigen specificity but could not directly compare degrees of reactivity to certain antigens. Additionally, through the use of the purified anti-SEA MSS- and HSS-Ids, we have the capacity to quantitatively measure specific antibody levels in our serum samples. Using these methods, we have shown that different levels of reactivity against SEA from MSS- or HSS-derived antibodies using Western blots of pooled Id and individual mice samples show both forms have subtle specificities to particular antigens which are unique and distinctive.

Firstly, we investigated the specific egg antigens that pooled samples of protective, CRI+ MSS-derived and CRI- HSS-derived antibodies reacted to. The data showed evidence of proteins that preferentially bind either MSS-Id and/or HSS-Id anti-SEA IgG (Table 4.1). Since these membranes were blotted against pooled sera, this shows that on average there is a difference between the binding specificities of MSS mice and HSS mice. Of the 77 reactive spots designated in SEA, 16 preferentially bound pooled MSS-Id and 11 bound pooled HSS-Id anti-SEA antibodies. This provided evidence that there are antibodies present in one clinical form that are not found in the other. The number of specific

antigen/antibody interactions found was also higher than the number found in the one-dimensional Western results (Chapter 3.3 and 3.4) which is most likely due to the improved separation of proteins using two-dimensional PAGE. The preferential antibody specificities could also be better determined due to the direct comparison of reactivity levels using ImageQuant, which was made possible by the equalisation of antibody added during Western blot.

Secondly, we investigated anti-SEA antibodies across all individual sera samples. Of the 77 reactive spots designated in SEA, ten preferentially bound MSS and five preferentially bound HSS-derived anti-SEA IgG as listed in Table 4.2. These results supported the pooled Id data showing antibodies present in one clinical form that are not found in the other. Furthermore, comparing the antigens recognized by the Id and sera, MSS and HSS mice revealed that similar antigens were identified as disease specific with these two approaches suggesting that our disease-specific candidates are robust. For MSS-specificity, these are spots 6, 29, 66 and 72. For HSS-specificity, these are spots 36 and 60. Finally, we also found spots that were consistently and highly reactive across both clinical groups; these are spots 1, 30, 31, 41, 70 and 71 (Table 4.3 and Figure 4.6).

Of all the candidate proteins, the most consistent spot is spot 36, which showed 100% reactivity to HSS anti-SEA in both the current experiments and previous individual mouse experiments (Figure 4.7). Despite ladder not being used during the DIGE experiments, it is likely that spot 36 is the 74kDa protein as seen in previous experiments

(Figure 3.5). This is due to its relative position to reactive spot 41, which is likely to be the universally reactive 71kDa band as seen in previous one-dimensional PAGE experiments (Chapter 3.4). A 70kDa protein has previously been isolated from schistosome eggs and was noted as being highly recognized by immunized mice.(Lanar, Pearce et al. 1985) This is heat shock protein 70, which has been suggested as a candidate for a schistosomal vaccine due to its ability to stimulate lymphocytes from mice vaccinated with radiation-attenuated cercariae.(Richter, Reynolds et al. 1993) It is possible that this uniformly reactive antigen at ~71kDa is this protein.

Interestingly, Attallah et al have shown that a target of a monoclonal IgG_{2a} antibody known to react to different developmental stages of *S. mansoni* is a 74kDa protein which was isolated from soluble worm antigen preparation and urine of infected individuals. Active immunization with the 74kDa protein produced a decreased number of granulomas and decreased worm burden after infection. However, although the number of granulomas decreased, the size of the granulomas was found to increase in immunised mice.(Attallah, Attia et al. 1999) If it turns out that this 74kDa antigen is the same protein as our HSS-specific protein isolated from SEA, then it would show that the immunoregulatory role of the HSS-specific antibodies is complex and merits further investigation.

We have shown that CRI+ anti-SEA IgG from MSS mice have different specificities than CRI- anti-SEA IgG from HSS mice supporting the idea that antibodies from both MSS

and HSS disease forms are immunologically distinct.(Henderson, Nix et al. 1993; Montesano, Colley et al. 2002) This also supports previous findings of antibodies that have distinct specificities in the human forms.(Correa-Oliveira, Oliveira et al. 1987) The data have shown that MSS-specific antibody/antigen interactions exist. Spots 6, 29, 66 and 72 highlight the antigens present in the SEA that are part of these interactions. These antigens are implicated in the protective role of CRI+ antibodies due to their specific binding to MSS antibodies. Such CRI+ antibodies are also found in human patients with the less severe INT form of schistosomiasis.(Montesano, Freeman et al. 1990^a) The protective abilities of CRI+ anti-SEA IgG have been demonstrated in previous studies such as its regulation of the cellular response to SEA (Montesano, Freeman et al. 1997; Secor and Freeman 2001), and its ability to protect newborns from severe pathology.(Montesano, Colley et al. 1999^a) Furthermore, HS schistosomiasis development, when compared to INT schistosomiasis development, is not solely due to infection intensity alone (Tanabe, Gonçalves et al. 1997; Mohamed-Ali, Elwali et al. 1999) and patients with the most severe fibrosis are not necessarily the individuals with the highest infection intensity, highlighting the importance of intensity-independent factors, such as the presence of these CRI+ protective antibodies, in the progression of severe disease.

Using our reactivity results, we also looked at whether there was a correlation between disease severity, determined by spleen to body weight ratios, and reactivity levels to specific antigens. Although it was clear that certain antigens in SEA were preferentially reactive to antibodies found exclusively in MSS or HSS mice, there was no evident link

between increasing spleen to body weight ratios, going from MSS to HSS mice, and gradually increasing or decreasing levels of specific reactivity by CRI+ and CRI- anti-SEA IgG. However, the use of a more quantitative technique than Western blotting to assess the absolute level of reactivity is required before any strong conclusions can be drawn.

Effective isolation of the disease form-specific antigens is the next important step in identifying what these antigens are. By knowing the MSS-specific antigens, we can increase understanding of how such antigens drive the production of protective antibodies. Furthermore, these can serve as candidate antigens for the development of vaccines that drive the protective immune response in patients with schistosomiasis. These can also serve as candidates for a diagnostic tool that can determine patients with INT or HS chronic disease, or patients at risk of developing HS disease. HSS-specific antigens have been found in spot 60 and, notably, spot 36. Although these antigens will have limited use in the development of a vaccine, these can serve as candidate antigens for diagnostic tools in the same manner as MSS-specific antigens. This is important as suitable candidates for such tools have not yet been found. Thus, after successfully verifying and isolating MSS-specific and HSS-specific antigens, our next step was to use mass spectrometry to identify these reactive proteins in SEA.

Chapter 5: Identifying reactive antigens in SEA

5.1 Introduction

CBA/J mice infected with *S. mansoni* present with moderate splenomegaly syndrome (MSS) or hypersplenomegaly syndrome (HSS) and the human forms of schistosomiasis, intestinal (INT) and hepatosplenic (HS) schistosomiasis, parallel the MSS and HSS forms respectively. The antibody repertoire of either form can be characterised by the presence of a cross reactive idiotype (CRI) which is present in INT/MSS forms, but absent in HS/HSS forms.(Henderson, Nix et al. 1993; Montesano, Colley et al. 2002) We have previously determined which proteins present in soluble egg antigen (SEA) specifically react with CRI+ or CRI- anti-SEA IgG from infected mice. These protein spots represent candidate antigens that may be used as the basis for diagnostic tools and vaccines against HS schistosomiasis. Furthermore, knowledge of these antigens can give us insight into the SEA constituents that shape the immune response during schistosomiasis infection. The reactive antigens in SEA were determined by differential in-gel electrophoresis (DIGE) analysis, and it was found that spots 6, 29, 66 and 72 were unique to MSS. Similarly, spots 36 and 60 were found to be HSS-specific. Several universally reactive antigens were also detected (spots 1, 30, 31, 41, 70 and 71).

Our next step was to determine the identity of these differentially-recognized proteins in SEA through matrix-assisted laser desorption/ionization (MALDI) using an α -cyano-4-hydroxycinnamic acid matrix in 1:1 ACN and MilliQ water with 0.25% TFA acid. Trypsin was used to cleave the proteins into fragments and time-of-flight (-TOF) mass spectrometry was used to determine mass fragment sizes. These fragment sizes provide a “fingerprint” that can be compared to expected fragment sizes for known proteins and allow identification of specific protein spots. An initial focus was placed on identifying antigens corresponding to reactive spots however all SEA proteins (visible in DIGE and under coomassie staining) underwent MALDI-TOF. Once identification of proteins was completed, proteins were matched to their spots on the membrane and their MSS or HSS-specificities were recorded. These identified antigens may be important due to their viability as diagnostic tools, candidate vaccine antigens, or their role in driving the differential immune response of schistosomiasis infection in mice.

5.2 Identified proteins present in SEA

Selected SEA protein spots were picked from preparative two-dimensional PAGE gels, digested with trypsin, and identified using MALDI-TOF. Figure 5.1 shows the combined information of many MALDI-TOF experiments using SEA samples that were suspended in lysis buffer (30mM TrisHCl, 2M Thiourea, 7M Urea, 4% (w/v) CHAPS) or SEA samples suspended in PBS (145mM NaCl, 10mM NaH₂PO₄). While both SEA preparations gave comparable two-dimensional PAGE results, the PBS-suspended SEA

required a protein precipitation treatment as described in Methods and so the use of samples in the lysis buffer was preferred, however the PBS-suspended SEA still yielded proteins that were identified with MALDI-TOF. The model gel used in Figure 5.1 shows two-dimensionally separated SEA which was suspended in lysis buffer. Identified reactive proteins were classified according to their designated spot number from our previous experiments, or classified alphabetically if they were non-reactive. Table 5.1 & Table 5.2 show a full list of identified proteins, listing spot number, name, mass and calculated pI.

Figure 5.1: Spot map of identified SEA proteins

Numbered items indicate identified SEA proteins reactive to anti-SEA IgG. Numbers correspond to assigned spot numbers. Alphabetised items indicate non-reactive proteins identified with MALDI-TOF.

Spot	Protein	Nominal mass (Da)	Calculated pI
1a	mucin [Schistosoma mansoni]	32491	5.28
1b	mucin [Schistosoma mansoni]	35967	4.99
10	mucin [Schistosoma mansoni]	32894	7.7
11	mucin [Schistosoma mansoni]	32894	7.7
34	heat shock protein 86	51420	5.37
35	heat shock protein 86	51420	5.37
36a	HSP70 [Schistosoma japonicum]	71613	5.12
36b	HSP70 [Schistosoma japonicum]	71613	5.12
38	SJCHGC09453 protein [Schistosoma japonicum]	89514	5.55
41a	heat shock protein 70	70231	5.42
41b	70,000 mol wt antigen/hsp70 homologue (619 AA) [Schistosoma mansoni]	68331	5.4
41c	heat shock protein 70	70231	5.42
41d	Heat shock 70 kDa protein homolog (HSP70) (Major surface antigen)	70231	5.42
43	SJCHGC04656 protein [Schistosoma japonicum]	26080	5.21
53	similar to GenBank Accession Number U54587 unknown in Schistosoma mansoni	15775	9.24
59	Major egg antigen (p40)	39575	6.23
60	Major egg antigen (p40)	39575	6.23
61	Major egg antigen (p40)	39575	6.23
62	Major egg antigen (p40)	39575	6.23
68	secretory glycoprotein k5 precursor [Schistosoma mansoni]	34929	7.25
69	secretory glycoprotein k5 precursor [Schistosoma mansoni]	34929	7.25
71	secretory glycoprotein k5 precursor [Schistosoma mansoni]	34929	7.25

Table 5.1: List of identified SEA proteins reactive to anti-SEA IgG with mass and pI

This table shows the numbered reactive spots and its corresponding protein identified by MALDI-TOF. Nominal mass and calculated pI are also shown.

Spot	Protein	Nominal mass (Da)	Calculated pI
A1	ER-luminal cysteine protease ER 60 [Schistosoma mansoni]	54785	6.3
A2	ER-luminal cysteine protease ER 60 [Schistosoma mansoni]	54785	6.3
B1	Calreticulin precursor (SM4 protein)	45540	4.69
B2	calreticulin	43163	4.73
C	protein disulfide isomerase homologue [Schistosoma mansoni]	54463	4.92
D	beta-tubulin [Schistosoma japonicum]	50048	4.84
E1	alpha tubulin	50660	4.97
E2	alpha tubulin	50660	4.97
E3	alpha tubulin	50660	4.97
F1	Actin-2	41999	5.3
F2	Actin-2	41999	5.3
G	30 kDa glycoprotein	18273	7.81
H	14-3-3 epsilon [Schistosoma mansoni]	28850	4.85
I	14-3-3 protein zeta [Schistosoma bovis]	28605	4.72
J1	thioredoxin peroxidase 1 [Schistosoma mansoni]	21315	6.1
J2	thioredoxin peroxidase 1 [Schistosoma mansoni]	21315	6.1
K	Adenylate kinase (ATP-AMP transphosphorylase) (AK)	22558	8.97
L	Glyceraldehyde-3-phosphate dehydrogenase (Major larval surface antigen) (P-37)	36640	8.16

Table 5.2: List of identified SEA proteins non-reactive to anti-SEA IgG with mass and pI

This table shows the alphabetised non-reactive spots and its corresponding protein identified by MALDI-TOF. Nominal mass and calculated pI are also shown.

Twelve non-reactive proteins were identified in SEA. Of the 77 spots designated to be reactive in SEA, 17 were identified. In this group, no MSS-specific reactive proteins were identified using MALDI-TOF. This low identification rate of reactive antigens present in SEA may be due to the high glycosylation of immunogenic schistosomal surface proteins (Jang-Lee, Curwen et al. 2007), making sample preparation and spectral analysis by MALDI-TOF difficult. Another explanation for the low rate of protein identification is that the proteins, while highly reactive, were not highly abundant.

Although none of the MSS-specific antigens were identified by MALDI-TOF, several HSS-specific antigens were. The HSS-specific spot 36, which was recognized by 100% of the HSS mice and none of the MSS mice, was identified as heat shock protein 70 (HSP70, 72kDa, pI 5.1). This protein is larger and more acidic than the highly reactive, non-specific HSP70 isoform (70kDa, pI 5.4) that was recognised by both MSS and HSS mice. Spot 60 was identified as major egg antigen (40kDa, pI 6.2) as were adjacent spots 59, 61 and 62. Interestingly while serum from all MSS and HSS mice recognized the major egg antigen (spots 59-62), the pattern of recognition of each individual spot was unique and some individual spots like spot 60 were recognized more readily by HSS mice. These HSS-specific antigens may be useful in developing diagnostic tools that can determine whether individuals contain HSS-specific antibodies and therefore have, or are at risk of developing, severe schistosomiasis.

Our analysis revealed several highly reactive protein spots that were recognized by all mice and were infection but not disease form-specific. Figure 5.2 shows the most reactive non-specific MSS/HSS proteins that were identified with MALDI-TOF. The most reactive was spot 1, which was identified as mucin (32kDa, pI 5.3). The highly reactive spots 70 and 71 were both identified as secretory glycoprotein k5 precursor (35kDa, pI 7.3), and spot 41 was identified as heat shock protein 70 (70kDa, pI 5.4). Identification of these proteins can help increase understanding of how certain antigens in SEA are involved and recognized by the immune response during schistosomiasis.

Combined with the protein mass data acquired from identified proteins, the previous one-dimensional PAGE experiments can also confirm the presence of disease form-specific antibodies and the proteins to which they bind (Figure 5.4). The protein labelled as spot 41, heat shock protein 70, can be seen. Bands of the correct size were not reactive for mouse 9C, and only weakly responsive with mouse 5C as shown. The slightly larger, HSS-Id specific spot 36 HSP70 is also present on HSS anti-SEA blotted membranes. Spot 70/71 as secretory glycoprotein k5 precursor lies at 35kDa. Mucin was highly reactive in all mice and may be the diffuse streak at ~50kDa. Furthermore, the low concentration of anti-SEA antibody from mouse 3C may explain its non-responsiveness for these experiments. The MSS-Id specific proteins, though unidentified, are approximated at sites corresponding to similar positions as those identified on the two-dimensional membranes (Figure 5.4).

A common issue with isoelectric focussing is that proteins towards the acidic or basic ends of the strip do not resolve as well, or as consistent, as proteins in the middle pI range of the strip (Figure 5.5). It can be seen that protein focussing at the acidic and basic ends varied across DIGE images. This had minor implications in determining reactive proteins as some were found in this variably resolved area. Furthermore, isolating MSS-specific protein spots 6 and 72 in these areas for mass spectrometry identification was more difficult than matching up proteins in the mid pI range. However, while difficult to analyze and identify, these spots, especially spot 72 which is more reactive to MSS sera, merit further investigation.

Legend:

Spot Volume	MSS	HSS
< 16500		
< 50000		
< 150000		
< 300000		
< 500000		
> 500000		

Reactivity	MSS HSS	HSS MSS
< 1		
< 2		
< 3		
< 4		
< 5		
> 5		

Figure 5.2: Non-specific and highly reactive spots on Western membrane against MSS mouse 7C
 Green arrows indicate spots that correspond to specific MSS-Id reactive proteins. Orange arrows indicate spots that correspond to non-specific highly reactive proteins. Red arrows indicate spots that correspond to specific HSS-Id reactive proteins.

Legend:

Spot Volume	MSS	HSS
< 16500		
< 50000		
< 150000		
< 300000		
< 500000		
> 500000		

Reactivity	MSS HSS	HSS MSS
< 1		
< 2		
< 3		
< 4		
< 5		
> 5		

Figure 5.3: HSS-specific and highly reactive spots on Western membrane against MSS mouse 7C
 Green arrows indicate spots that correspond to specific MSS-Id reactive proteins. Orange arrows indicate spots that correspond to non-specific highly reactive proteins. Red arrows indicate spots that correspond to specific HSS-Id reactive proteins.

Legend:

Spot Volume	MSS	HSS	Reactivity	MSS HSS	HSS MSS
< 16500			< 1		
< 50000			< 2		
< 150000			< 3		
< 300000			< 4		
< 500000			< 5		
> 500000			> 5		

Figure 5.4: Highly reactive spots confirmed on previous 1D-PAGE experiments

Orange arrows indicate bands that correspond to non-specific highly reactive proteins. Red arrows indicate spots that correspond to specific HSS-Id reactive proteins. Green arrows indicate approximate positions of specific MSS-Id reactive proteins.

Figure 5.5: Variable resolution at acidic and basic ends of the gel affected protein isolation

This image shows how protein resolution towards the basic end (towards the right) varies from gel to gel. Note in the left panel the well separated proteins (green spots) at the basic end, compared to the less resolved basic proteins in the right panel.

5.3 Discussion

The aim of this chapter was to identify proteins found in SEA using mass spectrometry. An initial focus was placed on identifying antigens corresponding to MSS-reactive antigens (i.e. spots 6, 29, 66 and 72) and HSS-specific antigens (i.e. 36 and 60; Figure 4.6). However all visible SEA proteins underwent mass spectrometry to more fully characterize the proteins present in SEA. Many reactive spots, especially MSS-specific spots, lay in areas of the gel showing low protein abundance under the coomassie stain, or near the acidic and basic ends of the gel where resolution was not as good. On areas of the coomassie-stained gel where there appeared to be very little protein present, a spot was taken from the position where reactivity was shown in the Western membranes. Despite our best efforts, no MSS-specific reactive spots were identified. In contrast, HSS-Id specific proteins isoforms of HSP70 (spot 36) and major egg antigen (spot 60) were identified. Other non-specific but highly reactive protein isoforms of mucin, heat

shock protein 70 and secretory glycoprotein k5 precursor were also successfully identified as well as some less reactive or non-reactive proteins.

CRI+ anti-SEA antibodies from MSS mice and humans contain idiotypes that regulate less severe chronic phase schistosomiasis.(Montesano, Freeman et al. 1990^b) Evidence for the existence of these protective antibodies was shown in both the one-dimensional and two-dimensional Western blotted membranes, as antibodies from MSS mice shared unique patterns of reactivity to proteins found in SEA. Spots numbered 6, 29, 66 and 72 corresponded to these proteins that preferentially bound the CRI+ anti-SEA antibodies. Thus these proteins may be part of the antigen/antibody interactions which drive the production of these protective antibodies and regulate less severe outcomes of disease. Although evidence for these protective antibodies was found, identification of the proteins recognized by these antibodies was more difficult. Reactive proteins were mainly low abundance or poorly resolved proteins. The apparent low abundance present on the coomassie stained gel suggests that the reactive proteins were highly glycosylated and diffusely resolved through the gel. This is likely, as the highly immunogenic schistosomal surface proteins are found to be highly glycosylated.(Jang-Lee, Curwen et al. 2007) As a consequence of this, no MSS-Id specific reactive proteins were identified using MALDI-TOF. Variable protein resolution at the acidic and basic ends of each gel also made MSS-specific antigen isolation and identification difficult (Figure 5.5) which may have contributed to the lack of MSS-specific antigen identification. In the acidic end of the gel, this includes MSS-specific spot 6, and MSS-specific spot 72 at the basic end of the gel.

Initially, HSS-Id specific anti-SEA antibodies were less of a focus as the initial hypothesis looked at the role of protective antibodies with the idea of developing a vaccine to induce protection against HS. Despite this, strong evidence for the existence of HSS-Id specific antibodies was shown in both the one-dimensional (Figure 5.4) and two-dimensional (Figure 5.3) Western blotted membranes. The HSS-specific protein spot 36, a 72kDa isoform of HSP70, was identified in MALDI-TOF. This spot is the 74kDa band seen in Figure 3.3 and discussed in Chapter 3. The other HSS-Id specific protein was identified as major egg antigen p40. The HSS-Id specific 72kDa isoform of HSP70 was the most robust example of idiotypic specificity between the MSS and HSS clinical forms as it was the only protein on the membrane to be consistently reactive or non-reactive to either form (Figure 4.7 & Figure 4.8).

The presence of HSS-specific antibodies suggests that these antibodies may be part of an antigen/antibody interaction that drives the detrimental immune response. This idea is the converse of our hypothesis that MSS-specific antibodies are protective but the mechanism by which this could occur is as yet unclear. The 70kDa HSP70 has previously been shown to be a major immunogen in schistosomiasis infection (Hedstrom, Culpepper et al. 1987) and from our experiments strong reaction to the 70kDa HSP70 occurred in all infected mice. However, the presence of the HSS-specific 72kDa isoform of HSP70 in HSS mice may only be indicative of a stress event that occurs in severe schistosomiasis. It is possible that the antibody is induced after an initial HSS-promoting immune event and is thus not part of a detrimental antigen/antibody reaction at all. Another possible but unlikely explanation for how anti-72kDa HSP70 could be

detrimental is that a cross-reactive antibody response causes an autoimmune reaction in human patients. Although an interesting suggestion, it is unlikely as, despite the highly conserved nature of HSP70 molecules, the antigenic sites of schistosomal HSP70 occur at divergent sites which are enough to elicit a discriminate antibody response.(Hedstrom, Culpepper et al. 1988)

To better understand whether the 72kDa HSP70 isoform antibody is a causative or indicative antibody for HSS disease in infected individuals, a better method to distinguish between this 72kDa HSP70 isoform and the known immunogenic HSP70 protein in determining antibody responses is needed. Whether or not the 72kDa HPS70 isoform directly promotes HSS or not, it is an interesting candidate antigen for a diagnostic tool that can determine what chronic form of schistosomiasis patients have. HSP70 has previously been tested for its usefulness as a diagnostic to determine early and late stages of schistosomiasis infection however it was seen to be of limited use as a diagnostic candidate in this regard.(Moser, Doumbo et al. 1990) It may be more useful in detecting individuals at risk of developing severe schistosomiasis, keeping in mind the distinct specificities between the 70kDa and 72kDa heat shock protein isoforms.

To establish whether this antibody arises early enough during the disease course to act as a marker to predict severe chronic disease later on, the current experiments can be replicated using sera taken from acutely infected mice. In acutely infected mice that eventually develop MSS, CRI are already detectable six weeks after initial infection

(Montesano, Colley et al. 2002), so a suitable early time-point to test for anti-HSP70 antibodies would be six weeks after initial infection to coincide with CRI+ antibodies. In mice that develop HSS, we would expect a rise in anti-72kDa HSP70 antibodies over time. If this turns out to be the case, then this would provide a strong candidate for a diagnostic marker to predict severe schistosomiasis in infected patients. Also, if the anti-72kDa HSP70 antibody is detrimental, we may see a rise and fall of the antibody in mice that eventually develop MSS. Further studies in humans could lead on from this.

The second spot that was preferentially reactive to HSS-derived antibodies against SEA was spot 60, and was identified as an isoform of major egg antigen p40. Major egg antigen p40 is found in the miracidium of mature schistosome eggs and will only become exposed to patient T-cells after disintegration of the egg.(Mathieson and Wilson 2010) The HSS-specific antibody response to this isoform may indicate an HSS stress event, or may be part of a detrimental immune response. Previous research has shown that it elicits strong immune responses in ~90% infected patients.(Nene, Dunne et al. 1986) Additionally, in the mouse model, it is highly immunogenic to mice that experience intense granuloma formation and is only weakly immunogenic to mice that develop less severe granulomas.(Hernandez, Trzyna et al. 1996) Furthermore, it has been suggested that major egg antigen p40 immune reactivity may be a risk factor for developing severe disease (Hernandez, Trzyna et al. 1996), and research in inhibiting the major egg antigen p40 response can develop ways to ameliorate the severe disease.(Stadecker and Hernandez 1998)

As we have found some antibodies against an isoform of major egg antigen p40 to be HSS-specific, this raises the possibility that antigen specificity may regulate granulomatous inflammation. The antigen may also be a suitable candidate for a diagnostic tool to show individuals at risk of developing severe schistosomiasis. However, before this idea is developed any further, a better method to distinguish between this major egg antigen p40 isoform and the other major egg antigen p40 proteins in determining antibody responses is needed. This is the same case as with the 72kDa HSP70 isoform as mentioned above. Distinguishing the isoform may be difficult as the other isoforms of major egg antigen p40 were identified as having the same molecular weight and pI.

In addition to the HSS-specific spots, we identified highly reactive, disease form non-specific proteins because these antigens may be useful in understanding the key antigens implicated in the immune response. The strongest reaction elicited by anti-SEA IgG from infected mice was against spot 1 which was identified as mucin. In mice, ~80% of all antibody binding was at this site on the membrane. Mucins are part of the mucus barriers on epithelial surfaces and have been found to be expressed on infective cercariae.(Theodoropoulos, Hicks et al. 2001) In the human-parasite system, mucins are highly immunogenic proteins but have been studied very little. They are suggested to play a role in host recognition and penetration due to its expression during miracidia and cercaria phases however its exact role is unknown.(Theodoropoulos, Hicks et al. 2001) Mucins have also been found in snail-parasite strain compatibility and it has been

hypothesised that mucin-like proteins could be recognized as “self” proteins used by the parasite to avoid recognition by its host.(Roger, Mitta et al. 2007)

Spot 41, which was also highly reactive in all mice, was identified as HSP70. It is smaller and more basic than the HSS-specific isoform and it plays a role in the immune response and is present throughout the schistosome egg's development.(Mathieson and Wilson 2010) It has been shown that HSP70 antibodies are detectable 5-6 weeks after the initial infection (Moser, Doumbo et al. 1990) and persist through to chronic infection, indicating that antibody responses are maintained during the course of infection.(Hedstrom, Culpepper et al. 1987) HSP70 has also been suggested as a candidate for a schistosomal vaccine due to its ability to stimulate lymphocytes from mice vaccinated with radiation-attenuated cercariae.(Richter, Reynolds et al. 1993) Our data shows that HSP70 is a highly immunogenic protein that may have a role in driving the immune response and is in agreement with these previous studies.

Secretory glycoprotein k5 precursor was also identified as the universally immunogenic protein spots 70 and 71. It has been found to be a primary target of the IgG and IgE antibody response against egg antigens in humans. Although the protein is found in both the egg and the miracidia stage of the parasite, its exact function is not yet known. One hypothesis is that secretory glycoprotein k5 precursor plays a role in human-parasite interactions.(Schramm, Hamilton et al. 2009) Further characterisation of this antigen, in conjunction with the other highly immunogenic proteins found in SEA, merits further

analysis as they may help increase our understanding of how certain antigens drive the immune response during schistosomiasis infection.

Chapter 6: General Discussion

6.1 Summary

Previous research has indicated that anti-SEA antibodies that have cross-reactive idiotypes (CRI) play a role in the regulation of the immune response to schistosome eggs present in the livers of infected patients.(Montesano, Colley et al. 2002) Because CBA/J mice with moderate splenomegaly syndrome (MSS) and hypersplenomegaly syndrome (HSS) share anti-SEA idiotypic repertoires to humans with intestinal (INT) and hepatosplenic (HS) forms, respectively (Montesano, Freeman et al. 1990^a), we have used the CBA/J mouse model of schistosomiasis to characterize and identify antigens in soluble egg antigen (SEA) that are specifically recognized by antibodies in the serum of mice with MSS, and/or mice with HSS. Knowing the reactive antigens present in SEA will be useful in increasing our understanding of the immunology of schistosomiasis and may lead to the development of new diagnostic or therapeutic tools.

Our primary aim was to determine the specific antigens in SEA that react to CRI+ anti-SEA antibodies using the CBA/J mouse model of disease. We tested differences in SEA specificity across mice with either MSS or HSS and showed that CRI+ anti-SEA antibodies found in MSS mice possess distinctly different patterns of specificity compared to the CRI- antibodies found in HSS mice. Through one-dimensional Western blots, most MSS mice recognized a band that migrated at 55kDa which was not reactive in any HSS mice, and all HSS mice recognised a band at 74kDa that was not reactive in

any MSS mice. This agrees with Correa-Oliveira *et al* who showed that there were differential antibody specificities in patients with INT and HS and further supports the hypothesis that antigenic recognition is distinct between the MSS/INT and HSS/HS disease forms.

Our next step was differential in-gel electrophoresis (DIGE), combining two-dimensional PAGE with Western blot analysis, using pooled Id and individual sera samples to better identify antibody-reactive proteins in the SEA preparations. Preliminary results indicated that DIGE was an effective way to isolate these antibody-reactive proteins. We also determined that anti-SEA IgG titres across individual mice greatly vary and cannot be indicative of disease form. This agreed with human studies that have shown that anti-SEA IgG ELISA titres vary widely across individuals with specific clinical presentations of disease.(Lima, Gazzinelli et al. 1986)

Further DIGE experiments used equal amounts of antibody during the Western blot step, giving us the ability to directly compare antibody reactivity and elucidate subtle differences in specificities to particular antigens that were unique and distinctive. We isolated these antigens and classified them according to their strong preference for MSS-Id anti-SEA, HSS-Id anti-SEA, or both. Comparing the antigens recognized by the affinity-purified, pooled Id and the individual sera revealed that similar antigens were identified as disease specific with these two approaches indicating that our disease-specific candidates were robust. Overall, of the 77 reactive antigens we found in SEA,

four were MSS-specific and two were HSS-specific. Additionally, we found six spots that were consistently and highly reactive across both clinical groups (Table 4.3 and Figure 4.6). Thus, we isolated six spots that were of significant interest as disease-specific candidates and a further six candidates that were infection-specific.

Using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) we successfully identified several of our candidate proteins in SEA. An initial focus was placed on identifying antigens corresponding to MSS-reactive antigens and HSS-specific antigens however all visible SEA proteins underwent mass spectrometry to more fully characterize the proteins present in SEA. Of the 77 spots designated to be reactive in SEA, 17 were identified. In this group, no MSS-specific reactive proteins were identified using MALDI-TOF despite our best efforts. In contrast, HSS-Id specific proteins isoforms of HSP70 and major egg antigen were identified. Other non-specific but highly reactive protein isoforms of mucin, heat shock protein 70 and secretory glycoprotein k5 precursor were also successfully identified as well as some less reactive or non-reactive proteins.

The HSS-Id specific 72kDa isoform of HSP70 was the most robust example of idiotypic specificity. HSS-Id was the only candidate to be consistently reactive to HSS, and non reactive to MSS-derived anti-SEA IgG (Figure 4.7 & Figure 4.8). The other HSS-specific protein identified in MALDI-TOF was an isoform of major egg antigen p40. In our studies, and in previous studies, HSP70 has been shown to be a major immunogen in

schistosomiasis infection (Hedstrom, Culpepper et al. 1987), and like HSP70, major egg antigen p40 also elicits strong immune responses, apparent in ~90% infected patients.(Nene, Dunne et al. 1986) It has been suggested that major egg antigen p40 immune reactivity may be a risk factor for developing severe disease (Hernandez, Trzyna et al. 1996), and research in inhibiting the major egg antigen p40 response can develop ways to ameliorate the severe disease.(Stadecker and Hernandez 1998) However, our results extend this previous work by identifying a unique isoform that is differentially recognized by mice with HSS.

The presence of HSS-specific antibodies suggests that these antibodies are part of an antigen/antibody interaction that drives the detrimental immune response or possibly that these antibodies are indicative of a stress event that occurs in severe schistosomiasis. To better understand whether the HSS-specific antibodies are causative or indicative antibodies for HSS disease in infected individuals, a better method to distinguish between the HSS-specific isoforms and the known immunogenic proteins in determining antibody responses is needed. Furthermore, to determine whether the HSS-specific isoforms are suitable candidate antigens for diagnostic markers, the current experiments should be replicated using sera taken from acutely infected mice to determine whether the HSS-specific antibodies arise early enough in the disease course to predict the outcome of chronic disease in infected individuals. Given that CRI+ anti-SEA antibodies are detectable in animals as early as six weeks post infection (Montesano, Colley et al. 2002), detection of the HSS-specific anti-Hsp70 or p40 antibodies at this early time point would support the development of these as possible diagnostic markers.

Importantly, our isolation of MSS-specific antigens fulfils another one of our aims which was to determine antigens that specifically react to protective, CRI+ anti-SEA antibodies. Isolation of these antigens is the first step in generating a vaccine antigen candidate that protects against severe schistosomiasis and outstanding work is to investigate ways of identifying these antigens. An effective vaccine lies in its ability to reduce morbidity by decreasing infection, schistosome fecundity or, in this case, pathology.(Siddiqui, Ahmad et al. 2008) Vaccine development strategies have mainly focussed on preventing schistosomiasis infection, however no vaccine used to routinely treat schistosomiasis exists as yet.(Bergquist, Utzinger et al. 2008) Developmental strategies which limit pathology that occurs during chronic disease is another approach that can lead to a vaccine which can help decrease overall morbidity in endemic populations. These vaccines would also be viable tools to be used in conjunction with existing chemotherapy programs that focus on decreasing morbidity in endemic populations.

We also successfully identified the highly reactive, disease form non-specific proteins mucin, HSP70 and secretory glycoprotein k5. These antigens support previous research which suggested their roles in disease progression (Hedstrom, Culpepper et al. 1987; Moser, Doumbo et al. 1990; Theodoropoulos, Hicks et al. 2001; Roger, Mitta et al. 2007; Schramm, Hamilton et al. 2009) and merit further analysis as they may be useful in understanding how key antigens drive the immune response during schistosomiasis infection. Furthermore, these antigens may prove useful in combination with the HSS-specific marker(s), as general markers of schistosome infection.

Overall, we determined that antibody specificities of infected mice differ between clinical groups and that infection specific antibody titres greatly differ across individual mice, irrespective of disease form. We demonstrated that DIGE is an effective method to detect the disease form-specific antibody/SEA interactions followed by MALDI-TOF to identify the candidate SEA proteins. Finally, we achieved our aim of determining differential antibody specificities in the serum of infected MSS and HSS mice to SEA and have identified candidate proteins that are recognized only in the severe form of disease. These results increase our understanding of the immune response during schistosomiasis and may be useful towards the development of diagnostic tools for the severe form of disease. Further research to determine the viability of the HSS-reactive antigens as diagnostic markers and to identify the novel MSS-reactive proteins may provide more candidates for diagnostic tools and vaccines in the future.

6.2 Significance

Outlined in the World Health Assembly resolution 54.19, May 2001, is the combined strategy to control morbidity in tropical countries. The belief is that sustained treatment utilizing current therapies in endemic areas will result in lower severity and subclinical morbidity in these populations. The Schistosomiasis Control Initiative and the Partnership for Parasite Control are such public-private partnerships that can implement such a strategy. Inherently, these praziquantel based chemotherapy treatment schemes are difficult to sustain indefinitely (Croce, Porazzi et al. 2010), thus the preferential

treatment of high risk groups within populations is important. Also, based on previous chemotherapy programmes attempting to control widespread disease (e.g. malaria) a more focused approach in treatment is important to avoid phenomena such as acquired drug resistance in the schistosome pathogen.(Ismail, Botros et al. 1999; Colley and Secor 2004; Hotez, Brindley et al. 2008)

We found evidence for antigens that are specific for moderate or severe disease form antibodies and the most straightforward use for these antigens is their use as a marker for susceptibility to severe disease. The World Health Organisation determines that the type of mass treatment a population will receive will be based on morbidity of individuals, rather than prevalence, since schistosome worms do not replicate in humans.(Colley and Secor 2004; Hotez, Brindley et al. 2008) Antigens that are markers for a disease form can serve as candidate antigens for the development of a diagnostic tool that can determine individuals who likely to develop severe schistosomiasis during the chronic phase of disease. By focusing treatment to these individuals, this can direct a reduced severe pathology in clinical cases of schistosomiasis and lessen disease burden in countries implementing chemotherapy based programmes.

Another issue that needs to be addressed is the fact that praziquantel is the only drug used to treat schistosomiasis, and there are currently issues with drug resistance (Ismail, Botros et al. 1999), treatment length (Siddiqui, Ahmad et al. 2008) and maintaining treatment programs in poorer, endemic countries.(Croce, Porazzi et al. 2010) A vaccine that is long

lasting and potent can contribute greatly to disease control and provide an alternative to chemotherapy treatment programs. Currently, no vaccine exists that is routinely used to treat schistosomiasis.(Bergquist, Utzinger et al. 2008) We found evidence for antigens that are specifically reactive to protective, CRI+ antibodies and these can be used as candidates for the development of a vaccine that can drive the production of these protective antibodies. This would limit pathology in infected individuals by regulating the immune response that determines disease outcome. We also found evidence of HSS-Id specific antigens and further analysis of these antigens may help increase understanding of severe disease induction in chronic patients, which can lead to ways of ameliorating severe disease.

6.3 Future Directions

There was some difficulty in identifying MSS-reactive spots as they were of low abundance or diffuse throughout the membrane. Approaches to identify these proteins focus on increasing the abundance of these proteins or improving the resolution of the focussing steps. Immunoprecipitation using beads coated in MSS-Id and loaded with excess SEA sample would result in a gel with high concentrations of MSS-Id specific antigens which would be more suitable for mass-spectrometry analysis. Additionally, many reactive spots were faint in the gel and appeared diffuse likely due to the antigens themselves being heavily glycosylated.(Jang-Lee, Curwen et al. 2007) Pre-treatment of

SEA to remove modifications and improve their two-dimensional gel focussing may also help in generating high abundance spots suitable for MALDI-TOF.

Another interesting avenue of research would be to look at reactive antigens present in soluble worm antigen preparations (SWAP) as it has been hypothesised that antigens present in both SEA and SWAP may be responsible for the induction of CRI+ anti-SEA antibodies that happen early on in schistosome infection.(Colley and Secor 2004) Elucidating disease form-specific antigens present in SWAP may help increase understanding of the induction of protective CRI+ anti-SEA antibodies, which would be important in terms of developing a vaccine. The role of SEA and SWAP in altering the immune response is supported by evidence of patients with repeated rounds of infection and praziquantel treatment developing a resistance to re-infection (Black, Mwinzi et al. 2010) which may be due to the effect of praziquantel, which kills both adult worms and mature eggs, releasing antigens from both of these stages of the parasite life cycle.(Colley and Secor 2004; Vereecken, Naus et al. 2007)

Another future step would be to look at the reactivity of antibodies found in the sera samples of human patients with chronic schistosomiasis. Using the same methods we had in analysing the CBA/J mouse model, human sera samples containing either INT-Id or HS-Id anti-SEA antibodies can be tested against SEA. This can determine antigens present in SEA that are reactive to human-derived antibodies. It can also test the relationship between the CBA/J mouse model of disease and the human system with

respect to antigen specificity. By finding robust antigens that react with human antibodies, these can serve as candidates for the development of vaccines and diagnostic tools that are based on these antigens.

After suitable candidate antigens have been found, there is still the development of diagnostic tools and/or vaccines to consider. An SEA enzyme-linked immunosorbent assay (ELISA) diagnostic tool exists, which is routinely used in non-endemic areas. However, these tests are limited by the fact that they fail to reliably distinguish between current and past infection, rendering them unsuitable for measuring treatment success. They also fail to pick up infections which are generally less than three months old. Additionally, they are mainly used clinically in non-endemic areas as there is less risk of co-infection with other helminths, which can cause cross-reactivity.(Chand, Chiodini et al. 2010) Thus, in order for the candidate protein to be worthwhile, testing the specificity of the candidate protein to be able to distinguish past and present infection should be undertaken using sera of infected and formerly infected patients. Also, infection specific-antibodies against the protein should be detectable in the serum of infected individuals early in infection. The efficacy of the candidate protein to detect antibodies early in infection can be tested using sera from infected mice early in infection. Because CRI+ antibodies are detectable in the serum of infected mice at six weeks of infection, it is possible that such a candidate protein can be found through our experimental protocol. Additionally, the protein must be *S. mansoni* specific, and testing against co-infected mice, or individuals from endemic populations where co-infection with other parasites is common, will be important to judge its specificity. Furthermore, to be used in the field,

the test should be rapid and accessible. An ELISA based approach requires specialised equipment however it is likely that such a diagnostic tool will take this form, as previous SEA assays before it. Once such a diagnostic tool exists, it will not only provide a valuable tool in distinguishing individuals at risk of schistosomiasis, but also a means of evaluating the efficacy of treatment programs over time in reducing schistosomiasis prevalence in endemic populations.

Vaccine development will also need to be pursued if a suitable candidate antigen is found which is specific to CRI+ antibodies. Montesano *et al* have shown that CRI+ anti-SEA antibody injections of neonatal mice increased their survival and decreased pathology eight weeks after subsequent infection with schistosomiasis.(Montesano, Colley et al. 1999^a) Experimental protocols can be replicated using active immunisations of the candidate antigen to determine whether it drives the production of protective antibodies that mediate the immune response. If successful, further analysis of its efficacy in reducing severe pathology, as well as suitable adjuvants and delivery methods, can be carried out. Clinical trials in endemic populations would be required to determine whether immunisation before infection is effective at reducing pathology in individuals after infection. If such a vaccine is found, it can reduce morbidity in endemic populations and improve quality of life. Also, vaccine development is not necessarily a question of providing an exclusive alternative to chemotherapy; if such a vaccine that drives the production of protective antibodies proves to be functional, it can be used in conjunction with praziquantel-based therapy programs to reduce morbidity in endemic populations undergoing treatment.(Bergquist, Leonardo et al. 2005)

Defining the pathogenesis of schistosomiasis in humans is a high priority in order to understand the overall disease course.(Cheever, Hoffmann et al. 2000) There are limited studies linking the humoral response with morbidity (Caldas, Campi-Azevedo et al. 2008) and finding a humoral basis for the disease course may provide a unified definition for each of the stages of disease. This can also help with future research and control strategies by providing clarity to the definitions of the different stages and forms of disease and more robust methods of distinguishing them. Finally, the current dearth in ways to efficiently diagnose and treat severe schistosomiasis reflects our limited understanding about how immune responses drive outcomes of disease. We believe that our study will benefit further research in developing diagnostic tools and vaccines, and increase understanding of helminth interactions in human biology.

Chapter 7: References

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Appendix A - Tables

Chapter 4 tables

Spot reactivity is displayed for all 77 reactive spots seen on the membranes.

	Individual Mice Sera												Pooled Sera			
Spot	1C	2C	3C	7C	9C	5C	6C	10C	12C	17C			M1	M2	H	Spot
1															1	
2															2	
3															3	
4															4	
5															5	
6															6	
7															7	
8															8	
9															9	
10															10	
11															11	
12															12	
13															13	
14															14	
15															15	
16															16	
17															17	
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22															22	
23															23	
24															24	
25															25	
26															26	
27															27	
28															28	
29															29	
30															30	

	Individual Mice Sera											Pooled Sera			
Spot	1C	2C	3C	7C	9C	5C	6C	10C	12C	17C	Reactivity	M1	M2	H	Spot
31															31
32															32
33															33
34															34
35															35
36															36
37															37
38															38
39															39
40															40
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63															63
64															64
65															65
66															66
67															67
68															68
69															69
70															70

	Individual Mice Sera												Pooled Sera			
Spot	1C	2C	3C	7C	9C	5C	6C	10C	12C	17C	Reactivity		M1	M2	H	Spot
71																71
72																72
73																73
74																74
75																75
76																76
77																77

Legend:

Spot Volume	MSS	HSS	Reactivity	MSS HSS	HSS MSS
< 16500			< 1		
< 50000			< 2		
< 150000			< 3		
< 300000			< 4		
< 500000			< 5		
> 500000			> 5		

Figure A.1: Spot reactivity corresponding to colour density

Spot refers to the assigned spot number given to a particular reactive protein found in SEA. 1C, 2C, 3C, 7C and 9C refer to membranes that were blotted with primary antibodies taken from MSS mouse sera. 5C, 6C, 10C, 12C and 17C refer to membranes that were blotted with primary antibodies taken from HSS mouse sera. M1 and M2 refer to membranes that were blotted with primary antibodies taken from the MSS-Id pooled sera. H refers to a membrane that was blotted with primary antibodies taken from HSS-Id pooled sera. Spot volume shows the absolute value (arbitrary units) for antibody bound to a particular protein and its corresponding colour. Reactivity relates to whether the protein preferentially binds MSS-Id or HSS-Id anti-SEA antibodies.

Chapter 5 tables

Spot	Protein	Matches	% Cover	Expect
1a	mucin [Schistosoma mansoni]	9	42	1.2E-06
1b	mucin [Schistosoma mansoni]	4	17	1.2E-02
10	mucin [Schistosoma mansoni]	10	41	1.9E-07
11	mucin [Schistosoma mansoni]	7	27	4.6E-04
34	heat shock protein 86	15	39	2.3E-13
35	heat shock protein 86	18	44	9.3E-12
36a	HSP70 [Schistosoma japonicum]	7	15	5.5E-06
36b	HSP70 [Schistosoma japonicum]	8	16	4.7E-07
38	SJCHGC09453 protein [Schistosoma japonicum]	19	25	2.8E-04
41a	heat shock protein 70	15	27	3.0E-12
41b	70,000 mol wt antigen/hsp70 homologue (619 AA) [Schistosoma mansoni]	11	20	1.9E-07
41c	heat shock protein 70	9	17	3.7E-02
41d	Heat shock 70 kDa protein homolog (HSP70) (Major surface antigen)	11	16	2.5E-04
43	SJCHGC04656 protein [Schistosoma japonicum]	8	35	1.3E-02
53	similar to GenBank Accession Number U54587 unknown in Schistosoma mansoni	4	48	7.8E-03
59	Major egg antigen (p40)	12	36	7.4E-08
60	Major egg antigen (p40)	7	23	2.3E-07
61	Major egg antigen (p40)	12	35	7.8E-06
62	Major egg antigen (p40)	6	23	9.6E-06
68	secretory glycoprotein k5 precursor [Schistosoma mansoni]	17	44	2.3E-08
69	secretory glycoprotein k5 precursor [Schistosoma mansoni]	15	38	9.3E-07
71	secretory glycoprotein k5 precursor [Schistosoma mansoni]	13	36	9.3E-08

Table A.1: List of identified SEA proteins reactive to anti-SEA IgG with matches, protein coverage and expectation values

This table shows the numbered reactive spots and its corresponding protein identified by MALDI-TOF. Protein matches, protein coverage (%) and expectation values are also shown.

Spot	Protein	Matches	% Cover	Expect
A1	ER-luminal cysteine protease ER 60 [Schistosoma mansoni]	8	20	6.3E-03
A2	ER-luminal cysteine protease ER 60 [Schistosoma mansoni]	9	17	2.1E-03
B1	Calreticulin precursor (SM4 protein)	8	15	5.9E-04
B2	calreticulin	7	10	2.2E-05
C	protein disulfide isomerase homologue [Schistosoma mansoni]	14	39	3.0E-07
D	beta-tubulin [Schistosoma japonicum]	10	20	1.3E-06
E1	alpha tubulin	7	20	1.7E-03
E2	alpha tubulin	6	18	1.2E-04
E3	alpha tubulin	8	21	3.7E-06
F1	Actin-2	7	23	3.70E-05
F2	Actin-2	6	20	3.2E-04
G	30 kDa glycoprotein	4	34	1.2E-02
H	14-3-3 epsilon [Schistosoma mansoni]	11	37	1.6E-05
I	14-3-3 protein zeta [Schistosoma bovis]	13	44	3.0E-11
J1	thioredoxin peroxidase 1 [Schistosoma mansoni]	6	35	3.7E-08
J2	thioredoxin peroxidase 1 [Schistosoma mansoni]	7	41	2.2E-06
K	Adenylate kinase (ATP-AMP transphosphorylase) (AK)	7	41	1.2E-04
L	Glyceraldehyde-3-phosphate dehydrogenase (Major larval surface antigen) (P-37)	7	23	1.9E-03

Table A.2: List of identified SEA proteins non-reactive to anti-SEA IgG with matches, protein coverage and expectation values

This table shows the alphabetised non-reactive spots and its corresponding protein identified by MALDI-TOF. Protein matches, protein coverage (%) and expectation values are also shown.

Appendix B – Calculations

Chapter 3 calculations

3.2 One-dimensional gel and Western Blot analysis for individual mouse sera

Figure B.1: Graph showing Migration (x) versus Protein size (y)

The formula for the graph is $y = 5376x^{-1.033}$ with $R^2 = 0.9962$ and shows the curve for the ladder proteins.

Lane	Migration	Derived size (kDa)
12C (HSS)	24	202
12C (HSS)	32	150
12C (HSS)	61.5	76
12C (HSS)	66	71
12C (HSS)	107.5	43
12C (HSS)	131	35
2C (MSS)	23.5	206
2C (MSS)	32	150
2C (MSS)	41	116
2C (MSS)	66	71
2C (MSS)	131	35
250kDa ladder	20	244
150kDa ladder	31	155
100kDa ladder	47	101
75kDa ladder	60	78
50kDa ladder	98	47
37kDa ladder	131	35
25kDa ladder	170	27

Table B.1: Table of estimated antigen sizes that bind MSS or HSS-specific antibodies

These estimated antigen sizes are derived from the membrane scan shown above. Note that the 75kDa ladder size is taken from the corresponding coomassie stained portion of the membrane (image not shown). The formula for the graph is $y=5376x^{-1.033}$ with $R^2=0.9962$ and was used as a guide to derive protein sizes present in the gel.

3.3 Extended individual mouse anti-SEA reactivity

Figure B.2: Graph showing Migration (x) versus Protein size (y)

The formula for the graph is $y=14554x^{-1.266}$ with $R^2=0.9901$ and shows the curve for the ladder proteins.

Lane	Migration	Derived size (kDa)
3C (MSS)	66	73
3C (MSS)	116	35
7C (MSS)	29	205
7C (MSS)	35	162
7C (MSS)	66	72
7C (MSS)	82	55
7C (MSS)	85	53
7C (MSS)	96	45
7C (MSS)	116	35
9C (MSS)	29	205
9C (MSS)	35	162
9C (MSS)	81	56
9C (MSS)	86	52
9C (MSS)	96	45
9C (MSS)	117	35
5C (HSS)	29	205
5C (HSS)	35	162
5C (HSS)	60	82
5C (HSS)	65	74
5C (HSS)	85	53
5C (HSS)	94	46
5C (HSS)	115	36
17C (HSS)	29	205
17C (HSS)	35	162
17C (HSS)	60	82
17C (HSS)	65	74
17C (HSS)	85	53
17C (HSS)	96	45
17C (HSS)	116	35
250kDa ladder	27	224
150kDa ladder	35	164
100kDa ladder	49	105
50kDa ladder	89	50
37kDa ladder	115	36

Table B.2: Table of estimated antigen sizes that bind MSS or HSS-specific antibodies

These estimated antigen sizes are derived from the membrane scan shown above. Note that the 75kDa ladder size is taken from the corresponding coomassie stained portion of the membrane (image not shown). The formula for the graph is $y=5376x^{-1.033}$ with $R^2=0.9962$ and was used as a guide to derive protein sizes present in the gel.

3.5 Anti-Soluble Egg Antigens Antibody Titres Across Disease Forms

Bradford assay data

Figure B.3: This BSA standard curve was used to determine the diluted concentrations of the HSS-Id and MSS-Id samples.

The BSA curve was generated from two sets of dilutions. The recorded values for HSS-Id and MSS-Id diluted samples lay in the linear range of the standard curve and were used to determine protein concentration.

The formula for the linear range of the BSA standard curve is:

$$y = 0.253608 + 0.0909266x$$

where y is the given absorbance value of a particular sera sample and x is the total protein concentration (mg/mL) of the sample.

The concentration of pooled MSS-Id sample is:

$$[MSS - Id] = \frac{(0.3723 - 0.253608)}{0.0909266} / 2$$

= 0.6527mg/ml (Note that 2µl MSS-Id sample was added to the starting well).

The concentration of pooled HSS-Id sample is:

$$[HSS - Id] = \frac{(0.2931 - 0.253608)}{0.0909266} / 18$$

= 0.02413mg/ml (Note that 18µl HSS-Id sample was added to the starting well).

Nanodrop data

Figure B.4: Nanodrop estimates support MSS-Id (Left) and HSS-Id (Right) true concentration values, of which there is a vast difference.

The true concentrations of the MSS-Id and HSS-Id are 0.6527mg/ml and 0.02413mg/ml respectively.

ELISA data

The formula for the MSS-Id standard curve is:

$$y = \min + \frac{\max - \min}{1 + \left(\frac{x}{EC_{50}} \right)^{Hillslope}}$$

where y is the given absorbance value of a particular sera sample and x is the anti-SEA IgG concentration (mg/mL) of the diluted sample.

$$\min = 0.0331891$$

$$\max = 0.255927$$

$$EC_{50} = 3.79092e^{-6}$$

$$Hillslope = -0.544136$$

The absorbance values y used for each sera sample was taken from the value that lay in the linear range of that sample's dose response curve. The absorbance value of that point also had to lie in the linear range of the MSS-Id dose response curve which was from 0.0603 to 0.213 in order to find the concentration (mg/mL) of the diluted sample x .

To find x , the equation is derived as follows:

$$y = \min + \frac{\max - \min}{1 + \left(\frac{x}{EC_{50}} \right)^{Hillslope}}$$

$$y - \min = \frac{\max - \min}{1 + \left(\frac{x}{EC_{50}} \right)^{Hillslope}}$$

$$1 + \left(\frac{x}{EC_{50}} \right)^{Hillslope} = \frac{\max - \min}{y - \min}$$

$$\left(\frac{x}{EC_{50}} \right)^{Hillslope} = \frac{\max - \min}{y - \min} - 1$$

$$\left(\frac{x}{EC_{50}} \right) = \sqrt[Hillslope]{\frac{\max - y}{y - \min}}$$

$$x = EC_{50} \sqrt[Hillslope]{\frac{\max - y}{y - \min}}$$

where y is the given absorbance value of a particular sera sample and x is the concentration (mg/mL) of the diluted sample.

Every 100 μ L starting solution in each well contained 0.957 μ L sera sample and was diluted accordingly. Most y values used for each sera sample were at the 125 dilution except for mouse 3C, 9C and 17C (where y was at the 25, 5 and 25 dilution respectively)

as the midpoint for these dose response curves lay outside the linear range of the MSS-Id standard.

To get the concentration of the original sample, this formula was used:

$$z = x \times \text{dilution} \times \frac{100\mu L}{0.957\mu L}$$

where z is the concentration of anti-SEA IgG (mg/mL) in the original sera sample.

Appendix C – Membrane scans

The images below are a complete list of antibody binding images showing anti-SEA IgG present in each sample. The left panel is DIGE. SEA (9.5µg/gel) was labelled with Cy3 (green), separated by two-dimensional PAGE, and transferred to a PVDF membrane before being incubated with MSS-Id or HSS-Id. Specific antibody binding was detected using Cy5-labelled goat anti-mouse IgG antibody (red, diluted 1:600). Yellow indicates sites of antibody binding in an area with high amounts of SEA. The right panel shows only antibodies present. Red circles indicate the position of the assigned reactive spot, and the area inside the circle was used to determine the volume of antibody binding.

Chapter 4 membrane scans

4.2 - Pooled sera reactivity results

Figure C.1: Image showing sites of anti-SEA IgG binding for MSS-Id (M1)
Anti-SEA IgG diluted 1:5000.

Figure C.2: Image showing sites of anti-SEA IgG binding for MSS-Id (M2)
Anti-SEA IgG diluted 1:5000.

Figure C.3: Image showing sites of anti-SEA IgG binding for HSS-Id (H)
Anti-SEA IgG diluted 1:5000.

4.3 - Individual sera reactivity results

Figure C.4: Image showing sites of anti-SEA IgG binding for MSS mouse 1C
Anti-SEA IgG diluted 1:5000.

Figure C.5: Image showing sites of anti-SEA IgG binding for MSS mouse 2C
Anti-SEA IgG diluted 1:5000.

Figure C.6: Image showing sites of anti-SEA IgG binding for MSS mouse 3C
Anti-SEA IgG diluted 1:5000.

Figure C.7: Image showing sites of anti-SEA IgG binding for MSS mouse 7C
Anti-SEA IgG diluted 1:5000.

Figure C.8: Image showing sites of anti-SEA IgG binding for MSS mouse 9C
Anti-SEA IgG diluted 1:5000.

Figure C.9: Image showing sites of anti-SEA IgG binding for HSS mouse 5C
Anti-SEA IgG diluted 1:5000.

Figure C.10: Image showing sites of anti-SEA IgG binding for HSS mouse 6C
Anti-SEA IgG diluted 1:5000.

Figure C.11: Image showing sites of anti-SEA IgG binding for HSS mouse 10C
Anti-SEA IgG diluted 1:5000.

Figure C.12: Image showing sites of anti-SEA IgG binding for HSS mouse 12C
Anti-SEA IgG diluted 1:5000.

Figure C.13: Image showing sites of anti-SEA IgG binding for HSS mouse 17C
Anti-SEA IgG diluted 1:5000.

Figure C.14: Image showing sites of anti-SEA IgG binding for normal mouse 3N
No anti-SEA IgG present in the uninfected sample.

Chapter 3 scans

3.4 - Two-dimensional gel and Western Blot analysis for pooled and individual mouse sera

For completeness, scans for preliminary Western blots have been included, however we have noted that many of these scans show poorly resolved SEA, as indicated in the Cy3 SEA visualisation. This was taken into account when we reasoned that low reactivity was possibly due to low antibody concentrations, rather than poor SEA resolution. For each Western blot, serum was added from each sample at 1:600 dilution. True dilution values of anti-SEA IgG added to each Western blot have been included in each figure, however this was not known at the time.

Figure C.15: Image showing sites of anti-SEA IgG binding for MSS-Id
Anti-SEA IgG diluted 1:900.

Figure C.16: Image showing sites of anti-SEA IgG binding for HSS-Id
Anti-SEA IgG diluted 1:25000.

Figure C.17: Image showing sites of anti-SEA IgG binding for MSS mouse 3C
Anti-SEA IgG diluted 1:97000. Low reactivity may be due to poorly resolved SEA.

Figure C.18: Image showing sites of anti-SEA IgG binding for MSS mouse 7C
Anti-SEA IgG diluted 1:15000.

Figure C.19: Image showing sites of anti-SEA IgG binding for MSS mouse 8C (1 of 2)
Anti-SEA IgG diluted 1:35000. Low reactivity may be due to poorly resolved SEA.

Figure C.20: Image showing sites of anti-SEA IgG binding for MSS mouse 8C (2 of 2)
Anti-SEA IgG diluted 1:35000. Low reactivity was likely due to low amounts of anti-SEA IgG.

Figure C.21: Image showing sites of anti-SEA IgG binding for MSS mouse 9C (1 of 2)
Anti-SEA IgG diluted 1:1900. Reactivity was present despite poorly resolved SEA, likely due to the high concentration of anti-SEA IgG added.

Figure C.22: Image showing sites of anti-SEA IgG binding for MSS mouse 9C (2 of 2)
Anti-SEA IgG diluted 1:1900.

Figure C.23: Image showing sites of anti-SEA IgG binding for HSS mouse 5C
Anti-SEA IgG diluted 1:18000.

Figure C.24: Image showing sites of anti-SEA IgG binding for HSS mouse 6C
Anti-SEA IgG diluted 1:15000.

Figure C.25: Image showing sites of anti-SEA IgG binding for HSS mouse 10C
Anti-SEA IgG diluted 1:30000.

Figure C.26: Image showing sites of anti-SEA IgG binding for HSS mouse 12C
Anti-SEA IgG diluted 1:9000. Low reactivity may be due to poorly resolved SEA.

Figure C.27: Image showing sites of anti-SEA IgG binding for uninfected mouse 5N
No anti-SEA IgG present in the uninfected sample.

Appendix D – Reagents

1% DTT Equilibration buffer (50mM Tris, 6M Urea, 30% Glycerol, 2% SDS, fresh 1% Dithiothreitol, with bromophenol blue)

2.5% IAA Equilibration buffer (50mM Tris, 6M Urea, 30% Glycerol, 2% SDS, fresh 2.5% Iodoacetamide, with bromophenol blue)

BD OptEIA™ TMB Substrate Reagent Set

Blocking buffer (5% foetal calf serum in PBS)

Bradford reagent (0.01% (w/v) Coomassie Blue Dye, 50mL, 5% (95%) ethanol, 10% (85% w/v) phosphoric acid, filtered before use)

Coating buffer (pH ~ 9.0 0.1M sodium carbonate / sodium bicarbonate buffer)

Colloidal staining solution (17% ammonium sulphate, 34% methanol, 3% phosphoric acid)

Fixer (50% ethanol, 3% v/v phosphoric acid)

MOPS NuPage working buffer (40 mL Stock MOPS, 760 mL MilliQ water)

Precipitation Agent (Calbiochem® ProteoExtract™ reagent catalogue number 539180)

Rehydration buffer (2M Thiourea, 7M Urea, 2% IPG buffer 3-10, 2% DTT, 4% (w/v) CHAPS, with bromophenol blue)

Running buffer (Tris 25mM, Glycine 192mM, SDS 0.1% (w/v))

SDS-PAGE gels (10% separating gel and 4% stacking gel, 1% SDS)

Stop solution (0.18M H₂SO₄)

T-TBS (Tris 20mM, NaCl 136mM, Tween-20 5% (v/v))

Valve 1 solution (50mM Ambic, 50% Methanol)

Valve 2 solution (20 mM Ambic)

Valve 3 solution (0.1% Trifluoroacetic acid, 50% Acetonitrile)

Wash buffer (0.05% Tween-20 in PBS)

Wash solution for protein precipitation (Calbiochem® ProteoExtract™ reagent catalogue number 539180)

Western Transfer buffer (25 mM Tris, 192 mM Glycine, 20% Methanol v/v)