"Carbohydrate-mediated antigenic cross-reactivity between *Schistosoma mansoni* antigens and environmental allergens"

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General Abstract

There has been an increase in allergic diseases such as asthma, which triggers an IgE allergic reaction, in countries with advanced health systems. However, in helminth-endemic countries, an inverse correlation between infection with parasitic helminths including schistosomes and allergic sensitisation has been observed. This has led authors to formulate the so-called hygiene hypothesis. Previous studies have shown that rabbit IgG antibodies against *Schistosoma mansoni* soluble egg antigens (SmSEA) cross-reacted with various allergens such as peanut, rubber latex and grass and tree pollens. Here we describe antigenic molecules that cross-react with rabbit anti-*S. mansoni* IgG antibodies in extracts of the Australian cockroach (ACR) *Periplaneta australasiae* and the house dust mite (HDM) *Dermatophagoides farinae*. Our investigation was carried out using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western immunoblotting using rabbit antisera raised against *S. mansoni* antigen. The purified material was subjected to Tandem mass spectrometry to identify *S. mansoni* antigen that is cross-reactive with molecules in an extract of the different allergens. We found the cross-reactive allergens as Der f 15 in HDM and two homologues of the *Periplaneta americana* cockroach allergen Cr-PI/Per a 3 in ACR. Acid elution of anti-SmSEA antibodies cross-reactive with the allergens reacted with the major egg antigens of *S. mansoni* namely IPSE-alpha 1, Kappa-5 and Omega-1. Moreover, rabbit anti-schistosome IgG antibodies eluted from HDM

reacted with a variety of plant extracts. Treatment with sodium meta periodate ablated most of the cross-reactivity of the antigen suggesting that it might be due to cross-reactive carbohydrate determinants (CCDs). In this work, we have also used the humanized Rat Basophilic Leukemia RS-ATL8 reporter system which is used to detect allergen-specific IgE in human serum. The reason for using such a system was to investigate whether anti-schistosome IgG antibodies that are cross-reactive with an allergen – *D. farinae* - can act as ‘blocking antibodies. We found that the two different donor serum samples used were able to sensitise the reporter cells upon challenge with anti-human IgE, but when challenged with the mite allergen there was only a very low response. This could mean that the molecule Der f 15, which is a minor HDM allergen, might not be the target of an allergen-specific IgE response in these two donors. Furthermore, we developed an *in vitro* system to study if recombinant IgG can block IgE mediated activation using Phl p 7 a well-known characterised allergen. This was carried out by expressing IgE and IgG protein in mammalian HEK 293 cells, the expressed IgG protein was purified using Affinity Chromatography ÄKTAstart system. Our data show that the two proteins were successfully expressed, and the IgG protein was also purified successfully. In conclusion, chapter 3 and 4 findings could provide a useful application for improved allergen-specific immunotherapy. Furthermore, we could use our reporter system to demonstrate that recombinant IgG antibodies can block the interaction of anti-Phl p 7 IgE antibody with Phl p 7 allergen, thereby blocking allergen-mediated crosslinking of IgE receptors in chapter 5.

Publication, Presentations and Poster

Marwa H. El-Faham, **Fatou Gai**, Joseph E. Igetei, Sarah Richter, Franco H. Falcone, Gabi Schramm and Michael J. Doenhoff.
‘’Antigenic cross-reactivity between *Schistosoma mansoni* and allergenic invertebrates putatively due to shared glycanic epitopes‘’. Scientific Reports, 2020.

Tatiane Teixeirade de Melo, Mariana Moreira Mendes, Clarice Carvalho Alves, Gardênia Braz Carvalho, Viviane Cristina Fernandes, Deborah Laranjeira Ferreira Pimenta, Marinade Moraes Mourão, **Fatou Gai**, Marina Kalli, Aline Coelho, Rosy Iara Macielde Azambuja Ribeiro, Franco H.Falcone, Rosiane Aparecida da Silva Pereira, Cristina Toscano Fonseca. The *Schistosoma mansoni* cyclophilin A epitope 107-121 induces a protective immune response against schistosomiasis. Molecular Immunology, 2019.

Use of the Humanized Rat Basophilic Leukaemia RS-ATL8 reporter assay for the demonstration of IgG blocking antibody (Drafted Manuscript).

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Table of Contents

General Abstract i

Publication, Presentations and Poster iii

Acknowledgment v

Table of Contents vi

List of Figures xii

List of Tables xv

Abbreviations xvii

Chapter 1: General introduction 1

1.1 Schistosomiasis 1

1.2 Epidemiology 2

1.3 The life cycle of the parasite 3

1.3.1 Egg production 4

1.3.2 Miracidium 5

1.3.3 Cercaria 6

1.3.4 Schistosomula 7

1.3.5 Adult 8

1.3.6 Granuloma formation around the egg antigen of *S. mansoni* 9

1.4 Characterized Schistosoma mansoni egg antigens 11

1.4.1 IPSE/alpha-1 11

1.4.2 Omega-1 14

1.4.3 Kappa-5 (K-5) 15

1.5 Helminths and allergens *16*

1.5.1 Parasitic helminths and the hygiene hypothesis 16

1.5.2 Helminths and allergens 18

1.6 Mast cells and basophils *24*

1.7 Cross-reactive carbohydrate epitopes *26*

1.8 RS-ATL8 reporter system *27*

1.9 Study aim and objectives *28*

Chapter 2: General materials & methods 31

2.1 Preparation of Schistosoma mansoni antigens *31*

2.1.1 Soluble egg antigen (SEA) 31

2.1.2 Snail infection to generate Cercarial homogenate (Ch) 33

2.1.3 Schistosomula extract 34

2.1.4 Adult worm homogenate (Wh) 35

2.2 Estimation of protein concentrations *35*

2.3 Allergen extract preparation *37*

2.3.1 *Dermatophagoides farinae* (HDM) crude extract 37

2.3.2 Cockroach 38

2.4 Preparation of Sodium Dodecyl Sulphate Polyacrylamide Gels (SDS-PAGE) *38*

2.5 Protein sample preparation for SDS-PAGE *40*

2.5.1 Loading of protein samples onto SDS-PAGE gels 40

2.6 Staining to visualize protein bands *41*

2.6.1 Coomassie brilliant blue staining technique 41

2.7 Protein extraction from SDS-PAGE gels 41

2.8 Western immunoblotting *42*

2.9 Acid-elution of cross-reactive antibodies *43*

2.10 Sodium meta-periodate treatment of nitrocellulose membrane *44*

2.11 Tandem mass spectrometry (TMS) *45*

2.12 Cell culture *46*

2.12.1 Defrosting cells 47

2.12.2. Routine passaging 47

2.12.3 Cryopreservation 48

2.13 Cellular assay *49*

2.13.1 Cell sensitisation 49

2.13.2 Cell stimulation 49

2.13.3 Luminescence measurement 50

2.14 List of materials used that were in stock in the lab *50*

2.15 Antibodies used in the study *51*

2.16 Software and database used in the study *51*

2.17 Statistical analysis used *51*

Chapter 3: Antigenic cross-reactivity between *Schistosoma mansoni* and *Dermatophagoides farinae* due to shared glycan epitopes and potential relevance to the hygiene hypothesis 52

Abstract *52*

3.1 Introduction *54*

3.2 Results *57*

3.2.1 Investigation on the cross-reactivity between extracts of *S.mansoni* and house dust mite using rabbit anti-*S.mansoni* egg antisera 57

3.3 Discussion *75*

3.4 RS-ATL8 cellular optimisation ... *80*

3.4.1 Introduction ... 80

3.4.2 Immunoglobulin E (IgE) ... 81

3.4.3 IgE receptors ... 82

3.4.4 Humanised reporter cell lines ... 84

3.4.5 Complement factor ... 86

3.5 Materials ... *88*

3.5.1 Cells ... 88

3.5.2 Human serum ... 88

3.5.3 House dust mite / *D. farinae* antigen ... 88

3.6 Methods ... *89*

3.6.1 Serum treatment ... 89

3.7 Results ... *89*

3.7.1 Serum optimisation ... 89

3.7.2 Optimisation of purified *D. farinae* antigen ... 91

3.8 Discussion ... *93*

Chapter 4: Antigenic cross-reactivity between *Schistosoma mansoni* and *Periplaneta* species ... 99

Abstract ... *99*

4.1 Introduction ... *100*

4.2 Results ... *105*

4.2.1 Investigations on the cross-reactivity between *S. mansoni* and cockroach extract using rabbit anti-*S. mansoni* egg antisera ... 105

4.3 Discussion ... *123*

Chapter 5: Investigation of Recombinant IgE and Phl p 7 allergen with Recombinant IgG (blocking antibody)................................ 126

Abstract 126

5.1 Introduction 128

5.1.1 Allergen Specific Immunotherapy (IgG blocking antibodies).......................... 130

5.1.2 Immunoglobulin G (IgG) 132

5.1.3. The structure of IgG 132

5.2 Materials and Methods 134

5.2.1 Mammalian cell culture 134

5.2.2 Maintenance and freezing of HEK293-6E cells 134

5.2.3 Phl p 7 allergen 135

5.2.4 DNA Preparation 136

5.2.5 Preparation of Polyethyleneimine (PEI)... 136

5.2.6 Mammalian cell expression of IgE and IgG 137

5.2.6.1 Small scale transfection of HEK 293-6E cells 137

5.2.6.2 Large-scale transfection of HEK 293-6E cells 141

5.2.7. Affinity Chromatography (AC) using ÄKTAstart system................................ 142

5.2.8. Protein quantification 142

5.3 Results 143

5.3.1 Recombinant IgE and IgG protein expression in HEK293-6E cells grown in suspension 143

5.3.2 Large-scale purification of recombinant IgG Protein using AC ÄKTA System .. 145

5.3.3 Proof-of-principle of Recombinant IgG (rIgG) blocking experiment using the RS-ATL8 cell line 149

5.3.3.1 Recombinant IgE (rIgE) optimisation 149

5.3.3.2 Recombinant IgG antibody blocking experiment 150

5.4 Discussion *152*

Chapter 6: Overall conclusion and future work 159

6.1 Cross-reactivity between S. mansoni and allergens *159*

6.2 Observation of complement factor optimisation and mite allergen on the RS-ATL8 reporter system *163*

6.3 Observation on the expression of recombinant IgE and IgG proteins *164*

6.4 Observations of the effect of recombinant IgG blocking antibodies using the reporter system (RS-ATL8) *165*

6.5 Future work *166*

References 167

Appendices 213

List of Figures

Figure 1. 1: Map showing the global distribution of schistosomiasis. 3

Figure 1. 2: The life cycle of human schistosome........................ 4

Figure 1. 3: *Schistosoma mansoni* egg. 5

Figure 1. 4: *Schistosoma mansoni* miracidium. 6

Figure 1. 5: *Schsitosoma mansoni* cercaria 7

Figure 1.6: Adult male and female schistosome worms ………… 9

Figure 1.7: Hepatic granuloma induced in either *Schistosoma mansoni* or *Schistosoma japonicum* infection ………………………………………11

Figure 3.1: Coomassie blue-stained 12% SDS PAGE (lane1) and western immunoblots (lanes 2 and 3) of HDM extract (44 µg/lane).. 58

Figure 3. 2: Western blot of a *D. farinae* extract probed with rabbit BR84 anti-SmSEA antiserum for acid-elution of antibodies reacting against a >90 kDa *D. farinae* antigen.. 60

Figure 3. 3: Immunoblot reactivity of acid-eluted rabbit anti-SmSEA antibodies that were cross-reactive with a >90 kDa HDM antigen.61

Figure 3. 4: Western blots of various plant water-soluble extracts probed with BR84 anti-*D.farinae* >90 kDa acid-eluted antibodies. 62

Figure 3. 5: structure of Immunoglobulin E (IgE). 82

Figure 3. 6: Diagramatic representation of the binding between IgE and the FcεRI α-chain.. 83

Figure 3. 7: Schematic diagram showing different stable transfected RBL cell lines with respect to human and rat FcεRI receptors............ 84

Figure 3. 8: Diagram illustrating the mechanism underlying the use of the humanized RS-ATL8 reporter system as a diagnostic tool for detection of sIgE in patients' sera. 86
Figure 3. 9: Complement factor optimisation for two different individual donor serum samples A and B. 90
Figure 3. 10: Antigen optimisation for the two donors A and B... 92

Figure 4.1: Coomassie blue-stained SDS-PAGE and Western immunoblots of ACR extract. 106
Figure 4. 2: A 8% Coomassie blue-stained SDS-PAGE of cockroach extract. 107
Figure 4. 3: Western immunoblots probed with rabbit anti-85/88 kDa cockroach proteins eluted from immunoblots of cockroach extract probed with anti-SEA antiserum 1025Z. 113

Figure 5. 1: Diagram representation of IgG structure 133
Figure 5. 2: Plasmid map of PVITRO1-102.1F10-IgE/λ............ 139
Figure 5. 3: Schematic representation of the expression cassette139
Figure 5. 4: Plasmid map of pVITRO1-102.1F10-IgG1/λ.......... 140
Figure 5. 5: Schematic representation of the expression cassette140
Figure 5. 6: Fluorescence microscopy image was taken 48 hours post-transfection of 293-6E HEK cells with pTTo vector using PEI transfection reagent at a ratio of 1:2 (w/w) (pTTo/GFPq vector to PEI)........ 143
Figure 5. 7: Western blot analysis of expression recombinant IgE and IgG 144

Figure 5. 8: AC purification of recombinant IgG protein. 146

Figure 5. 9: SDS PAGE analysis of the IgG protein eluted fractions. 147

Figure 5. 10: AC Purification of IgG protein 148

Figure 5. 11: Recombinant IgE optimisation: 149

Figure 5. 12: Recombinant IgG and Phl p 7 allergen: 15050

List of Tables

Table 3. 1: MASCOT search output of NCBI nr with the tandem MS data from the purified >90 kDa D. farinae gel band. 59

Table 3. 2: Amino acid sequence alignment of Der f 15 and Kappa- 5 using pair wise alignment tool 64

Table 3. 3: Amino acid sequence alignment of Der f 15 and IPSE/alpha -1 using pair wise alignment tool.. 66

Table 3. 4: Amino acid sequence alignment of Der f 15 and Omega-1 using pair wise alignment tool 68

Table 3. 5: Potential N-Linked Glycosylated site for Der f 15 71

Table 3. 6: Potential O-Linked Glycosylated site for Der f 15 72

Table 4. 1: MASCOT search output of NCBInr with the tandem MS data from the purified ~85 kDa cockroach gel band. 108

Table 4. 2: MASCOT search output of NCBInr with the tandem MS data from the purified ~88 kDa cockroach gel band. 110

Table 4. 3: Amino acid sequence alignment of Per a 3 and IPSE/alpha-1 using pair wise alignment tool 115

Table 4. 4: Amino acid sequence alignment of Per a 3 and Kappa- 5 using pair wise alignment tool 117

Table 4. 5: Amino acid sequence alignment of Per a 3 and Omega- 1 using pair wise alignment tool 119

Table 4. 6: Potential N-linked glycosylation sites for Cr-PI/allergen Per a 3. 122

Table 5. 1: Representation of IgG structure.............................. 134

Abbreviations

APC: Antigen Presenting Cell

APS: Ammonium Persulfate

BSA: Bovin Serum Albumin

CCDs: Cross-reactive carbohydrate determinants

Ch: Cercarial homogenate

CFA: Complete Freund's adjuvant

CR: cockroach

DPBS: Dulbecco's Phosphate Buffered Saline

EBNA1: Epstein-Barr virus nuclear antigen 1

EBV: Epstein-Barr virus

EDTA: Ethylenediaminetetra acetic acid

FBS: Fetal bovine serum

FcεRI-α: FcεRI receptor alpha chain

FcεRI-β: FcεRI receptor beta chain

FcεRI-γ: FcεRI receptor gamma chain

HDM: house dust mite

HEK-283: Human embryonic kidney cells-293

IgE: Immunoglobulin E

IgG: Immunoglobulin G

IL: interleukin

AC: Affinity chromatography

ITAMS: immunoreceptor tyrosine-based activation motif

MEM: Minimun Essential Medium

NCM: nitrocellulose membrane

NFAT: Nuclear factor of activated T-cells

Orip: Origin of replication

PBS: Phosphate Buffered Saline

PEI: Polyethyleneimine

RBL: Rat Basophilic Leukemia

SIT: Allergen-specific immunotherapy

SmSEA: *Schistosoma mansoni* Soluble Egg Antigen

TBST: Tris-Buffered Saline and Tween

TBS: Tris-Buffered Saline

TEMED: Tetramethylethylenediamin

TMS: tandem mass spectrometry

1-D SDS-PAGE: One dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis

Wh (worm homogenate)

Chapter 1: General introduction

1.1 Schistosomiasis

Schistosomiasis is a parasitic infection caused by trematode worms of the genus *Schistosoma* which can lead to acute and major chronic disease (Di Bella et al. 2018). They are transmitted by amphibious or aquatic snails which serve as the intermediate hosts. Schistosomiasis also known as Bilharzia is amongst the neglected tropical diseases occurring mainly in tropical and subtropical areas of Asia, Africa and Latin America (Hotez et al. 2016). Schistosomiasis remains a major public health concern, because people living in endemic areas continue to come into contact with contaminated water during their domestic activities. There are three common species namely *S. mansoni*, *S. haematobium* and *S. japonicum* which are a significant public health problem in humans (Di Bella et al. 2018). *Schistosoma mansoni* causes intestinal schistosomiasis, it uses *Biomphalaria* snails as intermediate hosts and is widely spread in Africa, South America and the Caribbean (Standley et al. 2012). S. *haematobium* causes urinary schistosomiasis, it uses *Bulinus* species snails as intermediate hosts and is endemic in Africa and the Middle East. *S. japonicum* causes intestinal schistosomiasis and it is a zoonotic parasite. It uses *Oncomelania* species amphibian snails as an intermediate host and they are found in South East Asia (Colley et al. 2014). However, there are other species of schistosome, including *S. guineensis*, *S. intercalatum* and *S. mekongi*,

all causing intestinal schistosomiasis, but they are of low significance to public health.

1.2 Epidemiology

Schistosomiasis, ranks as the second parasitic disease after malaria with regards to the number of people infected and those at risk. According to estimates, the disease causes the annual loss of 1.7 million disability-adjusted life years (DALYs) (King 2010).

Globally, more than 207 million people are infected and 779 million people are at risk from schistosome infections, with the highest number of cases in sub-Saharan Africa (Steinmann et al. 2006). According to the World Health Organization, an estimated 200,000 deaths are attributed to the disease annually (WHO., 2017). *S. mansoni* is the most prevalent species found in Brazil, where 25 million people are living in endemic areas and 4-6 million are infected (Lambertucci 2010). People become at greater risk of infection due to an increase in population size in endemic areas and disease transmission increases as a result of irrigation projects. In Senegal, Mali, Sudan and Ghana construction of dams has led to an increase in urinary schistosomiasis (Chitsulo et al. 2000). The main causes of disease transmission include poverty, which has been linked to a lack of basic needs such as drinking water, poor environmental conditions, lack of health facilities and poor sanitary installations, all of which contributed to infection of people living in endemic areas (Zakhary 1997). In non-endemic countries, tourists and migrants have also increased the spread of schistosomiasis (Guiguet

Leal et al. 2012). For example, the epidemiological study of *S. mansoni* infection in migrants and travelers in Spain found that 2.5% of Travelers and 15.1% of African immigrants were diagnosed with schistosomiasis (Roca et al. 2002). The global distribution of schistosomiasis (Figure 1.1)

Figure 1. 1: Map showing the global distribution of schistosomiasis. Diagram from (U Olveda 2013).

1.3 The life cycle of the parasite

The life cycle of schistosomes (Figure 1.2) requires a definitive vertebrate host in which sexual reproduction takes place and a second host which is the intermediate molluscan host for asexual reproduction.

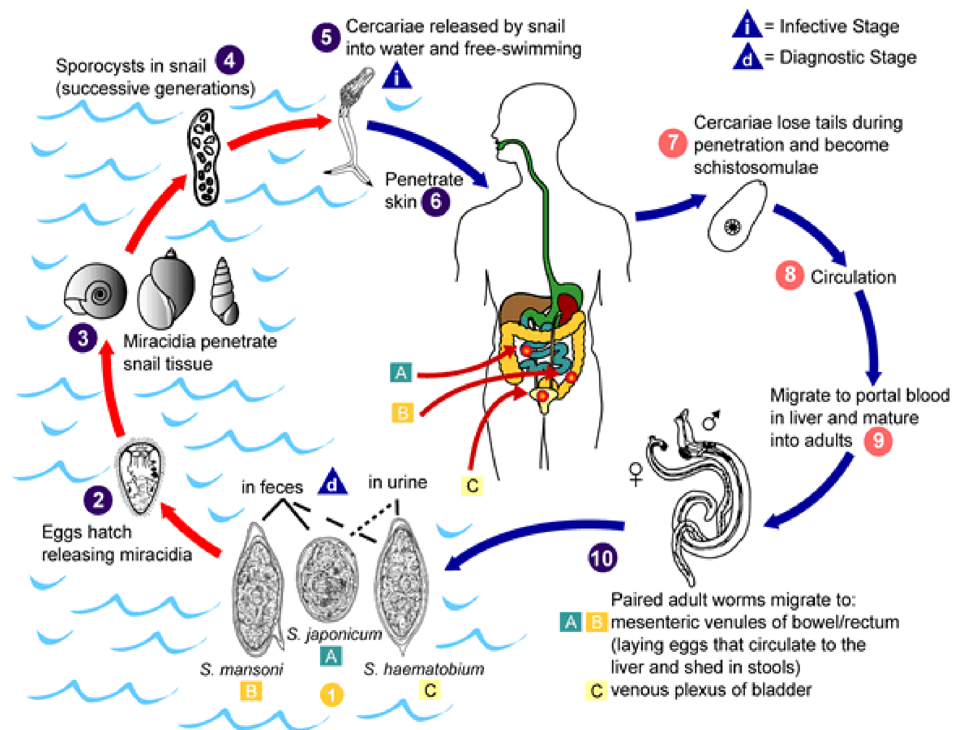


Figure 1. 2: The life cycle of human schistosome. (Image from CDC, 2012 http://www.cdc.gov/parasites/schistosomiasis/biology.html)

1.3.1 Egg production

S. mansoni eggs are oval-shaped (Figure 1.3) measuring 114 to 180 μm long and 45 to 70 μm wide; they have a prominent lateral spine near the posterior end and the anterior end is slightly curved (CDC, 2012). A female worm produces 300 eggs or more per day depending on the species (Pearce & Huang 2015). The ova contain miracidia larvae with cilia and enzymes that help in the migration of eggs into the bladder for *S. haematobium.* For *S. mansoni* and *S. japonicum,* the egg crosses into the lumen of the intestine. Eggs are excreted in the faeces or urine and can stay effective for up to seven days (Gryseels et al. 2006). The egg releases miracidia when it comes in contact with water (Colley et al.

2014). Miracidia penetrate the intermediate snail host, guided by light and chemical stimuli. Upon penetrating the snail, miracidia multiply asexually into sporocysts and later develop into cercarial larvae (Gryseels et al. 2006).

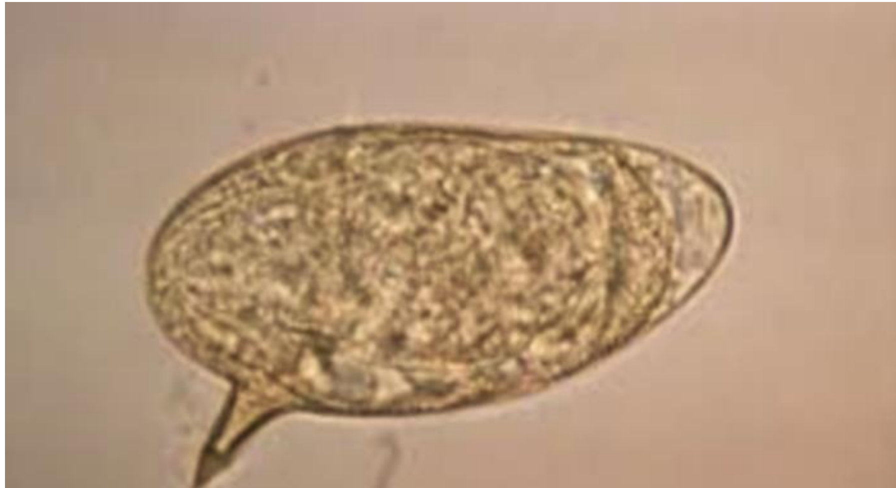


Figure 1. 3: *Schistosoma mansoni* egg. (Image from wikipedia: https://en.wikipedia.org/wiki/Schistosoma_mansoni**)**

1.3.2 Miracidium

The miracidium is a free-living motile organism covered with cilia (Figure 1.4). Miracidia can swim rapidly using their cilia and are able to locate a snail host from a distance of about 9 m and 97 m in standing and running waters, respectively (Upatham 1973). Studies have shown that its turning or swimming behaviour is photokinetic and they show chemokinetic responses towards a snail host. The miracidia of *Schistosoma* are attracted by macromolecular glycoconjugates of the snail body surface and can survive for 5 to 6 hours under favourable conditions (Haberl et al. 1995; Haberl & Haas 1992). The miracidium is

capable of host invasion; upon penetration, the parasite develops into a mother sporocyst that remains in the tissue of the host's cephalopodal region which is near the penetration site. The mother sporocyst generate daughter sporocysts which migrate to the digestive gland of the mollusc then their germinative cells generate cercariae which can infect a human host (Negrão-Corrêa et al. 2012; Wang et al. 2016)

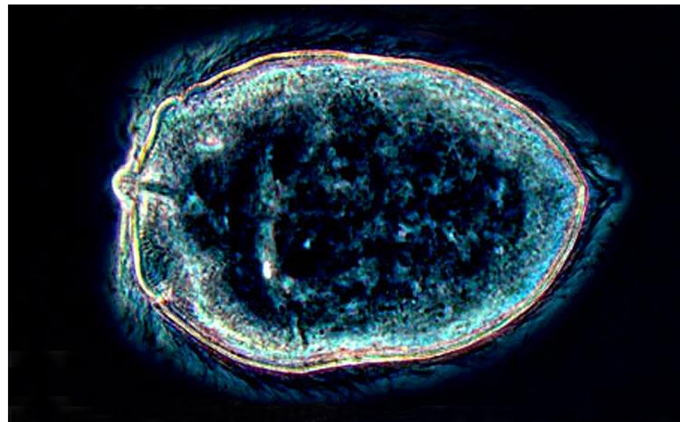


Figure 1. 4: *Schistosoma mansoni* miracidium. Image from Emery, (2011)

1.3.3 Cercaria

Four to six weeks after infection, cercariae start to leave the infected snail and can swim in water for up to 72 hours in order to try and locate a suitable definitive host (Gryseels et al. 2006). Shedding of cercarial is provoked by light and take place during daylight. The cercarial tail and body (Figure 1.5) are enclosed in a syncytial tegument covered with a carbohydrate-rich glycocalyx, and they have also ciliated sensory papillae that aid in host detection (Walker 2011). The cercaria has pre-acetabular glands that contain enzymes including protease that help in

skin penetration and the post-acetabular glands which secrete mucus helping the cercaria to adhere to the surfaces (Haas et al. 1997; Walker 2011). Snails infected by a miracidium can produce up to several thousand cercariae daily over periods lasting months (Gryseels et al. 2006). After finding a suitable host, the cercaria will penetrate the skin, transform into schistosomula, then migrate to the lungs and liver.

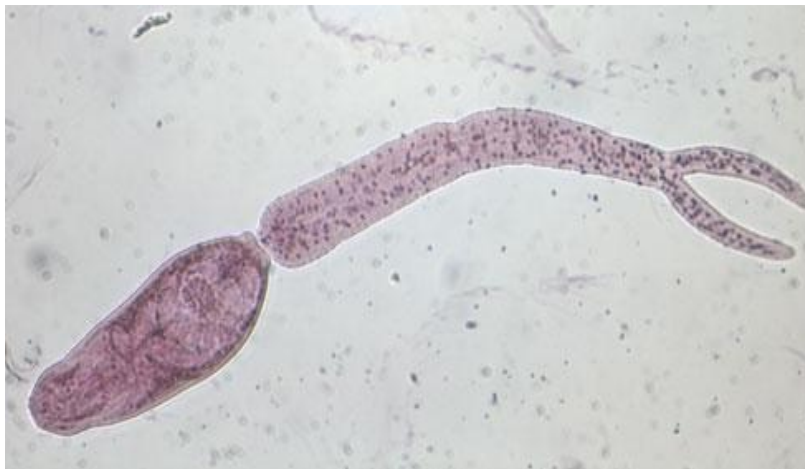


Figure 1. 5: *Schsitosoma mansoni* cercaria: Image from Emery, (2011).

1.3.4 Schistosomula

Schistosomula or young worms need about 5 to 7 weeks before reaching adulthood (Colley et al. 2014). The infective cercaria penetrates the skin of the definitive host and transforms into a schistosomulum. Schistosomula remain in the skin for 2-3 days (Kusel, Al-Adhami & Doenhoff 2007), entering the circulation via the lymphatics and venules wall to reach the lung five to seven days after skin penetration (Schwartz & Fallon 2018). From 7 to 10 days they are found in the hepatic portal vein, having become trapped there due to an increase in size or some

unknown change in their physiology. They now start developing into sexually mature adult males or females, though females remain relatively immature if they do not pair up with a partner of opposite sex (Schwartz & Fallon 2018).

1.3.5 Adult

Adult schistosomes are about 7-20 mm in length. They are white or greyish worms with a cylindrical body that have two terminal suckers, a tegument, reproductive organs and a digestive tract. They are sexually dimorphic worms. The adult male body forms a gynaecophoric canal, a channel holding the longer and more slender female (Figure 1.6) (Pearce & Huang 2015). The adult *S. haematobium* worm pairs live in blood vessels of perivesical tissue while *S. mansoni* and *S. japonicum* worms live in the mesenteric blood vessels. They all feed on blood and blood proteins. The adult female produces 300 to 3000 eggs per female depending on the species (Lu et al. 2016) in which the ovum contains a miracidium that secretes enzymes that help eggs to cross the tissues into the bladder and intestine for *S. haematobium* and *S. mansoni*, respectively.

Figure 1.6: Adult male and female schistosome worms. (Image from CDC 2012, https://www.cdc.gov/dpdx/schistosomiasis/index.html**)**

1.3.6 Granuloma formation around the egg antigen of *S. mansoni*

Granulomas are inflammatory cells such as lymphocytes, eosinophils, neutrophils and macrophages that are formed around the parasite eggs trapped in the liver and intestines during chronic infection with *S. mansoni* (Hams, Aviello & Fallon 2013; McManus et al. 2018). Granulomas that are formed around the parasite egg develop as a result of CD4+ T helper (Th) 2 immune response regulated by cytokines such as IL-4 and IL-13 (Schwartz & Fallon 2018). Mice injected with the soluble egg antigens (SEA) or *S. mansoni* eggs induces a Th2 immune responses (Vella & Pearce 1992). Granulomatous response is

responsible for disease pathology, causing tissues eosinophilia, portal hypertension, deposition of collagen, fibrosis and severe inflammation (Amaral et al. 2017; Wilson et al. 2007). A severe fibrosis in the liver may develop which disrupts blood flow and cause portal hypertension, leading to gastrointestinal bleeding, liver failure and hepatic encephalopathy (Amaral et al. 2017; Schwartz & Fallon 2018). Thus, much of the symptomatology of *S. mansoni* is correlated to the egg -induced granulomatous inflammatory response (McManus et al. 2018).

However, granuloma protect host tissues by isolating toxins secreted by the egg antigens essential for egg translocation into the gastrointestinal lumen and excretion in the feces (Amaral et al. 2017). They help in the translocation of bacterial from the intestine into the circulation of the host (Schwartz & Fallon 2018) The major features of granuloma form around eggs trapped in the liver is shown in (Figure 1.7).

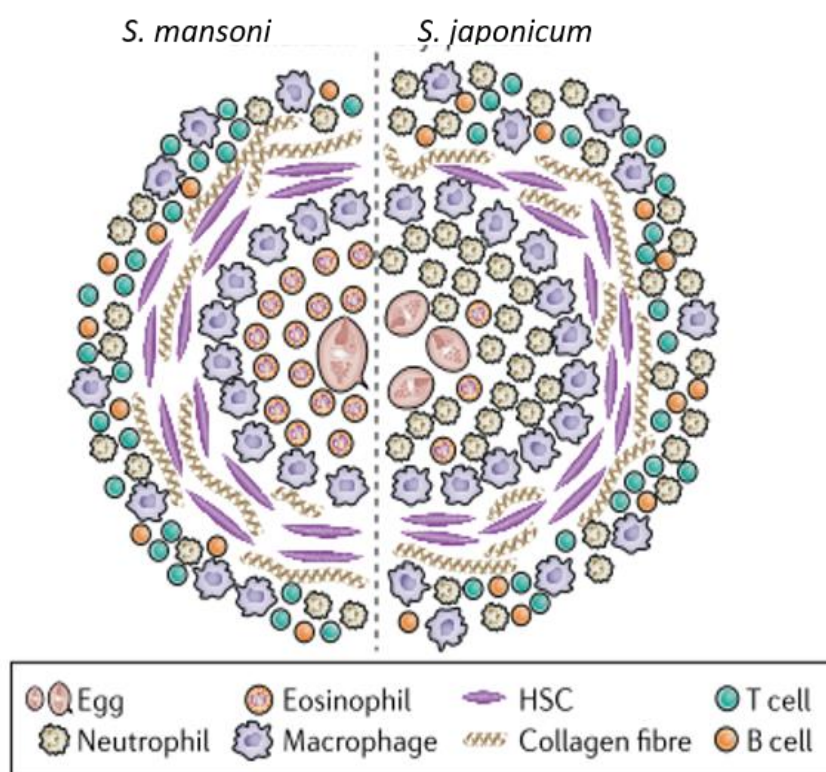


Figure 1.7: Hepatic granuloma induced in either *Schistosoma mansoni* or Schistosoma japonicum infection. Image from McManus et al. (2018).

1.4 Characterized *Schistosoma mansoni* egg antigens

There are only three major *S. mansoni* egg antigens that have been found to be the primary targets of the egg-directed antibody response of the host, namely IPSE/alpha-1, kappa-5 and omega-1 (Schramm et al. 2009).

1.4.1 IPSE/alpha-1

A cation-exchange chromatography fraction (CEF6) of *S. mansoni* soluble egg antigen (SmSEA) contains alpha-1 (α_1), a dimeric

glycoprotein, having a molecular weight of 41 kDa and 36 kDa under a non-reducing condition in sodium dodecyl sulphate polyacrylamide gel electrophoresis (Dunne et al. 1981; Dunne, Jones & Doenhoff 1991). In the presence of 2-mercaptoethanol, the 41 kDa is reduced to 22 kDa, while the 36 kDa band is reduced to 18 kDa subunit (Dunne, Jones & Doenhoff 1991). Schramm and co-authors found that IPSE and previously described molecule alpha-1 were identical, hence the term IPSE/alpha -1 (Schramm et al. 2006). IPSE/alpha-1 is a glycosylated protein of *S. mansoni* eggs that induces the release of interleukin 4 cytokine from human basophils (Schramm et al. 2003). IPSE/alpha-1 is a major secretory/excretory glycoprotein of the *S .mansoni* egg that contributes to the enlargement of hepatic granulomas (Fahel et al. 2010). Previous studies have found that IPSE/alpha-1 was involved in the control of schistosomiasis inflammation by releasing IL-4 and IL-13 from human and murine basophils (Schramm et al. 2003, 2007). Moreover, a form of IPSE/alpha-1 identified as *S. mansoni* chemokine binding protein (SmCKBP) can block certain chemokine activity and inflammation in mice (Smith et al. 2005).

IPSE/alpha-1 is involved in the downregulation of the T helper type 1 (Th1) immune response which is induced in the early stage of infection with *S. mansoni.* After several weeks of infection, the Th1 immune response is down-regulated and then switched to Th2 responses, coinciding with the onset of egg-deposition in the liver. Studies have demonstrated that a Th2 response is produced when naïve animals are injected with the egg antigen of *S. mansoni* (Okano et al. 1999; Vella &

Pearce 1992). Falcone and co-authors incubated basophils from healthy human donors with the SEA of *S. mansoni* in the presence of IL-3 (a basophil priming factor) and found that the SEA triggered basophil degranulation and released histamine and, most importantly, released high levels of IL-4 (Falcone et al. 1996). IPSE/alpha-1 has immunoglobulin binding properties and binds with the highest affinity to IgE: structural analysis revealed that it belongs to the beta-gamma crystalline superfamily (Meyer et al. 2015).

IPSE/alpha-1 contains a Nuclear Localisation Sequence (NLS) at the C-terminus, which consists of a small cluster of positively charged amino acids preceded by a proline (Kaur et al. 2011). NLS is important for nuclear translocation. The glycosylation of IPSE/alpha-1 was investigated using mass spectrometry combined with enzymatic degradation and it was found that it contains two N-glycosylation sites and a Lewis X –motif (Wuhrer et al. 2006).

Proteomic analysis identified IPSE/alpha-1 as a major hepatotoxin secreted protein of *S. mansoni* egg (Abdulla et al. 2011). An ion-exchange fraction analysis of *S. mansoni* egg secreted proteins (ESP) yielded a hepatoxic fraction. The presence of IPSE/alpha-1 was identified in that fraction. An *in vitro* study using pure recombinant IPSE/alpha-1 that has been applied to mouse hepatocytes displayed dose-dependent hepatotoxicity and depletion of IPSE/alpha-1 using monoclonal antibodies diminished the cytotoxicity of soluble-egg antigen and egg-secreted protein by 35% and 32% respectively (Abdulla et al. 2011).

1.4.2 Omega-1

Omega-1 (ω_1) was isolated by ion-exchange chromatography from *S. mansoni* soluble egg antigen (SEA) as a constituent of cationic exchange fraction 6 (CEF6); that is, CEF6 was comprised of two antigens, ω_1 and IPSE-alpha-1 (Dunne, Jones & Doenhoff 1991). Omega-1 is a glycoprotein from *S. mansoni* eggs that can cause cell-mediated granulomatous immune responses (Schwartz & Fallon 2018). Mice infected with *S. mansoni* have been found to suffer from microvesicular hepatocyte liver damage (Dunne, Jones & Doenhoff 1991). Omega-1 is a monomeric antigen with a molecular weight of 31 kDa under non-reducing conditions and a pI of more than 9.0 (Dunne, Jones & Doenhoff 1991). Monospecific anti-ω_1 sera were passively transferred into T cell-deprived mice infected with *S. mansoni* and the study found that the antibodies were able to prevent mircovesicular hepatocyte damage in the mice. Omega-1 has also been described as a stage and species-specific antigen which could be a useful serodiagnostic tool; antibodies against it are only found in animal sera injected with the egg of *S. mansoni***,** but not other life stages of the parasite, namely, worms and cercariae (McLaren *et al.*, 1981; Dunne, Jones and Doenhoff, 1991). Moreover, mice infected with *S. haematobium* and *S. japonicum* do not produce omega-1 antibodies (Dunne et al. 1981, 1984).

Omega-1 is a member of the T2 ribonuclease (RNase) protein family (Fitzsimmons et al. 2005), a class of enzymes that has been found in a wide range of organisms from bacteria to mammals. They play biological

roles such as modulating host immune responses and they are intracellular or extracellular cytotoxins (Luhtala & Parker 2010).

Omega-1 has been found to play an important role in polarizing Th2 responses *in vitro* (Everts et al. 2009). The carbohydrate present in *S. mansoni* SEA has been found to promote strong Th2 responses (Thomas et al. 2003), and the glycosylation of omega-1 might play a role in its Th2-polarizing activities. In addition, the egg antigens of *S. mansoni* have been shown to trigger basophils to produce IL-4 and induce the expansion of alternatively activated macrophages (Donnelly et al. 2008). These factors can contribute to Th2 responses after egg antigen exposure.

1.4.3 Kappa-5 (K-5)

Kappa-5 is one of the three cell antigens of *S. mansoni* egg that is immunogenic in infected hosts (Schramm 2009). Characterization of K-5 showed that it is one of the major antigenic glycoproteins of *S. mansoni* eggs (Schramm et al. 2009), similarly to IPSE/alpha-1 and ω_1 that are both also found in the eggs of *S. mansoni.* Schramm and colleagues found that K-5 was present also in the miracidium of the parasite at the mRNA and protein levels, but with differences in the number of glycosylation sites. In the miracidial isoform of kappa-5 four N-glycosylation sites were occupied while in the egg both three and four occupied N- glycosylation sites were detected (Schramm et al. 2009).

Kappa-5 has been reported to be associated with antibody responses of IgE and IgG isotypes in sera of African individuals infected with *S. mansoni,* unlike IPSE/alpha-1 and ω_1 which are associated only with IgG antibodies (Schramm et al. 2009).

Under reducing conditions in SDS-PAGE, kappa-5 occurs as a dimer with a double band between 50 kDa and 40 kDa, while under non-reducing conditions it appears as a single band with a molecular weight of 100 kDa (Schramm et al. 2009). An estimated pI of 8.23 has been observed for K-5 (Schramm et al. 2009).

The glycan structure of kappa-5 was composed of GalNAcβ1-4GlcNAc (LDN) termini, structures attributed with immunogenic properties, together with four fully occupied N- linked glycosylation sites made up of xylosylated and difucosylated core regions which are the targets of kappa-5 specific IgE antibodies in sera of individuals infected with *S. mansoni* (Meevissen et al. 2011).

1.5 Helminths and allergens

1.5.1 Parasitic helminths and the hygiene hypothesis

There has been a steep rise in the prevalence of auto-immune diseases such as type 1 diabetes and allergic diseases such as asthma; an estimated 300 million people are suffering from asthma worldwide and this could increase as populations become more urbanized as allergic disease is rarer in rural areas (Flohr et al. 2010; To et al. 2012). There

are differences in allergic disease prevalence within countries; for example, families of children farmers who were involved in full-time farming activities compared with families with part time farming activities, the study found that there was a reduced prevalence of allergic diseases in children whose families were running the farm in a full time activity. The study concluded that environmental exposure to pathogens and traditional living conditions on a farm can play a key role in the reduction of allergic disorders in children (Von Ehrenstein et al. 2000). Another observational study between rural and urban settings in Ethiopia found that the prevalence of allergic disorders was greater in an urban setting than in rural areas, a difference attributed to an urban lifestyle (Yemaneberhan et al. 1997). Stimulation of the immune system by pathogens such as bacteria, parasites and viruses can thus seemingly protect the development of inflammatory diseases and less exposure to infectious agents may contribute to an increase in autoimmune and allergic diseases in westernized countries (Yazdanbakhsh & Matricardi 2004).

The hygiene hypothesis was originally formulated by Strachan (1989) in a study in which he found that hay fever was less common in children from larger families than in first-born children. Strachan suggested that the lower incidence of allergies in children from larger families might be due to acquiring infection from an older sibling in early childhood (Strachan 1989). As a result, many studies started to examine the relationship between allergy and infection. The International Study of Asthma and Allergies in Childhood (ISAAC) conducted surveys in low-

and middle-income countries (LMIC) such as Estonia, Albania and Nigeria compared with high-income countries which included Finland, the United Kingdom, Austria and West Germany (Björkstén et al. 1998; Priftanji et al. 2001). The study focused on parameters that served as markers for allergy/allergic sensitisation such as IgE levels, airway function, health standards and occupational lifestyle. The conclusion drawn from this survey was that allergies are most common in industrialised countries and rare in developing countries (Beasley et al. 1998). Reduced exposure to pathogens, improved hygienic conditions, good health facilities and lifestyle, all contributed to the high prevalence of allergic disorders in industrialised countries.

1.5.2 Helminths and allergens

Both helminths and allergens induce the activation of T-helper 2 type (Th2) responses associated with the release of cytokines such as IL-4, IL-5, and IL-13, peripheral blood eosinophilia, intestinal mastocytosis, and high levels of IgE antibody (Allen & Maizels 2011; Licona-Limón et al. 2013). According to many authors, IgE and its cellular responses and receptors have not evolved to target innocuous non-parasitic materials such as dust-mites, dog and cat dander or pollens from plants, but instead, are thought to have evolved as part of anti-parasitic defence (both endo- and exoparasites). Allergy could, therefore, be a mis-directed

anti-parasite response in hypersensitive individuals (Artis & Maizels 2012).

However, the opposing hypothesis called the toxin hypothesis of allergy, proposes that the IgE–mediated allergic response evolved as a defense against venoms and other noxious environmental substances rather than macro-parasites (Profet 1991). Profet noted that a common feature of most allergens such as plants and venoms is that they contain toxins. Profet further proposed that the symptoms of IgE mediated allergic response such as diarrhea, coughing and vomiting evolved as defense mechanisms which permit the sensitised host to respond immediately and to get rid of noxious substances that triggered such responses from the body. Mast cells and IgE are associated with allergic reactions and an individual sensitive, for example, to venom allergen can develop such reaction (Annila 2000). Higginbotham suggested that many mast cells might be present in the skin of different animals such as rodents and humans and that these cells might enhance resistance to Russell's viper venom (Higginbotham 1965). Higginbotham further reported that heparin, a proteoglycan stored in mast cells that is highly anionic, can neutralize venom toxicity by binding to a venom component such as melittin which is highly cationic. Many studies have supported this toxin hypothesis; thus Metz and colleagues in their study have shown that mast cells can enhance resistance to environmental noxious factors such as bee stings and snake bites in mice (Metz et al. 2006). Marichal *et al.*, in their study show that Th2 immunity induced in mice by injection with honeybee venom can significantly increase the resistance of the mice to

the hypothermia and mortality caused by subsequent challenges with potentially lethal doses of venom (Marichal et al. 2013). The authors further concluded that IgE antibodies and the IgE receptor FcεRI were important for such acquired resistance to honeybee venom and that IgE involved can protect against noxious substances. Another study by Palm *et al.*, (2013) investigated how phospholipase A2 (PLA2) a major allergen in bee venom induces Th2 immune responses in mice. This study found that Th2 immune responses induced by injections of PLA2 bee venom allergen can protect mice challenged with a lethal dose of PLA2. The ability of the bee venom PLA2 to induce Th2 responses in mice is dependent on its enzymatic activity through the cleavage of membrane phospholipids to produce lysophospholipids and a release of interleukin-33 (Palm et al. 2013).

Allergy occurs in individuals with atopy, a genetic predisposition to produce prolonged IgE antibody responses, and they are detrimental immune responses against a wide range of environmental antigens (Johansson et al. 2001; Marichal et al. 2013). Inflammatory responses to allergy require a type 2 immune response which is characterized by CD4+ T helper type (Th2) cells and the production of allergen-specific IgE antibodies (Fujita et al. 2012). $CD4^{+}$ Th2 cells produce cytokines such as interleukin (IL) 4 and IL-13, which are important in the induction of class switching to synthesis of immunoglobulin E heavy chain in B cells and the generation of allergen-specific IgE antibodies (Pulendran & Artis 2012). Allergen-specific IgE binds to the high-affinity Fc-epsilon receptor (FcεR1) on the surface of mast cells and blood basophils,

crosslinking the receptor and triggering degranulation. This results in the release of inflammatory mediators such as histamine, which is responsible for the early phase response (Orengo et al. 2018). Late phase responses occur several hours after allergen exposure. This phase includes cytokine release and prostaglandin (mast cells) or leukotriene (basophils) production (Cady et al. 2010; Galli, Tsai & Piliponsky 2008).

In chronic infections, helminths induce immunoregulatory cytokines produced by regulatory T cells, such as transforming growth factor β (TGFβ) and interleukin 10 (IL-10) and also an increase in IgG4 antibody (Weinstock 2012). Th2 responses mediate protective immunity against parasites and are characterized by increases in the levels of interleukin-4 (IL-4) and other cytokines such as IL-5. These cytokines can downregulate inflammatory responses (Anthony et al. 2007). The carbohydrate molecules in schistosomes stimulate the production of anti-inflammatory cytokines by antigen-presenting cells (APCs) (Okano et al. 2001). The ability of chronic helminth infections to suppress inflammation is correlated with protection against allergic diseases and studies have revealed that exposure to certain helminths, including schistosomes, protects against inflammatory diseases (Cooper 2009; Hartgers et al. 2008; Smits et al. 2010). According to Van den Biggelaar and co-authors, a study on Gabonese children revealed that an increase in IL-10 induced by infection with schistosomes was negatively associated with sensitivity to house dust-mite (Van Den Biggelaar et al. 2000). Another study also investigated the course of asthma in two different groups, one coming

from a highly endemic area for schistosomiasis and the other group not. This study found that in the schistosomiasis-affected group skin test-positivity in response to indoor allergen was less pronounced than in the other group, suggesting that infection with the parasite was associated with a reduced risk of asthma (Medeiros et al. 2003). A number of studies suggested that long-term anti-helminth treatment can increase the risk of allergic disorders (van den Biggelaar et al. 2004; Lynch et al. 1993). This observation was seen also in a randomized placebo-controlled trial on Vietnamese school children in which anti-helminthic therapy was associated with a higher risk of skin sensitisation to allergen (Flohr et al. 2010). The results of these studies are in contrast to other studies in which helminth infection increased the prevalence of asthma and atopic disease (Hagel et al. 2007; Wördemann et al. 2008). A cross-sectional study of 439 children with asthma concluded that sensitisation to *Ascaris lumbricoides* was associated with increased asthma morbidity and severity among children in Costa Rica (Hunninghake et al. 2007).

It is important to note that only a relatively small number of protein families include allergens and that the antigens targeted by IgE in helminth infection are present in these allergen families (Fitzsimmons *et al.*, 2014). Such allergen families include tropomyosin, a muscle protein which is an important IgE target in many helminth infections including anisakiasis, ascariasis, onchocerciasis and schistosomiasis (Asturias et al. 2000; Jenkins et al. 1998; Santos et al. 2008). In most allergies to invertebrates, the IgE cross-reactivity between *Ascaris* and dust mites is due to tropomyosin (Acevedo et al. 2009). The allergen (Bla g7)

cockroach tropomyosin has shown strong IgE cross-reactivity with *Ascaris* (Santos et al. 2008).

Paramyosin, another allergen protein family from invertebrates is targeted in IgE responses against *S. japonicum* (Jiz et al. 2009) and *Ascaris lumbricoides* (Valmonte, Cauyan & Ramos 2012). A study has revealed that *Ascaris* paramyosin shows IgE cross-reactivity with dust-mite paramyosin allergen (Blo t 11) (Valmonte, Cauyan & Ramos 2012). In another allergen protein family, that of tegumental allergen-like (TAL) molecules, the EF-hand proteins are one of the largest groups of allergens. TAL proteins are predominantly found in *S. mansoni* and an IgE response of the TAL family has been associated with resistance to *S. mansoni* re-infection (Dunne et al. 1997; Fitzsimmons et al. 2012). Glutathione S-transferases (GSTs) are another allergen protein family which is targeted by IgE during infections (Santiago et al. 2012). The GST of nematode species is homologous with other members of the GST allergen family (A11FAM) which includes allergens in cockroach (Bla g 5) and house dust mite (Der p 8) and also IgE- antigen in grass (Deifl et al. 2014). A GST homologue in *Wuchereria bancrofti* binds IgE raised against Bla g 5 (Santiago et al. 2011).

Venom-allergen like (VAL) proteins in helminths are another family targeted by IgE. Hookworms secrete a VAL-family member known as *Ancylostoma* Secreted Protein-2 (ASP-2) which has shown to be a target of IgE antigen in human studies conducted in Brazil and China (Bethony et al. 2005; Diemert et al. 2012). Immunity is related to the IgE response

to this protein (Bethony et al. 2005). The VAL protein belongs to the larger group of pathogen-related-1 family, VAL proteins that have been found in tapeworms (Tsai et al. 2013), *S. japonicum* (Chen et al. 2010) *and S. mansoni* (Chalmers et al. 2008) and groups 3 and 5 insect venom allergens.

1.6 Mast cells and basophils

Mast cells and basophils were first described by Paul Ehrlich in 1878 and 1897 respectively (Varricchi et al. 2018). Mast cells and basophils are important effector cells in Immunoglobulin (E) IgE mediated allergic reactions such as asthma and autoimmune disorders (Rivellese et al. 2017; Schroeder 2011). Mast cells and basophils originated from $CD34^{+}$ haemopoietic stem cells (Stone, Prussin & Metcalfe 2010). In addition, basophils typically mature in the bone marrow and circulate in the peripheral blood, unlike basophils, mature mast cells do not circulate in the blood they matured in connective tissues (Wedemeyer & Galli 2000). Mast cell and basophils have a different life span, basophil life span is short which last for days while mast cells have a long lived lasting for months (Stone, Prussin & Metcalfe 2010). Both mast cells and basophils possess high-affinity IgE receptors (FcεR1), crosslinking of the receptor-bound IgE with antigens (allergens) will result in the release of preformed mediators such as histamine and cytokines (Falcone, Haas & Gibbs 2000). Basophils activation of the FcεR1 dependent can induce the *de novo* production and leukotriene C_4 (LTC_4) secretion. Studies have

reported that mature basophils can release IL-4 and IL-13 in response to the receptor dependent activation (Brunner, Heusser & Dahinden 1993; Gibbs et al. 1996). Mast cell development and growth requires the presence of stem cell factor (SCF) receptor c-kit /CD117 expressed on mast cell surface (Dahlin et al. 2017). The importance of SCF in the generation of mast cell has been investigated in murine rodents, human and non-human primates. A study have shown that treatment of mice with SCF induce an increase in the number of mast cells and maturation of mast cells proliferation of more mature mast cells (Tsai et al. 1991). Other cytokines such as Interleukin-4 (IL-4), IL-10 also regulate mast cell development and differentiation (Wedemeyer & Galli 2000). Unlike basophils express Interleukin-3 (IL-3) α chain (CD123) which is responsible for their development and survival (Varricchi et al. 2018). The cytoplasmic granules of basophils and mast cells stain metachromatically with certain dyes which reflects their content of proteoglycans, both cells have chondroitin sulphates and heparin is only present in mast cells (Wedemeyer & Galli 2000). Chondroitin sulphates contribute to the storage of neutral proteases and histamine, in which basophils are the most histamine found in human blood.

Basophils and mast cells play an important role in innate immune response to pathogens (Abraham SN and Michel Arock 1998). Microorganism induce mediator or cytokine release from mast cells and basophils. For example, Protein A of *staphylococcus aureus* and Protein L *of Pneumococcus magnus* are bacterial superantigens, that can bind to the IgE region and induce release of mediators from mast cells and

basophils (Wedemeyer & Galli 2000). Patella and co-authors reported that basophils and mast cells can be activated by viral proteins. Protein Fv (pFv) released in the intestine of patients with viral hepatitis, as well as the HIV-1 glycoprotein gp 120 have been shown to induce IL-4 and IL-13 production from mast cells and basophils by interacting with the V_H3 region of IgE (Patella et al. 1998, 2000). In addition, *S. mansoni* soluble egg antigen (SmSEA) induce the release of IL-4 from basophils of nonimmune donors (Falcone et al. 1996). Mast cells play an important role in the early phase of IgE mediated allergic reactions (Galli 1993). Basophils play a key role during IgE- associated late phase reactions (LPRs) in the nose, skin and airway of humans (Wedemeyer & Galli 2000).

1.7 Cross-reactive carbohydrate epitopes

Plant N-glycans containing core α1,3-fucose are found in insect glycoproteins such as venoms, plant foods and also trematodes such as *Schistosoma* species (Bencúrová et al. 2004; Homann, Schramm & Jappe 2017). IgE has been shown to bind to carbohydrates containing β1,2-xylose and especially the plant N glycan α1,3-fucose which is the main structural element for antibody binding (Bencúrová et al. 2004; Van Ree et al. 2000). Studies have found that N-glycans were mainly involved in IgE binding to phospholipase A2, a major allergen of honeybee venom and thus α1,3-fucose is the structure responsible for the cross-reactivity of IgE with plant and insect glycoproteins (Tretter et al. 1993; Weber et

al. 1987). Aalberse and co-authors have shown that IgE from sera of a patient cross-reacted with different foods and insect venom extracts (Aalberse, Koshte & Clemens 1981). These carbohydrate structures, especially the plant N –glycans, are responsible for most of the cross-reactive epitopes of many allergens and are termed cross-reactive carbohydrate determinants (CCDs) (Wicklein et al. 2004). Many glycans are common in all mammals, which is why mammalian glycoproteins are not immunogenic as their immune systems develop tolerance to their own structures (Aalberse & van Ree 1997). Conventional measurements work by measuring the levels of allergen-specific IgE by measuring the binding of IgE, not the ability to crosslink the receptor. CCDs do bind IgE but are in most cases monovalent; that is they cannot crosslink the receptor on mast cells or basophils (Van Der Veen et al. 1997). In many cases, anti-CCD IgE antibodies do not cause clinical allergy symptoms and they also can cause false-positive test results for food allergy (Mari et al. 2008; Van Ree 2002).

1.8 RS-ATL8 reporter system

Nakamura and co-authors (2010) developed a new humanized rat basophilic leukaemia cell line, named RS-ATL8. This cell line is used to detect the presence of allergen-specific IgE, through the crosslinking of specific IgE bound to the high-affinity receptor on mast cells in the presence of an allergen (Nakamura et al. 2010). In this assay, rat basophilic leukaemia cells (RBL-SX38) generated by Wiegand and

colleagues (1996) were used. However, RBL does not bind human IgE so they need to be transfected with the α subunit cDNA or the three subunit genes α,β and γ of the human FcεR1 receptor (Wiegand et al. 1996). Moreover, Nakamura *et al.* (2010) transfected RBL-SX38 cells with nuclear factor of activated T cells (NFAT)-responsive firefly luciferase reporter gene. Hence the cells were named RS-ATL8 and the method was called EXiLE (IgE cross-linking-induced luciferase expression). This method uses a patient's IgE for measuring the sensitisation of mast cells and the cells are stimulated with an allergen (the principle of this assay is illustrated in more detail in chapter 3).

1.9 Study aim and objectives

Countries with advanced health systems are experiencing an increased prevalence of IgE-mediated immune disorders such as allergies and asthma and a reduced prevalence of chronic parasite infections may be responsible for this. A possible explanation for this is the 'hygiene hypothesis'. The blocking antibody (IgG) hypothesis has gained wider attention for potential use as immunotherapy for allergies and may provide an alternative explanation for the hygiene hypothesis. Allergen-specific immunotherapy induces allergen-specific regulatory T cells (Tregs), and these cells play a vital role in regulating immune responses to an allergen (Hussey Freeland et al. 2016). Treg cells produce anti-inflammatory cytokines such as IL-10 which has a dampening effect on allergy (Couper, Blount & Riley 2008). IgG, especially IgG4, antibodies

are produced in patients undergoing allergen immunotherapy (Van De Veen et al. 2013; Vickery et al. 2013). However, helminth infection is associated with a high level of polyclonal IgE which could saturate FcεR1 on basophils and mast cells and 'block' the cross-linking of such receptors by allergen-induced specific IgE antibody (Quinnell et al. 2018). In this work our results are consistent with the blocking antibody idea that is since many plant proteins and helminths are glycoproteins, the glycan epitopes can induce IgE antibodies (Homann, Schramm & Jappe 2017), which are known to be antigenically cross-reactive commonly called cross-reactive carbohydrate determinants (CCDs) (Wicklein et al. 2004). However, glycan epitopes can also induce IgG antibody production and therefore anti-*S. mansoni* IgG antibodies that are cross-reactive with invertebrate allergens could act as a blocking antibody. This research has been divided into two parts, the aim of the first part of this work was to investigate the potential cross-reactivity between *Schistosoma mansoni* and environmental allergens which include house dust mite (HDM) *Dermatophagoides farinae*, and the Australian cockroach (ACR) *Periplaneta australasiae.* In order to achieve this aim, the main specific objectives were as follows:

1. Purify and identify the antigens in both house dust mite and cockroach extracts that are antigenically cross-reactive with *S. mansoni.* This will be done by probing electroblots of these aqueous allergen extracts with rabbit antisera raised against *S. mansoni* egg antigens.

2. Purify rabbit anti-schistosome IgG antibodies reactive with the house dust mite and cockroach antigens from rabbit anti-schistosome antisera. Cross-reactive anti-schistosome IgG antibodies were eluted from immunoblots and tested for reactivity against the different life stages of *S. mansoni* egg antigens. SDS-PAGE will be used to purified both *S. mansoni* antigens and the allergens will then be subjected to mass spectrometric analysis to identify schistosome cross-reactive molecules.

3. Investigate whether the purified rabbit anti-schistosome IgG antibodies block reactivity of allergen-specific IgE using the reporter cell line RS-ATL8.

The second part of this work was to show that IgG antibody can block reactivity of allergen-specific IgE using the RS-ATL8 reporter assay and the main objectives were as follows:

1. To develop an *in vitro* system and demonstrate that recombinant IgG antibodies can block IgE binding to Phl p 7 and inhibit allergen induce basophil degranulation.

2. Plasmids which encode human IgE and IgG that are specific for Phl p7 will be used, both proteins being expressed by a mammalian HEK cell line in order to produce recombinant proteins.

3. Recombinant IgG protein will be purified using Affinity Chromatography (AC) ÄKTA system.

Chapter 2: General materials & methods

Ethical approval for the mice work and Human serum

Experiments using mice for production of *S. mansoni* antigens and rabbits for production of antisera were approved by the Animal Welfare and Ethical Review Board of the University of Nottingham in which these materials were produced and the work was carried out in accordance with the regulations set out in the UK Animals (Scientific Procedures) Act, 1986, (Project licence numbers PPL 40/3024 and 40/3595). For the human serum ethical approval was given by the University of Nottingham's School of Pharmacy Research Ethics Committee (ref 047-2019).

2.1 Preparation of *Schistosoma mansoni* antigens

2.1.1 Soluble egg antigen (SEA)

Antigenic extracts were prepared from an isolate of *S. mansoni* originating from Puerto Rican strain *S. mansoni* soluble egg antigens were extracted from the eggs in tissues of infected mice as described by (Doenhoff et al. 1988). Each mouse was injected with 2.5 mg hydrocortisone acetate 3 days prior to perfusion to inhibit granuloma formation (Lucas et al. 1980) and facilitate separation of eggs from host tissue. After perfusion of approximately 40 infected mice, the livers and intestines were macerated in a food blender in approximately 3 volumes of 1.8% saline solution followed by incubation with 2 g/litre pig pancreatic

trypsin (Sigma, Poole, Dorset, UK) for 2 hours at 37°C. Eggs were retrieved by filtration through metal sieves of varying mesh sizes (600, 300 and 150 μm). Egg suspensions were subsequently subjected to repeated rinsings and gravity sedimentations in 1.8% saline solution until the suspending fluid was seen to be free of liver and intestinal tissues. Excess fluid was removed from the sedimented eggs and the pellet was frozen at -80°C. Between 2 x 10^6 and 3 x 10^6 eggs from approximately 40 infected mice were isolated routinely. For the preparation of SmSEA several egg pellets were thawed and homogenized using three times their volume of isotonic saline solution and centrifuged at 20000 x g for 4 hours at 4°C and the supernatant was aliquoted stored until use.

For the immunization of rabbits using egg antigen of *S. mansoni*, the egg antigen solutions were emulsified in an equal volume of complete Freund's adjuvant as described by Dunne et al. (1986). 1 mL total volume of injection containing approximately 5 mg protein, were administered to rabbits in 0.1 mL quantities both subcutaneously and intramuscularly at multiple dorsal sites of the antigen once a week to a total of 1 mL (Doenhoff et al. 2016). The injection continued until a strong antibody response was reached against the SEA immunogen in immunoelectrophoresis. Rabbits were terminally anaesthetised and exsanguinated by cardiac puncture and sera were collected and stored at -20°C until used.

2.1.2 Snail infection to generate Cercarial homogenate (Ch)

The life cycle of a Puerto Rican strain of *S. mansoni* was maintained in laboratory *B. glabrata* snails and random bred mice as previously described (Doenhoff et al. 1978). Snails were kept in transparent, 12-litre capacity, plastic tanks each containing 10 litres of deionized water. Tanks were constantly oxygenated using an aeration system with each tank provided with a sintered air filter. The room in which the snails were kept was maintained at 26 ± 2°C with alternating, daily 12-hour light and dark periods.

Adult snails (10-12 mm shell diameter) were mass infected with the freshly hatched miracidia obtained as described by (Doenhoff et al. 1988). The snails to be infected were placed in a clean tank containing 3 litres of deionized water and the suspension containing hatched miracidia was added. The snails were exposed overnight (approximately 12 hours) in the tank without artificial aeration and food. The following day, the snails were rinsed with fresh water, transferred into clean tanks with 10 litres of fresh water and maintained as normal with food and aeration. Snails were induced to shed cercariae by exposing them to a light source 35 days after exposure to *S. mansoni* miracidia.

For the preparation of cercarial homogenate, the method used was adapted by Doenhoff et al. (1988). Briefly, cercariae shed from snails were pooled into a suspension and the suspension was concentrated in 10 mL of water by filtration. Cercariae were gravity-sedimented for 2

hours at 4°C. The excessive fluid was removed, and the pellet stored at -20°C. To prepare cercarial homogenate (CH) 4 or 5 frozen pellets containing from 250000 to 750000 cercariae were thawed and pooled, cercarial bodies were disrupted by ultrasonication and the resulting homogenates were centrifuged at 4000 x g at room temperature. The supernatant was stored at -80°C.

2.1.3 Schistosomula extract

A schistosomula extract was prepared from larvae that remained after production of cercarial transformation fluid (CTF), (Smith et al. 2012). CTF was prepared by removing excess fluid from freshly chilled and sedimented unfrozen cercariae. The cercariae were resuspended in 4 times their volume in tissue culture medium 199 (Wellcome Re-agents, Beckenham, Kent) and transformed them by aspirating 14-20 times through a 19G syringe needle (Doenhoff et al. 1988). This resulted in the majority of cercarial bodies separating from their tails. The suspension was incubated at 30°C for one hour and centrifuged. The pellet of schistosomula remaining after removal of the CTF was dissolved in 1x Phosphate buffer saline (PBS). The suspension was agitated for 10 minutes at room temperature and then centrifuged. The supernatant was collected and stored at -80°C until used.

2.1.4 Adult worm homogenate (Wh)

Adult worm homogenate was prepared from perfused worms from mice infected with 400 *S .mansoni* cercariae approximately 45 days previously and injected with 2.5 mg hydrocortisone acetate 3 days previously (Doenhoff et al. 1978; Smithers & Terry 1965) . Worms were rinsed in perfusion fluid (citrated and heparinized isotonic saline) at room temperature until traces of mouse erythrocytes had been removed. The worms were sedimented by gravity and the pellet frozen at -80°C. To prepare worm homogenate several batches of worms were thawed and pooled and mechanically homogenized in PBS buffer using a ground glass tube with teflon plunger. The homogenate was centrifuged at 20000 x g for 4 hours at 4°C and the supernatant was stored until required.

2.2 Estimation of protein concentrations

Protein concentration estimation for the allergen extracts was carried out using the Bio-Rad DC Lowry assay method adapted from Lowry et al. (1951), bovine serum albumin was used as a standard.

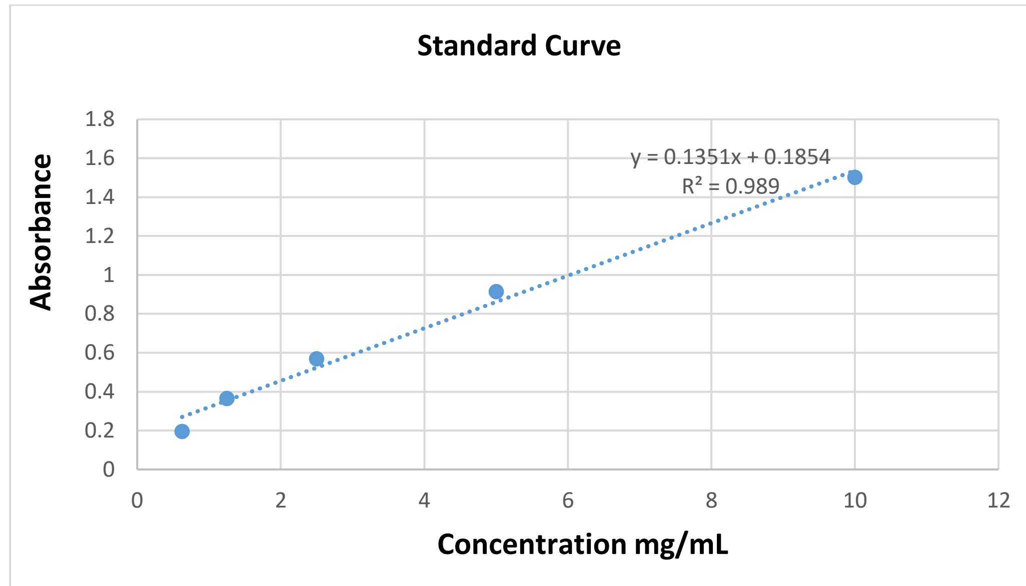
At least 15 minutes before using the spectrophotometer was switched on, the deuterium light was turned off and absorbance set to a wavelength of 750 nm. A cuvette was used containing 10 µL of distilled water to serve as a blank. Bovine serum albumin (BSA), 20 mg/mL, was serially diluted in distilled water in five cuvettes. Each protein sample was

done the same way to be estimated. 100 µL of Bio-Rad DC protein assay reagent A and 800 µL of Bio-Rad DC protein assay reagent B (Hercules, CA 94547, USA) was added into each cuvette. The mixture in each cuvette was incubated at room temperature for 15 minutes. The blank was placed in the spectrophotometer and set to zero. Hence, the absorbance in each of the five cuvettes containing the serially diluted BSA was measured at 750 nm and recorded. Each of the serially-diluted protein samples was measured and recorded. An equation was generated from Microsoft excel for the standard curve for both standard and samples and used to estimate the amount of protein from their absorbance values.

Preparation of Protein for concentration measurement (dilution series)

Curvet number	1	2	3	4	5
Working concentration	10 mg/mL	5 mg/mL	2.5 mg/mL	1.25 mg/mL	0.625 mg/mL
Standard (BSA) µL	10	10	0	0	0
Deionised H_2O (µL)	0	10	10	10	10
Reagent A (µL)	100	100	100	100	100
Reagent B (µL)	800	800	800	800	800

Equation showing protein concentration of *D. farinae* and cockroach allergen extracts.



D. farinae		Cockroach
Absorbance	0.78	2.952
Concentration (mg/mL)	4.4	20

2.3 Allergen extract preparation

2.3.1 *Dermatophagoides farinae* (HDM) crude extract

House dust mite powder was kindly donated by Dr Beverley Lees of Allergy Therapeutics. The powder was mixed with phosphate-buffered

saline (PBS), pH 7.4 (50 mg/mL) in a 15 mL Falcon tube. The tube was mixed by inverting several times until the powder was evenly distributed. The suspension was agitated gently for 30 minutes and centrifuged at 10,000 x g for 10 minutes, all at room temperature. The supernatant containing 4.4 mg/mL protein was collected and stored at -20°C in the short term (2 weeks) and at -80°C in the longer term and they were used within 4-8 weeks of preparation.

2.3.2 Cockroach

Dead adults of a local infestation of cockroaches, identified as *Periplaneta australasiae* (ACR), were macerated in a mortar and pestle, suspended in an equal volume of PBS, agitated gently for 30 minutes at room temperature, sonicated on ice with 3 x 10-second pulses separated by 10-second breaks and centrifuged for 10 minutes at 4000 x g at room temperature. The supernatant containing 20 mg/mL protein was stored as similar to that of HDM.

2.4 Preparation of Sodium Dodecyl Sulphate Polyacrylamide Gels (SDS-PAGE)

The method for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was adapted from Laemmli (1970) and performed as described by Studier 1973. The reagents or materials used were: Bio-rad Minigel SDS system (Bio-rad Laboratories, California

USA), 2 glass plates (small and big), comb, spacers, deionised water, butanol, 1.5 M Tris-HCl pH 8.8, SDS 10%, 30% polyacrylamide solution, 0.5 M Tris HCl, pH 6.8, tetramethylethylenediamine (TEMED) and 10% freshly prepared ammonium persulphate (APS).

Casting equipment, spacer, combs and glass plates were cleaned with a spray of 70% ethanol and wiped with a piece of tissue paper. The gel-casting rig was unscrewed in order to insert the two glass plates, which were separated by a spacer on each side, the larger plate was first laid in the casting rig and then a spacer on the two edges and finally, the smaller plate was laid over them. In order to avoid leakage, the casting rig was adjusted before screwing the plates together. Water was added to the gel casting material and observed for 5-10 minutes for any leakage before adding the resolving gel solution.

A 12% resolving gel solution was prepared in a 10 mL Falcon tube (see Appendix). The resolving gel solution was mixed for a few seconds by inverting the tube, a pipette was used to add the resolving gel between the glass plates covering 2/3 the area of the smaller glass plate. Butanol was added on the surface of the gel in order to ensure a smooth surface. The gel was left at room temperature for polymerisation.

A stacking gel was then prepared on top of the resolving gel and the comb inserted. See (Appendix) for reagent preparation.

2.5 Protein sample preparation for SDS-PAGE

Protein samples were prepared for SDS-PAGE by thawing protein solutions at room temperature and aliquoting the required volume into an Eppendorf tube placed on ice. The protein sample was treated with an equal volume of either non-reducing or reducing 2 x loading buffer (the latter containing 4% β-mercaptoethanol). All samples were boiled for 5 minutes in a 100°C water bath or heating block before loading into wells.

2.5.1 Loading of protein samples onto SDS-PAGE gels

Before protein samples were loaded onto SDS-PAGE gels, the casting gel tank was clamped into the unit and placed in the gel rig. A running buffer (see appendix) was poured into the inner chamber to fill it up. The comb was removed, and bubbles were displaced by pipetting few microliters of running buffer into the well. Gel-loading pipette tips of appropriate size were used to load protein samples into wells. Blue Ray Prestained Molecular marker solution 8 µL (kDa, Jena Biosciences Germany) with a range of 10-180 kDa was loaded on the first lane to determine the molecular weight of protein bands in test samples. Empty wells were loaded with the same volume of non-reducing buffer. Gel electrophoresis was set to run at 120 V for about 90 minutes.

2.6 Staining to visualize protein bands

Coomassie brilliant blue (CBB) staining technique were used in order to visualise protein bands.

2.6.1 Coomassie brilliant blue staining technique

After gel electrophoresis and, prior to staining, gels were microwaved for 20 seconds, the process is repeated 3 times for the gel to stain faster. Then, the pre-prepared Coomassie brilliant blue protein staining solution (Thermo Scientific) was added to cover the gel in order to visualise the separated proteins. The gel with the stain was left on the rocker for 2 hours, after which gels were rinsed with deionised water and destained overnight to visualise protein bands clearly.

2.7 Protein extraction from SDS-PAGE gels

Protein extraction was done according to Beyer et al (2008). Gel slices containing the protein band at 93 kDa were excised from the gel and chopped into tiny pieces, incubated with an extraction buffer (10% SDS 0.06 M Tris-HCl, pH 7.0) at 37°C overnight in 1-mL Eppendorf tubes. After overnight incubation, the samples were centrifuged for 30 minutes at 14,000 x g at 37°C and the supernatant collected and stored at -20°C.

2.8 Western immunoblotting

The western immunoblotting method was adapted from (Maizels et al 1991) and described by (Towbin, Staehelin & Gordon 1979). Gels containing electrophoresed proteins were transferred onto a nitrocellulose membrane using Mini trans-blot electrophoretic transfer cells (Bio-Rad, California, US). Gels were incubated in cold 1x transfer buffer (100 mL of 10 x transfer buffer, 100 mL methanol, and deionised water) for 5-10 minutes. Nitrocellulose membrane was cut according to the size of the gel, 4 sheets of filter paper and 4 fibre pads being used, all incubated in cold 1x transfer buffer for 5-10 minutes. A gel sandwich was made, and the tank was filled with the cold buffer placed on a cooling unit. A small magnetic stirring bar was inserted into the tank and electrophoresis was set up at 50 volts to run for two hours.

Nitrocellulose membrane carrying electro-blotted proteins was incubated with a blocking buffer solution which contained 2.5 g semi-skimmed milk powder in 50 mL Tris-buffered saline tween (TBST). The membrane was incubated overnight at 4°C or alternatively, the membrane was blocked for 1 hour at room temperature on the rocker. After incubation, the blocking solution was removed, and the membrane was washed in 1 x TBST for 3 washings and each washing was for 5 minutes. The membrane was incubated overnight at 4°C in primary antibody diluted 1:100 in TBS solution. The membrane was washed in 1x TBST mentioned above to remove any unbound primary antibody.

For secondary antibody, horseradish peroxidise (HRP)-conjugated goat anti-rabbit IgG (Sigma, UK) was used to detect primary antibody. The membrane was incubated in 5 mL diluted goat anti-rabbit IgG antibody in a 1:1000 tris-buffered saline (TBS) solution for 1½ hours at room temperature.

Antigen-antibody reactivity was detected using a developing solution containing 20 mg 4-chloro-1-naphthol substrate (Sigma UK), which was dissolved in 4 mL ethanol. 20 mL TBS solution was warmed in a microwave oven for 20 seconds and added to the developing solution. Finally, 10 µL hydrogen peroxide (Sigma, UK) was added to the solution and spread on the membrane. The membrane was left on the rocker for 5-10 minutes until protein bands appeared. Deionised water was used to wash the membrane in order to stop the reaction.

2.9 Acid-elution of cross-reactive antibodies

The protocol for acid-elution was adapted from Rybicki (1986). The gel containing separated protein extract containing the antigen of interest was transferred onto a nitrocellulose membrane, which was incubated in primary antibody as described previously. Thin strips of membrane were then cut from each side of the membrane, washed in 1x TBST 3 times for 5 minutes each and incubated in secondary antibody, developed to reveal the position of antigen molecules reacting with primary antibodies on the strips. The remaining membrane was retained in the primary antibody at 4°C. The side strips were re-aligned with the retained

membrane and the position of the band of interest on the membrane was identified. The band of interest was then excised, incubated with 1 mL, 0.1 M glycine buffer, pH 2.8 agitated on a rocker for 10 minutes. The low pH of 2.8 dissociates antibodies bound to specific antigens on the membrane. The elution buffer was collected on a 15 mL Falcon tube and was neutralised using 100 mL, 1M Tris, pH 8.0. The solution containing eluted antibodies was removed and stored at -20°C. The Strips were rinsed three times in phosphate buffer saline (PBS) and re-incubated in the same primary antibody for 2 hours at room temperature or overnight in the fridge. The process of incubation with primary antibody and acid-elution was repeated four to five times.

2.10 Sodium meta-periodate treatment of nitrocellulose membrane

This technique was adapted by Doenhoff et al. (2016) and described by (Eberl et al. 2001; Hamilton et al. 1999). Nitrocellulose membranes carrying electroblotted proteins were incubated with 0.05 M sodium acetate buffer, pH 4.5, for 30 minutes on the rocker at room temperature, one of the membranes being treated with 20 mM sodium meta-periodate dissolved in the 0.05 M sodium acetate buffer, pH 4.5 in a dark place to destroy the carbohydrate determinants. The control was treated in sodium acetate buffer without meta-periodate. Each membrane was washed in 1x PBS and followed by washing in 1x TBST for three times. After the treatment process, primary antibody was added and incubated

overnight in the fridge on the rocker and the process continued as in Western blotting.

2.11 Tandem mass spectrometry (TMS)

MS analysis was performed at the Biomedical Sciences Research Complex Mass Spectrometry core facility at the University of St Andrews. A slice containing a protein of interest was excised from the SDS-PAGE gel and cut into 1 mm cubes. These were then subjected to in-gel digestion, using a ProGest Investigator in-gel digestion robot (Genomic Solutions, Ann Arbor, MI) using standard protocols (Shevchenko et al. 1996).

Briefly, the gel cubes were destained by washing with acetonitrile and subjected to reduction and alkylation before digestion with trypsin at 37°C. The peptides were extracted with 10% formic acid and concentrated down to 20 µL using a SpeedVac (ThermoSavant). The peptides were then placed on an Acclaim PepMap 100 C18 trap and an Acclaim PepMap RSLC C18 column (ThermoFisher Scientific), using a nanoLC Ultra 2D plus loading pump and nanoLC as-2 autosampler (Eksigent). The peptides were eluted with a gradient of increasing acetonitrile, containing 0.1 % formic acid (5-40% acetonitrile in 6 minutes, 40-95 % in a further 2.5 minutes, followed by 95% acetonitrile to clean the column, before re-equilibration to 5% acetonitrile). The eluate was sprayed into a TripleTOF 5600 electrospray tandem mass spectrometer (ABSciex, Foster City, CA) and analysed in Information Dependent

Acquisition (IDA) mode, performing 250 msec of MS followed by 100 msec MSMS analyses on the 20 most intense peaks seen by MS. The MS/MS data file generated via the 'Create mgf file' script in PeakView (Sciex) was analysed using the Mascot search algorithm (Matrix Science), against the NCBInr database (Oct 2014) with no species restriction (93482448 sequences), trypsin as the cleavage enzyme and carbamidomethyl as a fixed modification of cysteines and methionine oxidation as a variable modification. The peptide mass tolerance was set to ± 0.05 Da and the MSMS mass tolerance to ± 0.1 Da.

TMS-identified proteins were further investigated and analysed on the NCBI database (National Centre for Bioinformatics information, USA) using the Basic Local Alignment Search Tool (BLAST) http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Alignment of two amino acid sequences was done using the EMBOSS Needle pairwise sequence alignment tool (http://www.ebi.ac.uk/Tools/psa/)

The prediction of potential glycosylation sites on TMS-identified allergens was done using the CBS Software Prediction Servers NetNGlyc and NetOGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/& http://www.cbs.dtu.dk/services/NetOGlyc/) respectively.

2.12 Cell culture

All cell culture work was carried out in a microbiological cabinet class II. A sterile condition was very important for maintaining the cells. 70%

Industrial methylated spirit (IMS) was used routinely for wiping the cabinet and other equipment to maintain an aseptic environment.

2.12.1 Defrosting cells

Frozen stocks of cells were defrosted using the water bath to fully defrost cells. 1 mL of cells were transferred to a centrifuge tube, and 9 mL of cell culture medium was added, this medium containing 10% (v/v) heat-inactivated foetal bovine serum (FBS) (Thermo Fisher Scientific), 1% 2 mM L-glutamine (2 μg/mL), and 1% penicillin-streptomycin(100 μg/mL) (Sigma-Aldrich UK). Cells were centrifuged at 300 x g for 5 minutes to remove the supernatant which contains DMSO (dimethyl sulfoxide, cell culture grade) and cell culture medium (MEM), 10% and 90% respectively. The cell pellet was re-suspended in 1 mL of pre-warmed medium, transferred to a 25 cm² flask which contained 9 mL of MEM and incubated at 37°C in a humidified 5% CO_2 incubator.

2.12.2. Routine passaging

To maintain RS-ATL8 cells expressing human FcεRI and the NFAT–luciferase reporter gene, 1 mg/mL G418 (Thermo Scientific Fisher) and 600 μg/mL Hygromycin (Thermo Fisher) was added to the medium every two weeks respectively. When cells were confluent (80-100%) between 2 to 3 days, they needed to be passaged or split. The old medium was removed and a washing buffer consisting of 10 mL Dulbecco's

phosphate-buffered saline (DPBS) without Ca^{2+} or Mg^{2+} (Sigma –Aldrich UK) added to the cells. The DPBS was removed and 2 mL trypsin-EDTA added to the flask to detach the cells, which were then incubated for 10 minutes in a 5% CO_2 incubator at 37˚C until clumps of cells were visible. The flask was tapped in the corners gently and 8 mL of warm medium was added. 1 mL of cell suspension was retained in the flask and 9 mL of medium added to the flask and incubated in the CO_2 incubator for further experiments. The RS-ATL8 grows at the bottom of the flask and splitting is performed depending when cells are confluent. 1/5 splitting carried out for 2 days, 1/10 for 3 days, 1/20 for 4 days.

2.12.3 Cryopreservation

After the splitting step, the harvested cells were re-suspended in the freezing medium (10% DMSO and cell culture media only) (Dimethyl sulfoxide, cell culture grade) at a density of $5x10^6$ cells/mL and aliquoted into cryogenic labelled vials. DMSO helps to prevent the formation of ice crystals that might destroy cells when freezing the cells. The cryovials were transferred to a Mr Frosty freezing container (filled with isopropanol) stored at -40°C for an hour and then transferred to -80°C overnight. The next day the vials were moved to a liquid nitrogen vapour phase and stored until used.

2.13 Cellular assay

2.13.1 Cell sensitisation

To assess the presence of parasite-specific IgE or the presence of allergen-specific IgE in tested serum, cells of the reporter cell line were sensitised overnight or for a minimum of 8 hours, a procedure which increases the level of IgE receptor expression. During the process of sensitisation, the IgE binds to the FcεRI receptor. Some sera are toxic to the cells due to complement activation, therefore, it is important to inactivate these complements, hence the serum had to be treated (refer to section 3.6.1 for serum treatment).

From the passage cultures, 10 μL of cells were added to the haemocytometer slide for cell counting and the remaining cells were transferred to a centrifuge tube which was centrifuged for 5 minutes at 300 x g. Cells were re-suspended with the calculated amount of cell culture medium. The cells were mixed using the desired dilution of serum in excess, a 96-well plate having been used and a 50 μL of treatment being added into the plate in triplicate. Plates were incubated for 16-18 hours in a cell culture incubator.

2.13.2 Cell stimulation

The IgE binds to the human FcεRI receptor and crosslinking by the cognate allergen which will reveal the presence of specific IgE in the

serum during cell stimulation. Preparation of selected allergen/antigen dilutions and the positive control (anti-IgE) was necessary.

The old medium was removed from the plate and a 50 µL of the treatment (allergen/antigen concentrations or the positive control (anti-IgE) were added to the cells.

2.13.3 Luminescence measurement

After 4 hours of stimulation, 50 µL of ONE-Glo Luciferase Assay (Promega) was added to each well in a 96-well plate. 100 µL of the mixture was transferred from the transparent plate to a 96 white well plate and the luminescence response was measured in a Tecan Spark 10M plate reader.

2.14 List of materials used that were in stock in the lab

1. Soluble egg antigen (SEA)
2. Cercarial homogenate (Ch)
3. Schistosomula extract
4. Adult worm homogenate (Wh)
5. Latex extract
6. Tomato extract
7. Peanut extract
8. Avocado extract
9. Kiwi fruit extract

2.15 Antibodies used in the study

Primary antibodies	**Source**	**Dilution buffer**	**Dilution**
BR84	rabbit	Tris buffered saline (TBS)	1:100
1025Z	rabbit	Tris buffered saline (TBS)	1:100

Secondary antibody	**Source**	**Dilution buffer**	**Dilution**
HRP conjugated goat anti-rabbit IgG	Sigma UK	Tris buffered saline (TBS)	1:1000

2.16 Software and database used in the study

1. National Centre for Bioinformatics Information (NCBI)

2. European Molecular Biology Open Software Suit (EMBOSS) Needle Pairwise Sequence Alignment

3. CBS software Prediction Servers NetNGlyc and NetOGlyc

2.17 Statistical analysis used

1. One -way ANOVA using Graph Pad Prism 8 software. $P < 0.05$ was considered statistically significant.

Chapter 3: Antigenic cross-reactivity between *Schistosoma mansoni* and *Dermatophagoides farinae* due to shared glycan epitopes and potential relevance to the hygiene hypothesis

Abstract

In recent decades, in countries with advanced health systems, there has been a marked increase in diseases attributed to immunological disorders such as asthma and allergies. However, people infected with parasitic helminths including schistosomes have been found to suffer less from allergy. This has led authors to formulate a helminth parasite variant of the so-called 'hygiene' or 'old friends' hypotheses which was proposed by Graham et al (2003), stating that the mammalian immune system depend on certain microbes that evolved together with human organism, that the absence of these microbes may cause abnormal functionality of the immune system (Rook, Martinelli & Brunet 2003) . Previous studies have found that rabbit IgG antibodies raised against *Schistosoma mansoni* egg antigens cross-react with allergens such as peanut, grass pollen and natural rubber latex. In this work, we describe how rabbit IgG antibodies raised against *S. mansoni* soluble egg antigens (SmSEA) are cross-reactive with molecules in somatic extracts of the house dust mite (HDM) *Dermatophagoides farinae.* A cross-reactive molecule from HDM with approximate mass of 98kDa was identified by tandem mass spectrometric (TMS) analysis to be the allergen Der f 15. Rabbit anti-schistosome IgG antibodies eluted from the

HDM molecule in an immunoblot reacted with the three major glycosylated *S. mansoni* egg antigens IPSE/alpha-1, omega-1 and kappa-5. Moreover, anti-*S. mansoni* egg antibodies that had been eluted from the cross-reactive HDM antigen also reacted with antigenic constituents of a variety of plants which are known to be allergenic in humans. This extensive cross-reactivity was ablated by sodium meta-periodate treatment of the membrane carrying the plant antigens, indicating it was due to cross-reactive carbohydrate determinant (CCDs). Amino acid sequence analysis of the allergen indicated it had potential N- and O-linked glycosylation sites. These findings are novel and provide a possible explanation for the hygiene hypothesis and a potential starting point for improved allergen-specific immunotherapy. In this work, we have also used the humanized Rat Basophilic Leukaemia RS-ATL8 reporter system which is used to detect allergen-specific IgE. RS-ATL8 cells were sensitised overnight with low concentrations of serum of two different individual donors allergic to HDM and stimulated with a wide range of purified HDM antigen concentrations the next day. Luminescence was measured 4 hours after stimulation. Interestingly it was found that these two different serum samples were able to sensitize the reporter cells upon challenge with anti-human IgE, but when challenged with the mite allergen there was only a very low response. This could be because the molecule Der f 15, which is a minor HDM allergen, might not be the target of an allergen-specific IgE response in these two donors. The HDM molecules to which these donors were allergic were not further investigated.

3.1 Introduction

There has been a steep rise in the prevalence of allergies, asthma and other disorders of the human immune system in areas with advanced health systems (Okada et al. 2010). The hygiene hypothesis offered an explanation that improved lifestyles, prevention of infection by vaccinations and the use (or over use) of antibiotics and parasiticidal drugs meant that populations were suffering less from pathogenic infections (Rook, Raison & Lowry 2014) and the patterns of maturation of the human immune system were therefore now different from those that pertained to pre-hygienic, infection-rife eras (Guarner et al. 2006). In helminth-endemic countries, the negative correlation that has often (though not always) been observed between helminth infection, including schistosomes, and sensitization to allergies (Rujeni, Taylor & Mutapi 2012; Hermelijn H. Smits et al. 2010) has supported the hygiene hypothesis.

Immune responses that cause allergic reactions are so-called Th2-type responses (Licona-Limón et al. 2013; Oliphant, Barlow & Mckenzie 2011) and Th2 cell-driven immunity is also induced by helminth infections (Harris & Gause 2011; Matthew Fitzsimmons et al. 2014).Th2-biased immune responsiveness is however downregulated during chronic helminth infection in human hosts and this, in turn, has a suppressive effect on allergic sensitization in such individuals (Fallon & Mangan 2007; Maizels 2016; Yazdanbakhsh & Matricardi 2004). How this occurs has not been fully elucidated, though one explanation invokes activation of

regulatory T cells (Tregs) and production of IL-10 and TGF-β, anti-inflammatory cytokines which have a dampening effect on atopic diseases such as allergy and asthma (Couper, Blount & Riley 2008; Doetze et al. 2000). Blocking antibodies may provide an alternative, not mutually exclusive explanation for the hygiene hypothesis. For example, the IgE blocking hypothesis in which parasitic helminth infection induce the production of large amount of polyclonal IgE level which is not specific to parasite antigen which could saturate Fcε receptors on mast cells and basophils and 'block' the cross-linking of such receptors by allergen-induced specific IgE antibody (Jarrett & Bazin 1974; Mitre, Norwood & Nutman 2005; Pritchard et al. 2007; Yazdanbakhsh, Kremsner & Van Ree 2002). The results presented here are consistent with an alternative 'blocking antibody'. Many proteins of plants, parasites such as helminths and invertebrates (insect venom) shared common carbohydrate structures, these glycan have IgE binding properties (Homann, Schramm & Jappe 2017), some of which are known to be antigenically cross-reactive, so called cross-reactive carbohydrate determinants (CCDs) (Aalberse & van Ree 1997). Glycan epitopes can also induce the production of IgG antibodies (Van Ree & Aalberse 1995). The possibility that anti-*S. mansoni* IgG antibodies that cross-react with allergens could, therefore, block allergic reactions is discussed as an explanation for the hygiene hypothesis.

Previous work has investigated the antigenic cross-reactivity between *S. mansoni* and allergens and has shown cross-reactivity between some

helminth molecules and the allergen Hev b 7 from latex (Doenhoff et al. 2016), Ara h 1 from peanut (Igetei et al. 2017) and several plant pollen allergens (Igetei et al, 2018). Invertebrates are an important trigger of allergic reactions, particularly asthma and hypersensitive skin reactions, so the aforementioned studies have been extended to an exploration of cross-reactive antigenic relationships between *S. mansoni* and an extract from house dust mite (HDM) which is the most important source of indoor allergens for humans and responsible for sensitization in >50% of allergic asthma, rhinitis and dermatitis in humans (Vrtala, Huber & Thomas 2014). We have used rabbit IgG antibodies reactive against *S. mansoni* egg antigens (anti-SmSEA) to probe Western immunoblots carrying water-soluble constituents of HDM in order to identify schistosome-cross-reactive antigens/allergens.

Protein bands containing a cross-reactive molecule in the allergen extract are isolated using SDS-PAGE and the purified material analysed in tandem mass spectrometry (TMS). Anti-SmSEA IgG antibodies that were cross-reactive with the allergen molecule were purified separately by acid-elution from immunoblots and the acid-eluted antibodies were used to probe immunoblots of SmSEA to identify the *S. mansoni* egg antigens that may have induced production of the allergen cross-reactive antibodies in the rabbits.

3.2 Results

3.2.1 Investigation on the cross-reactivity between extracts of *S. mansoni* and house dust mite using rabbit anti-*S. mansoni* egg antisera

The cross-reactivity between *S. mansoni* antigens and HDM was investigated by electrophoresing aqueous extracts of HDM in SDS-PAGE and subsequently probing electroblots of the extracts with a rabbit anti-SmSEA serum. Figure 3.1 shows the results on the HDM extract electrophoresed in a 12% polyacrylamide gel. Lane 1 in the Coomassie blue-stained gel shows a protein band of molecular size >90 kDa, which appears to be reactive with rabbit antibodies in western immunoblot lane 2. Normal rabbit serum gave no reactivity against HDM in lane 3, which is the negative control.

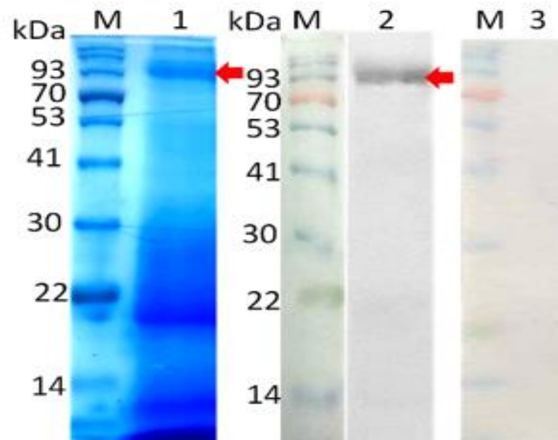


Figure 3.1: Coomassie blue-stained 12% SDS PAGE (lane1) and western immunoblots (lanes 2 and 3) of HDM extract (44 µg/lane). Blot were probed with anti- *S. mansoni* SEA antiserum BR84 on lane 2 or serum from a rabbit injected with Complete Freund's adjuvant alone lane 3. The >90 kDa protein band cross-reactive with the anti- *S. mansoni* SEA antiserum is arrowed. M: Molecular weight marker. Three independent experimental repeats were performed.

Identification of schistosome-cross-reactive house dust mite molecule by tandem mass spectrometry (TMS)

A sample of the >90 kDa band in the Coomassie-stained gel that appears cross-reactive with the rabbit anti-SmSEA antibodies was submitted for further analysis by tandem mass spectrometry, i.e., the band in lane 1 in Figure 3.1 highlighted with a red arrow.

The cross-reactive >90 kDa protein band reactive in the HDM extract was thus excised from a replicate of a Coomassie blue-stained gel and subjected to TMS. The results are summarized in Table 3.1 and the protein present in the HDM extract was identified as the Der f 15 (gi 5815436) allergen molecule. The TMS did not detect any peptides other than those from Der f 15.

Table 3.1: MASCOT search output of NCBI nr with the tandem MS data from the purified >90 kDa *D. farinae* gel band.

gi: 5815436 **98 kDa HDM allergen [*Dermatophagoides farinae*]**

Mass: 63882 Da **Score:** 175 **Matches:** 11 (4) **Sequences:** 10 (4) **emPAI[a]:** 0.35

Peptide match	Score	Expect	Rank	Unique
R.IVCYVGTWSVYHK.V	81	1.2e-06	1	U
K.VDPYTIEDIDPFK.C	101	8.3e-07	1	U
K.YSDMAANPTYR.Q	57	0.0016	1	U
K.IDKQNYLALVR.E	68	0.00056	1	U
K.QNYLALVR.E	57	0.58	1	U
R.ELKDAFEPHGYLLTAAVSPGK.D	9	2.6	4	U
K.DAFEPHGYLLTAAVSPGKDK.I	2	0.85	1	U
K.LVMGVPFYGR.A	21	0.3	1	U
K.LVMGVPFYGR.A + Oxidation (M)	26	4.3	3	U
R.AWSIEDR.S	36	0.39	1	U
K.EGDIPHPTNIHK.Y	22	0.077	1	U

Percentage sequence coverage: 18 %. Matched peptides shown in bold and underlined.

1 MKTIYAILSI	MACIGLMNAS	IKRDHNDYSK	NPMR**IVCYVG**	**TWSVYHKVDP**
51 **YTIEDIDPFK**	CTHLMYGFAK	IDEYKYTIQV	FDPYQDDNHN	SWEKRGYERF
101 NNLRLKNPEL	TTMISLGGWY	EGSEK**YSDMA**	**ANPTYR**QQFI	QSVLDFLQEY
151 KFDGLDLDWE	YPGSRLGNPK	**IDKQNYLALV**	**RELKDAFEPH**	**GYLLTAAVSP**
201 **GKDK**IDRAYD	IKELNKLFDW	MNVMTYDYHG	GWENFYGHNA	PLYKRPDETD
251 ELHTYFNVNY	TMHYYLNNGA	TRDK**LVMGVP**	**FYGRAWSIED**	**R**SKLKLGDPA
301 KGMSPPGFIS	GEEGVLSYIE	LCQLFQKEEW	HIQYDEYYNA	PYGYNDKIWV
351 GYDDLASISC	KLAFLKELGV	SGVMVWSLEN	DDFKGHCGPK	NPLLNKVHNM
401 INGDEKNSFE	CILGPSTTTP	TPTTTPTTPT	TTPTTPSPTT	PTTTPSPTTP
451 TTTPSPTTPT	TTPSPTTPTP	TTPTPAPTTS	TPSPTTTEHT	SETPKYTTYV
501 DGHLIKCYK**E**	**GDIPHPTNIH**	**K**YLVCEFVNG	GWWVHIMPCP	PGTIWCQEKL
551 TCIGE				

[a]emPAI, the exponentially modified protein abundance index

Purification of rabbit anti-schistosome egg antibodies cross-reactive with HDM by acid elution from an electroblot of HDM extract

Illustration of the procedure used to purify rabbit anti-SmSEA IgG antibodies that were cross-reactive with the >90 kDa molecule by acid elution from electroblot of HDM aqueous extract (Figure 3.2).

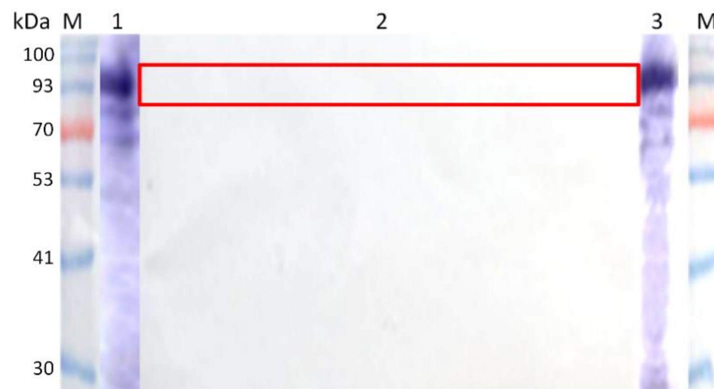


Figure 3. 2: Western blot of a *D. farinae* extract probed with rabbit BR84 anti-SmSEA antiserum for acid-elution of antibodies reacting against a >90 kDa *D. farinae* antigen. 200 µl *D. farinae* extract containing 660 µg protein was loaded into a single, wide lane in a 12% SDS-PAGE gel and electro-transferred to a nitrocellulose membrane. The membrane was incubated with rabbit anti-SmSEA antiserum BR84. Lanes 1 and 3 were then removed, washed and incubated with horse-radish peroxidase-conjugated goat anti-rabbit IgG antibodies and chromogenically stained. The red-outlined box indicates the position of the undeveloped lane 2 which was used for acid-elution of antibodies against the >90 kDa antigen by incubation with glycine/HCl buffer (pH 2.8). M: Molecular weight marker. Three independent experimental repeats were performed

Reactivity of acid–eluted antibodies and periodate-sensitivity

The purified acid-eluted rabbit anti-*S. mansoni* SEA antibodies cross-reactive with the >90 kDa HDM antigen were next used to probe blots of HDM extracts and schistosome extracts from different life stages of the parasite (Figure 3.3). There was intense reactivity against SmSEA, but low reactivity against cercarial or adult worm extracts (Figure 3.3 A).

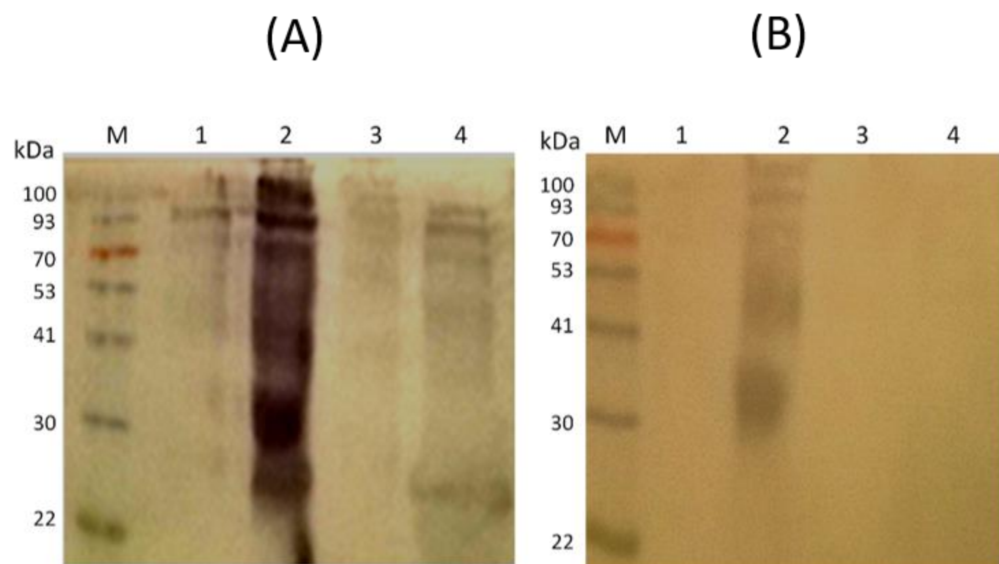


Figure 3. 3: Immunoblot reactivity of acid-eluted rabbit anti-SmSEA antibodies that were cross-reactive with a >90 kDa HDM antigen. (A) Eluted antibodies were used to probe Lane 1, *D. farinae* extract (44µg/lane); 2, SmSEA (20 µg/lane); 3, *S. mansoni* cercarial homogenate (20 µg/lane); 4, *S. mansoni* adult worm homogenate (20 µg/lane). (B) the same as (A) but treated with 10 mM Na-metaperiodate for one hour prior to application of primary antibody. Na-metaperiodate is an oxidizing agent which has destroyed the carbohydrate epitopes except in lane 2 which was not destroy by the treatment. M = Molecular weight marker. Three independent experimental repeats were performed.

Purified acid-eluted rabbit anti-*S.mansoni* SEA IgG antibodies cross-reactive with the molecules in HDM >90 kDa antigen were then used to

probe blots of different plant extracts which was made available in the lab and invertebrate extract that are associated with allergy (Figure 3.4).

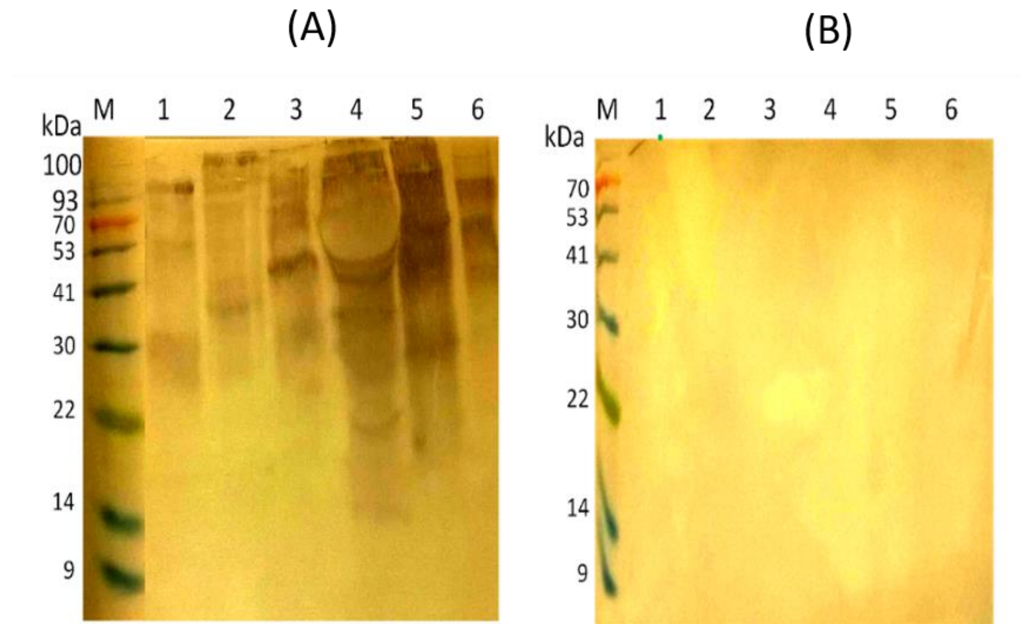


Figure 3. 4: Western blots of various plant water-soluble extracts probed with BR84 anti-*D.farinae* >90 kDa acid-eluted antibodies. (A) Eluted antibodies were used to probe: 1, *D. farinae* (44 µg/lane); 2, latex (157.5 µg/lane); 3, tomato (188 µg/lane); 4, peanut (167 µg/lane); 5, avocado (220 µg/lane); 6, kiwi fruit (94 µg/lane). (B) the same as (A), but after treatment with 10 mM Na-metaperiodate for one hour prior to application of primary antibody. In Figure (3.4 B), the application of Na-metaperiodate treatment destroyed the carbohydrate epitopes. M = Molecular weight marker. Three independent experimental repeats were performed.

The amino acid sequences of the egg antigens of *S. mansoni* which include, Kappa-5, IPSE/alpha-1 and Omega-1 were obtained from the National Centre for Bioinformatics Information (NCBI) database with accession numbers AAX83114, AAK26170, and ABB73003 respectively. To identify the level of similarity between the three egg antigens of *S.*

mansoni and Der f 15, an alignment was performed using the EMBOSS Needle Pairwise Sequence Alignment (European Molecular Biology Laboratory-European Bioinformatics Institute). The results are shown in table 3.2, 3.3 and 3.4 and revealed that the amino acid sequences of Der f 15 and Kappa-5 have 8.3% identity and 14.3% similarity (Table 3.2), Der f 15 and IPSE/alpha-1 show 3.8% identity and 6.2% similarity (Table 3.3), while Der f 15 and Omega-1 share 8.0% identity and 13.6% similarity (Table 3. 4). Furthermore, in Table 3.2, 3.3 and 3.4 the amino acid range (4-300), (1-134) and (101-255) is where similarities are found for Der f 15 and kappa, Der f 15 and IPSE/alpha-1 and Der f 15 and Omega-1 respectively.

Table 3. 2: Amino acid sequence alignment of Der f 15 and Kappa- 5 using pairwise alignment tool

```
# Length: 676
# Identity:      56/676 (8.3%)
# Similarity:    97/676 (14.3%)
# Gaps:         497/676 (73.5%)
# Score: 34.0

Derf15             1 -----------------------------------------------MKT      3
                                                                    :..
kappa5             1 MLQEYNLLINGVTLLISLSGLICEAQSPTNDEMHATISEYGRLYITNIHI     50

Derf15             4 IYAILSIMACIGLM--------NASIKRDHNDYSK------NPMRIVCYV     39
                     .|.:| |||....|        |...|.:.....|      |..|
kappa5            51 HYRLL-IMALSPNMKFTPGEADNILHKSEEEHQVKWALNYLNAAR-----     94

Derf15            40 GTWSVYHKVDPYTIEDIDPFKCTHLMY-GFAK--IDEYKYTIQVFDPYQD     86
                     .||.         :|:.|.||.|...| |..|  :..:..|:     .|.
kappa5            95 STWK---------LENEDMFKKTMSSYVGVNKTIVPNFCMTM-----LQQ    130

Derf15            87 DNHNSWEKR-------GYE---RFNNLR---LKNPELTTMISLGGWYEG-    122
                     .:..:|:..       |.|   :::.|:   .|..:|.|:..|.|..|.
kappa5           131 SSARNWDTNIQKDITYGCEVLKKYSELKWGARKKLDLMTIRWLNGSDENG    180

Derf15           123 -SEKYSDMAANPTYRQQFI---QSVL----------------DFL-----    147
                      |:..||...|...:::::   |||:                :||
kappa5           181 QSQHISDKGFNYHSKKEYLECAQSVMKIHRTKAEVDCRSTVGEFLKLQQV    230

Derf15           148 --------QEYKFDGLDLDWEYP----GSRLGNPKIDKQNYLALVRELK-    184
                             |..:||.::.::...    ..|:.|.:|::.|||..:..::
kappa5           231 KDTPSSKTQAIQFDKINENFNKSLVELKRRVENLEINRINYLKFMNPMER    280

Derf15           185 -----DAFEPHGYLLTAAVSPGKDKIDRAYDIKELNKLFDWMNVMTYDYH    229
                          ||.|              :::|..|.:..
kappa5           281 VISVIDAME--------------EEVDHKYGVYM----------------    300

Derf15           230 GGWENFYGHNAPLYKRPDETDELHTYFNVNYTMHYYLNNGATRDKLVMGV    279

kappa5           301 --------------------------------------------------    300

Derf15           280 PFYGRAWSIEDRSKLKLGDPAKGMSPPGFISGEEGVLSYIELCQLFQKEE    329

kappa5           301 --------------------------------------------------    300

Derf15           330 WHIQYDEYYNAPYGYNDKIWVGYDDLASISCKLAFLKELGVSGVMVWSLE    379

kappa5           301 --------------------------------------------------    300

Derf15           380 NDDFKGHCGPKNPLLNKVHNMINGDEKNSFECILGPSTTTPTPTTTPTTP    429

kappa5           301 --------------------------------------------------    300

Derf15           430 TTTPTTPSPTTPTTTPSPTTPTTTPSPTTPTTTPSPTTPTPTTPTPAPTT    479

kappa5           301 --------------------------------------------------    300
```

```
Derf15          480 STPSPTTTEHTSETPKYTTYVDGHLIKCYKEGDIPHPTNIHKYLVCEFVN    529

kappa5          301 --------------------------------------------------    300

Derf15          530 GGWWVHIMPCPPGTIWCQEKLTCIGE    555

kappa5          301 --------------------------    300
```

http://www.ebi.ac.uk/Tools/services/web/toolresult.ebi

Key

(I) Conserved sequences

(:) Semi-conserved mutations

(.) Non-conservative mutations

Table 3. 3: Amino acid sequence alignment of Der f 15 and IPSE/alpha -1 using pairwise alignment tool

```
# Length: 612
# Identity:      23/612 (3.8%)
# Similarity:    38/612 (6.2%)
# Gaps:         535/612 (87.4%)
# Score: 20.0


Derf15             1 --------------------------------------MKTIYAILSI-     10
                                                           ::...::.|:
IPSE/alpha-1       1 MFLIAVLSYTLISQLGITTSDSCKYCLQLYDETYERGSYIEVYKSVGSLS     50

Derf15            11 ------MACIGLMNASIKRDHNDYSKNPMRIVCYVGTWSVYHKVDPYT--     52
                           ..|:..:| ..||: ..|             |.::..|: ||
IPSE/alpha-1      51 PPWTPGSVCVPFVN-DTKRE-RPY-------------WYLFDNVN-YTGR     84

Derf15            53 ---------IEDIDPFKCTHLMYGFAKIDEYKYTIQVFDPYQDDNHNSWE     93
                              |:|.       ...||..|...|..||..|...:..:....
IPSE/alpha-1      85 ITGLGHGTCIDDF-------TKSGFKGISSIKRCIQTKDGKVECINQPKR    127

Derf15            94 KRGYERFNNLRLKNPELTTMISLGGWYEGSEKYSDMAANPTYRQQFIQSV    143
                     :|.|.||
IPSE/alpha-1     128 RRTYCRF-------------------------------------------    134

Derf15           144 LDFLQEYKFDGLDLDWEYPGSRLGNPKIDKQNYLALVRELKDAFEPHGYL    193

IPSE/alpha-1     135 --------------------------------------------------    134

Derf15           194 LTAAVSPGKDKIDRAYDIKELNKLFDWMNVMTYDYHGGWENFYGHNAPLY    243

IPSE/alpha-1     135 --------------------------------------------------    134

Derf15           244 KRPDETDELHTYFNVNYTMHYYLNNGATRDKLVMGVPFYGRAWSIEDRSK    293

IPSE/alpha-1     135 --------------------------------------------------    134

Derf15           294 LKLGDPAKGMSPPGFISGEEGVLSYIELCQLFQKEEWHIQYDEYYNAPYG    343
```

```
IPSE/alpha-1      135 --------------------------------------------------    134


Derf15            344 YNDKIWVGYDDLASISCKLAFLKELGVSGVMVWSLENDDFKGHCGPKNPL    393


IPSE/alpha-1      135 --------------------------------------------------    134


Derf15            394 LNKVHNMINGDEKNSFECILGPSTTTPTPTTTPTTPTTTPTTPSPTTPTT    443


IPSE/alpha-1      135 --------------------------------------------------    134


Derf15            444 TPSPTTPTTTPSPTTPTTTPSPTTPTPTTPTPAPTTSTPSPTTTEHTSET    493


IPSE/alpha-1      135 --------------------------------------------------    134


Derf15            494 PKYTTTYVDGHLIKCYKEGDIPHPTNIHKYLVCEFVNGGWWVHIMPCPPGT    543


IPSE/alpha-1      135 --------------------------------------------------    134
Derf15            544 IWCQEKLTCIGE    555


IPSE/alpha-1      135 ------------    134
```

Table 3. 4: Amino acid sequence alignment of Der f 15 and Omega-1 using pairwise alignment tool

```
# Length: 612
# Identity:      49/612 (8.0%)
# Similarity:    83/612 (13.6%)
# Gaps:         444/612 (72.5%)
# Score: 46.5

Derf15             1 MKTIYAILSIMACIGLMNASIKRDHNDYSKNPMRIVCYVGTWSVYHKVDP     50

Omega-1            1 --------------------------------------------------      0

Derf15            51 YTIEDIDPFKCTHLMYGFAKIDEYKYTIQVFDPYQDDNHNSWEKRGYERF    100

Omega-1            1 --------------------------------------------------      0

Derf15           101 NNLRLKNPELTTMISL------GGWYEGSEKYSDMAAN--PTYRQQFIQS    142
                        .::...|..::|:      .|:.:.|..|...:..  |||.:.....
Omega-1            1 ---MMRAQSLCFLVSILVTILHVGYSQNSWDYYVFSVTWPPTYCESIQCR     47

Derf15           143 VLDFLQEYKFDGLDLDWE--YPGSR---LGNPKIDKQNYLALVREL----    183
                     :...|:::...||   |.  :|..:   .|:.:.|.:....:..||
Omega-1           48 LPRGLRDFTIHGL---WPTIFPNRQPNCTGSLRFDIRRLQGIRNELDLMW     94

Derf15           184 -------------KDAFEPHGYLLTAAVSP-----------GKDKIDRAY    209
                                  |..||.||  |.|...|           |...:.:..
Omega-1           95 PHLKNYRESPSFWKHEFEKHG--LCAVEDPQVFNQYGYFKFGIQLMQKLN    142

Derf15           210 DIKELNKL---------FDWMNVMTYDYHGGWENFYGHN--APLYKRPD-    247
                     .:|.|.|.         :|.:|:|..     .|..:|:|  |...::|.
Omega-1          143 LLKTLMKYRISPHDSRQYDTINLMNV-----LEREFGYNGSANCIRKPGR    187

Derf15           248 ----ETDELHTYFNVNYTMHYYLNNGATRDKLVMGVPFYGRAWSIEDRSK    293
                         ..:|:|...|   ..|.::|           .||.|..
Omega-1          188 RGMYHLEEVHVCLN---RKHEFMN-----------CPFLGNC--------    215

Derf15           294 LKLGDPAKGMSPPGFISGEEGVLSYIELCQLFQKEEWHIQYDEYYNAPYG    343
                          |.|.:.||..
Omega-1          216 -----PKKFIFPPFQ-----------------------------------    225
```

```
Derf15           344 YNDKIWVGYDDLASISCKLAFLKELGVSGVMVWSLENDDFKGHCGPKNPL    393

Omega-1          226 --------------------------------------------------    225

Derf15           394 LNKVHNMINGDEKNSFECILGPSTTTPTPTTTPTTPTTTPTTPSPTTPTT    443

Omega-1          226 --------------------------------------------------    225

Derf15           444 TPSPTTPTTTPSPTTPTTTPSPTTPTPTTTPTPAPTTSTPSPTTTEHTSET    493

Omega-1          226 --------------------------------------------------    225

Derf15           494 PKYTTYVDGHLIKCYKEGDIPHPTNIHKYLVCEFVNGGWWVHIMPCPPGT    543

Omega-1          226 --------------------------------------------------    225

Derf15           544 IWCQEKLTCIGE    555

Omega-1          226 ------------    225
```

http:// www.ebi.ac.uk/Tools/services/web/toolresult.ebi

The prediction of potential N- and O-linked glycosylation sites on *D. farinae* was done using the CBS software prediction servers NetNGlyc and NetOGlyc (http://www.cbs.dtu.dk/services/NetNGlyc) and (http://www.cbs.dtu.dk/services/NetOGlyc) respectively.

The results revealed that the amino acid sequence of Der f 15 potentially has 3 N-linked and 54 O-linked glycosylation sites, tables 3. 5 and 3. 6, respectively. The predicted glycosylation site (Asn-Xaa-S/T) where Asn is Asparagine (N); Xaa can be any residue except proline; Serine (S); Threonine (T). Asparagine predicted to be N -glycosylated highlighted in bold on the sequence of Table 3.5. In Table 3.6, O-linked glycosylation sites are found in threonine/proline sequence of amino acids between 415-498 towards the C-terminal.

Table 3. 5: Potential N-Linked Glycosylated sites for Der f 15.

>Der f15 Length = 555

MKTIYAILSIMACIGLM**N**ASIKRDHNDYSKNPMRIVCYVGTWSVYHKVDPYT
IEDIDPFKCTHLMYGFAKIDEYKYTIQVFDPYQDDNHNSWEKRGYERFNNLR
LKNPELTTMISLGGWYEGSEKYSDMAA**N**PTYRQQFIQSVLDFLQEYKFDGLD
LDWEYPGSRLGNPKIDKQNYLALVRELKDAFEPHGYLLTAAVSPGKDKIDRA
YDIKELNKLFDWMNVMTYDYHGGWENFYGHNAPLYKRPDETDELHTYFNV**N**Y
TMHYYLNNGATRDKLVMGVPFYGRAWSIEDRSKLKLGDPAKGMSPPGFISGE
EGVLSYIELCQLFQKEEWHIQYDEYYNAPYGYNDKIWVGYDDLASISCKLAF
LKELGVSGVMVWSLENDDFKGHCGPKNPLLNKVHNMINGDEKNSFECILGPS
TTTPTPTTTPTTPTTTPTTPSPTTPTTTPSPTTPTTTPSPTTPTTTPSPTTP
TPTTTPTPAPTTSTPSPTTTEHTSETPKYTTYVDGHLIKCYKEGDIPHPTNIH
KYLVCEFVNGGWWVHIMPCPPGTIWCQEKLTCIGE

Position	Residue	Score*	Jury agreement∞	N-Glyc result$
18	**N**AS	0.6181	(9/9)	++
132	**N**PT	0.6447	(8/9)	+
259	**N**YT	0.7652	(9/9)	+++

*The score is the averaged output of nine neural networks.

∞The jury agreement column indicates how many of the nine networks support the prediction.

The N-Glyc Result$ shows one of the following outputs for predictions indicating *glycosylated* sites:

+ Potential > 0.5

++ Potential > 0.5 AND Jury agreement (9/9) OR Potential>0.75

+++ Potential > 0.75 AND Jury agreement

++++ Potential > 0.90 AND Jury agreement

Table 3. 6: Potential O-Linked Glycosylated sites for Der f 15

>Der f15 Length = 555

MKTIYAILSIMACIGLMNASIKRDHNDYSKNPMRIVCYVGTWSVYHKVDPYT
IEDIDPFKCTHLMYGFAKIDEYKYTIQVFDPYQDDNHNSWEKRGYERFNNLR
LKNPELTTMISLGGWYEGSEKYSDMAANPTYRQQFIQSVLDFLQEYKFDGLD
LDWEYPGSRLGNPKIDKQNYLALVRELKDAFEPHGYLLTAAVSPGKDKIDRA
YDIKELNKLFDWMNVMTYDYHGGWENFYGHNAPLYKRPDETDELHTYFNVNY
TMHYYLNNGATRDKLVMGVPFYGRAWSIEDRSKLKLGDPAKGMSPPGFISGE
EGVLSYIELCQLFQKEEWHIQYDEYYNAPYGYNDKIWVGYDDLA**S**ISCKLAF
LKELGVSGVMVW**S**LENDDFKGHCGPKNPLLNKVHNMINGDEKN**S**FECILGP**S**
TTTP**T**P**TTT**P**TT**P**TTT**P**TT**P**S**P**TT**P**TTT**P**S**P**TT**P**TTT**P**S**P**TT**P**TTT**P**S**P**TT**P
TP**TT**P**T**PAP**TTST**P**S**P**TTT**EH**TS**E**T**PKY**TT**YVDGHLIKCYKEGDIPHPTNIH
KYLVCEFVNGGWWVHIMPCPPGTIWCQEKLTCIGE

Position	Residue	Score*	Prediction
304	**S**	0.746906	
416	**S**	0.97124	
417	**T**	0.978311	
418	**T**	0.980472	
419	**T**	0.98408	
421	**T**	0.992813	
423	**T**	0.998455	
424	**T**	0.998705	
425	**T**	0.997559	
427	**T**	0.999381	
428	**T**	0.99506	
430	**T**	0.99884	
431	**T**	0.998708	
432	**T**	0.998308	
434	**T**	0.998612	

435	**<u>T</u>**	0.998758	O-Glycosylated
437	**<u>S</u>**	0.992194	
439	**<u>T</u>**	0.99598	
440	**<u>T</u>**	0.988899	
442	**<u>T</u>**	0.994358	
443	**<u>T</u>**	0.995248	
444	**<u>T</u>**	0.99669	
446	**<u>S</u>**	0.978851	
448	**<u>T</u>**	0.992388	
449	**<u>T</u>**	0.978779	
451	**<u>T</u>**	0.989288	
452	**<u>T</u>**	0.992476	
453	**<u>T</u>**	0.994422	
455	**<u>S</u>**	0.967404	
457	**<u>T</u>**	0.987643	
458	**<u>T</u>**	0.968631	
460	**<u>T</u>**	0.9806	
461	**<u>T</u>**	0.98968	
462	**<u>T</u>**	0.99234	
464	**<u>S</u>**	0.987485	
466	**<u>T</u>**	0.992013	
467	**<u>T</u>**	0.983368	
469	**<u>T</u>**	0.975937	
471	**<u>T</u>**	0.994849	
472	**<u>T</u>**	0.986307	
474	**<u>T</u>**	0.989304	
478	**<u>T</u>**	0.997451	

479	**T**	0.988791	
480	**S**	0.994586	
481	**T**	0.992045	
483	**S**	0.992686	O-Glycosylated
485	**T**	0.979075	
486	**T**	0.971932	
487	**T**	0.989194	
490	**T**	0.980241	
491	**S**	0.988864	
493	**T**	0.950198	
497	**T**	0.931241	
498	**T**	0.685178	

* Residues with scores ≥ 0.5 were predicted as glycosylated.

3.3 Discussion

Previous studies have demonstrated antigenic cross-reactivity between *S. mansoni* and allergens from different organisms, including allergens Ara h 1 from peanut *Arachis hypogaea* (Igetei et al. 2017), Hev b 7 from natural rubber latex *Hevea brasiliensis* (Doenhoff et al. 2016) and 5 allergenic molecules in plant pollens, 2 from Timothy grass *Phleum pratense* and 3 from the birch tree *Betula verrucosa* (Igetei et al. 2018).

In this work, we observed a cross-reactive antigenic relationship between *S. mansoni* and an extract from the HDM, which is an important cause of allergy. First of all, on a western immunoblot, the allergen extract was probed with anti-*S. mansoni* antisera to identify the cross-reactive molecule. Secondly, we purified the *S. mansoni* cross-reactive antibodies by acid–elution. Thirdly the purified cross-reactive antibodies were used to probe electroblots of *S. mansoni* extracts and different allergen extracts that are known to be allergenic in humans. Treatment with sodium meta-periodate was used to destroy carbohydrate epitopes (Bobbitt 1956; Woodward, Young & Bloodgood 1985). Lastly, the schistosome cross-reactive HDM molecule was identified by mass spectrometry as Der f 15.

House dust mite allergen sensitisation in the general population varies from 65 to 130 million people worldwide (Calderón et al. 2015). They are the most important sources of indoor allergens for humans and responsible for sensitization in >50% of patients with allergic asthma, rhinitis and dermatitis. There are two major groups (1 and 2) of mite

allergens in *D. farinae*; these are cysteine protease and epididymal protein, respectively, in which 80% of humans with HDM allergy have IgE antibody to the group 1 of mite allergen and more than 90% to the group 2 mite allergen (An et al. 2013; Trombone et al. 2002). About 20% of patients with HDM allergy have no IgE antibody to group 1 and 2 allergens (Thomas, Smith & Hales 2004), but have IgE antibodies to other HDM (minor) allergens.

However, we have demonstrated that rabbit anti-SmSEA has cross-reactivity with a molecule of approximately 90 kDa in aqueous extracts of HDM (Figure 3.1). The probing of electrophoresed HDM with a rabbit anti-SmSEA antiserum (BR84) showed reactivity in western immunoblotting (Figure 3.1, lane 2) with a molecule that was evident as a protein band in a Coomassie-stained SDS-PAGE gel containing HDM extract (Figure 3.1, lane 1). Figure 3. 2 illustrates the procedure used to purify rabbit anti-SmSEA antibodies that are cross-reactive with >90 kDa HDM molecule by acid-elution from an electroblots of the HDM aqueous extract. A control experiment was performed using serum from rabbits injected with Complete Freund's adjuvant as a negative control: it showed no reactivity with the HDM (Figure 3.1 lane 3).

Mass spectrometry was performed to identify the cross-reactive molecule and the allergen was Der f 15, a >90 kDa chitinase-like molecule found to be reactive with anti-SmSEA antibodies. The chitinase-like Der f 15 and Der f 18 are minor allergens for humans but Der f 15 is a major allergen for dogs (Hales et al. 2013; McCall et al. 2001). The majority of dogs with HDM-associated atopic dermatitis had

IgE antibodies to two proteins of apparent molecular weight 98 kDa and 109 kDa (McCall et al. 2001). The two proteins had identical amino acid sequences which predicted a protein of 63.2 kDa (McCall et al. 2001) and which TMS analysis for the >90 kDa HDM protein studied here gave as 63.8 kDa (as shown in Table 3.1).

Rabbit anti-SmSEA IgG antibodies here reacted against only one molecule between 93 kDa and 100 kDa, presumably the smaller of the two molecules identified as Der f 15 by McCall et al, (2001). The 30-40 kDa difference between the size predicted by the 555 amino acid-long sequence and that indicated herein immunoblots was attributed to extensive glycosylation (McCall et al. 2001). Our analysis of potential sites for glycosylation using the CBS software prediction servers indicates that Der f 15 may be glycosylated at numerous O-linked sites (Table 3. 6), particularly in a threonine/proline-rich section of sequence (amino acids 415-498) found towards the C-terminal end of the molecule and three N-linked sites (Table 3. 5), though only one N-linked site was predicted in the analysis by McCall et al, (2001).

Alignment of linear amino-acid sequences of Der f 15 with those of three SmSEA molecules (Kappa-5, IPSE/alpha-1 and Omega-1) which have also previously been implicated in antigenic cross-reactivity with other allergen molecules (Doenhoff et al. 2016; Igetei et al. 2017, 2018) as in (Table 3. 2, 3. 3 and 3. 4) to identify the level of similarity which has shown to be only 14.3%, 6.2% and 13.6% respectively.

The reactivity of the eluted antibodies against the HDM extract itself and schistosome egg antigens was abrogated by the oxidizing agent sodium

meta-periodate treatment, except for some reactivity that remained in the SmSEA (Figure 3.3 (B)). The reason for this residual activity after meta-periodate treatment is not fully understood but has been observed in previous studies (Doenhoff et al. 2016; Hamilton et al. 1999). This could be due to the known immunoglobulin binding activity of IPSE/alpha-1 mediated by a unique loop in the crystalline structure including aromatic and positively-charged amino acids (Meyer et al. 2015). The IPSE/alpha-1-immunoglobulin interaction is particularly strong with regards to IgE, but the egg-derived molecule does also react with IgG (Meyer et al. 2015). Another explanation could be that the purified antibodies were reacting with IPSE/alpha-1 glycan backbone rather than the fucose–containing epitopes which are oxidised by sodium meta-periodate. A similar observation of periodate-resistance of other glycoproteins has been described (Hino et al. 2009). Among a wide size range of cross-reactivity, two molecules of ~100 kDa and around 30 kDa in SEA appeared to be particularly reactive with antibodies eluted from Der f 15 (Figure 3. 3 (A)). These SEA molecules may be, respectively, the immunodominant *S. mansoni* egg antigens kappa-5 (Schramm 2009), IPSE/alpha-1 (Schramm et al. 2006) and/or omega-1 (Fitzsimmons et al. 2005). O-linked glycans have not been found on the above-mentioned SEA proteins, so some of the cross-reactivity of the rabbit anti-SmSEA antibodies with Der f 15 may be associated with glycosylation of the N-linked site(s) on the SEA molecules. None of the other SmSEA molecules reacting with the acid-eluted anti-Der f 15 antibodies have been investigated, but O-linked glycans thereon (Smit et al. 2015) may

have induced antibodies cross-reactive with Der f 15. The notion that the cross-reactivity between Der f 15 and antigens in SmSEA is due to shared cross-reactive carbohydrate determinants (CCDs) (Aalberse & van Ree 1997; Altmann 2007) is supported by the observation here that the anti-SmSEA antibodies eluted from Der f 15 reacted against not only SmSEA, but also against various plant extracts that are known to cause allergy in humans (Figure 3. 4 (A)). Furthermore, all the reactivity against the plant extracts was abrogated completely by prior treatment of the material electroblotted onto the nitrocellulose film with sodium meta-periodate as in (Figure 3. 4 (B)) which destroys carbohydrate determinants (Bobbitt 1956; Woodward, Young & Bloodgood 1985).

3.4 RS-ATL8 cellular optimisation

3.4.1 Introduction

The crosslinking of specific IgE bound to the high-affinity receptor FcεR1 on mast cells and basophils in the presence of a matching antigen (allergen) will result in fast degranulation and release of mediators, such as histamine and prostaglandins, leukotrienes, cytokines and chemokines, which in turn causes pathology and inflammation associated with allergy (Falcone, Haas & Gibbs 2000). Therefore, the measurement of allergen-specific IgE levels in the blood of allergic individuals is a key element for allergy diagnosis.

Serological tests have been used for many years to detect the interaction between an allergen and specific IgE. The most commonly used assay is the skin prick test (SPT) which is quick, reproducible and simple (Haahtela et al. 2014). Although alternative techniques have been developed such as the basophil activation test (BAT) for determining the activation of basophil granulocytes in whole blood, which only requires a small amount of blood and is not affected by other medications affecting SPTs, use of the BAT remains confined to specialized research units (Falcone et al. 2015).

The use of humanized rat basophilic leukemia (RBL) cell lines sensitised with patient serum is a diagnostic assay for the detection of allergen-specific IgE (Falcone et al. 2015). This assay has the advantages that it can detect allergen-specific IgE but does not require expensive and

specific equipment. There is still a need for further experimental work to verify that the humanized RBL assay will improve the diagnosis of clinical symptomatic allergy rather than only measuring the amount specific IgE level, as common methods such as UniCAP an *in vitro* test used to measure allergen-specifIc IgE or more recently allergen arrays do, therefore minimizing the chances of false-positive results. These cells are not yet used routinely for diagnostic purposes.

3.4.2 Immunoglobulin E (IgE)

Immunoglobulin E was discovered in 1966 by Ishizakas (Johansson 2016), Bennich and Johansson also contributed to the discovery. IgE is produced by B cells and plasma cells (Kelly et al. 2016). IgE only occurs in mammals (Thornton et al. 2003). Interleukin (IL-4) and (IL-13) induce immunoglobulin class switching from other isotypes to IgE (Lebman 2004; Punnonen & de Vries 1994). There is a very low concentration of IgE present in serum: it has the lowest concentration of all immunoglobulins and the half-life of free IgE is much shorter than that of other immunoglobulins (Kelly et al. 2016). IgE is approximately 190 KDa in size and has 2 heavy and 2 light chains joined by disulphide bonds (Kelly et al. 2016). The light chain has two domains, one variable and one constant. There are 5 domains that make up the heavy chain, one variable and four constant domains (Cε1-4) (Figure 3. 5).

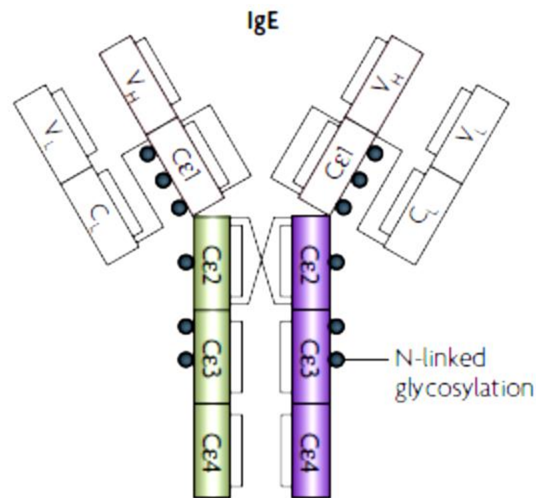


Figure 3. 5: structure of Immunoglobulin E (IgE): Image from Gould and Sutton (2008).

IgE is found circulating in the blood and attaches to different cell types through one of its receptors. The Cε2 to Cε3 region binds to these receptors on cell surfaces. IgE can remain bound to its high-affinity receptor (FcεRI) for many weeks (Gould et al. 2003; Kubo et al. 2014).

3.4.3 IgE receptors

FcεRI and CD23 (FcεRII) are the two known receptors for IgE. IgE binds with the high-affinity receptor (FcεRI) on mast cells and basophils and the resulting complex plays an important role in IgE-mediated allergic responses. The human receptor (FcεRI) can be expressed as both trimeric ($\alpha\gamma_2$) and tetrameric ($\alpha\beta\ \gamma_2$) forms (Kinet 1999). The α chain contains the IgE binding site and both the β and γ chains are responsible for cell signalling and receptor stability (Gould & Sutton 2008). The structure of the IgE molecules bound to the extracellular regions of the FcεRIα chain with the two domains of Cε2 and Cε3 shown in figure 3.6.

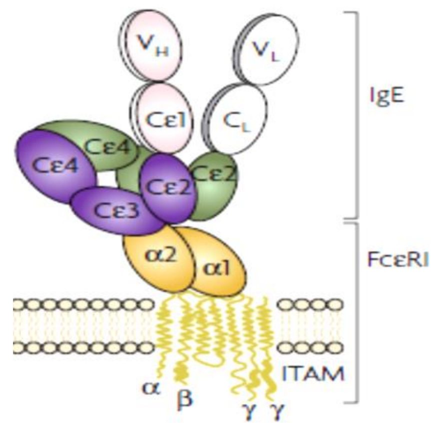


Figure 3. 6: Diagramatic representation of the binding between IgE and the FcεRI α-chain. The image also shows both γ and β chains of FcεRI with immunoreceptor tyrosine-based activation motifs**.** Image from Gould and Sutton (2008).

Rat Basophilic Leukaemia (RBL) cells are composed of a tetrameric FcεRI: one α, one β and two disulphide-linked γ chains (Metzger 1992). The rat FcεRI α chain does not bind human IgE and it, therefore, needs to be humanised with the human α chain which is responsible for IgE binding; i.e., wildtype RBL cells are not suitable for screening human sera, so in order to overcome this limitation many studies have been done with stable RBL transfectants which express the human FcεRI α chain or all three human chains (Passante E 2014) Figure 3. 7.

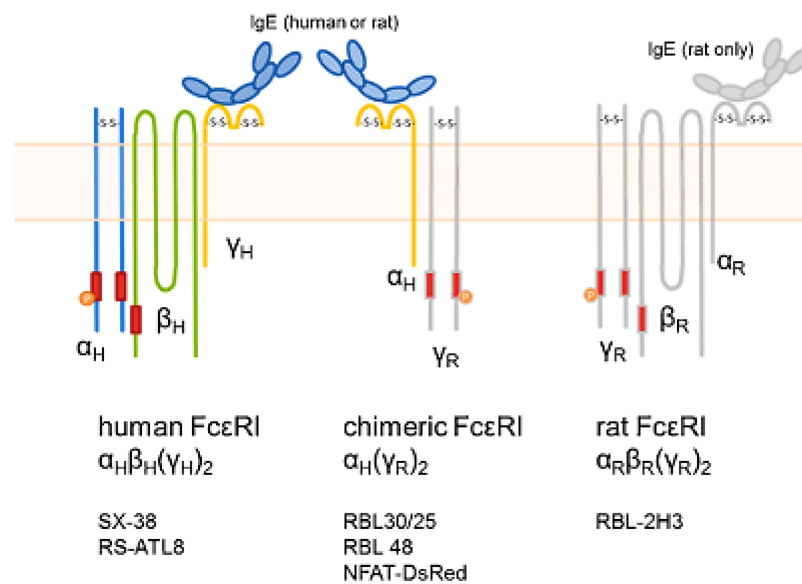


Figure 3. 7: Schematic diagram showing different stable transfected RBL cell lines with respect to human and rat FcεRI receptors. Diagram from Falcone et al. (2015). RBL-SX-38 and RS-ATL8 were stably transfected with human alpha and gamma chain. RBL-NFAT-DsRed and RBL 30/25 were transfected with human alpha chain. The grey represents the rat chains

3.4.4 Humanised reporter cell lines

A new assay called EXiLE (IgE crosslinking (x)-induced luciferase expression) for the detection of allergen-specific IgE was developed by Nakamura *et al.*, (2010). Taudou and co-authors pointed out some limitations when using the chimeric rat cells, such as toxicity of some sera when used at higher concentrations (Taudou et al. 1993). Ladics also pointed out that the low ratio of specific IgE (sIgE) to total IgE in some humans sera and low α_H expression levels might also result in insufficient sensitisation when using RBL cells (Ladics et al. 2008). To overcome these limitations, Nakamura et al. (2010) stably transfected RBL SX-38 with a nuclear factor of activated T cells (NFAT)-responsive luciferase

reporter gene for cell activation in the presence of allergen-specific IgE and cognate allergen. The cells are known as RS-ATL8 (Figure 3.8).

NFAT is a consequence of calcium release-activated channel (CRAC) dependent on Ca^{2+} influx, activating a calmodulin-dependent enzyme, resulting in calcineurin activation and dephosphorylation of NFAT (Hogan et al. 2003). Dephosphorylation of 13 phosphorylation sites of NFAT leads to exposure of a nuclear localization sequence (NLS) and masking of a nuclear export signal (NES) (Okamura et al. 2000). The NFAT nuclear translocation leads to binding of the NFAT to specific promoters, which will result in reporter gene activation to produce luciferase mRNA, in turn, translated into luciferase protein after reaching the cytosol and which can be measured using a suitable chemiluminescent substrate, as shown in Figure 3. 8.

The use of this NFAT-dependent reporter gene has significant advantages over traditional beta-hexosaminidase methods. As in beta-hexoaminidase, a high spontaneous release is observed which results in high background with the assay (Nakamura et al. 2012). A novel and convenient method, RS-ATL8 cell line uses a very sensitive detection with less background noise for detecting the interaction between allergen or parasite antigen with specific IgE in human sera.

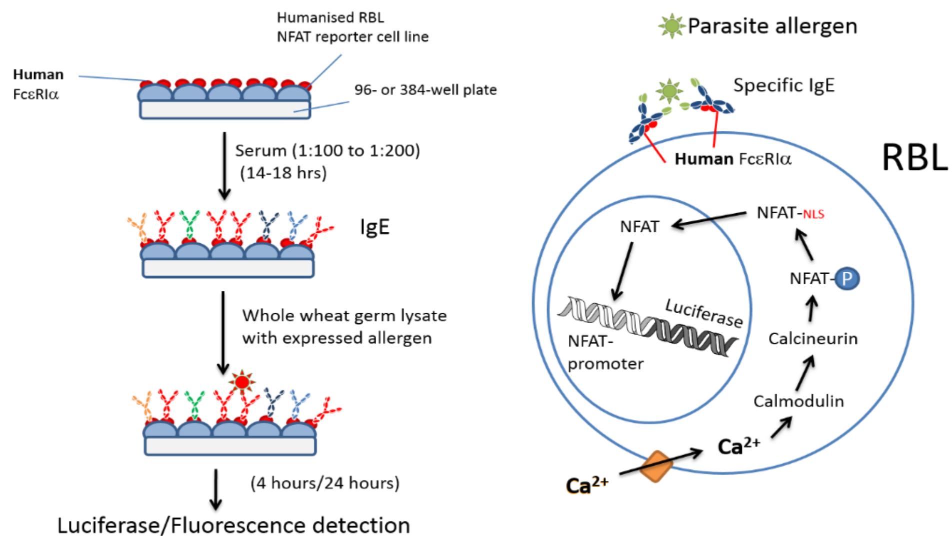


Figure 3. 8: Diagram illustrating the mechanism underlying the use of the humanized RS-ATL8 reporter system as a diagnostic tool for detection of sIgE in patients' sera. Diagram from Falcone et al. (2015).

RS-ATL8 cells are incubated overnight with the serum of a patient who has an allergy to dust mite (sensitization). The next day the sensitized cells are stimulated with the purified dust mite allergen and in the presence of specific IgE (sIgE), the allergen will cross-link with the FcεRI receptors (stimulation). This will result in activation of a signal transduction cascade resulting in nuclear translocation of NFAT which will activate the reporter gene, the protein product which can be measured after 3-4 hours using luciferase reagent.

3.4.5 Complement factor

Complement is an important part of the innate immune system. It comprises a set of plasma proteins and plays an important role in host defence against pathogens such as fungi, bacteria, viruses, and parasites, and in the inflammatory response that helps to fight infection

(Rus, Cudrici & Niculescu 2005). The system is composed of many proteins, most of which are found in serum, while other proteins are membrane-bound or receptors. Many complement proteins are proteases activated by proteolytic cleavage; such enzymes are zymogens that are widely distributed in body fluids. The complement system is activated in an enzyme cascade, the activation of one protein enzymatically cleaving and thus activating the next protein in the cascade (Janeway et a*l.* 2001).

A study by Dibbern *et al.*, (2003) studied sera from peanut-sensitive patients and found that some human serum can have toxicity effects on the Rat Basophilic Leukemia cells, which can spontaneously increase the level of beta-hexosaminidase. Complement proteins from human serum used to sensitize the RBL cells were responsible for the cytotoxicity of the cells. Dibbern *et al.*, (2003) removed cytotoxic factor by absorbing the sera to wild-type RBL-2H3 cells.

Alternatively, complement can be inactivated by heating serum at 56˚C for 30 minutes, though this treatment also denatures IgE and strongly diminishes its ability to bind to its receptors (Binaghi & Demeulemester 1983). This heat denaturation can be prevented by heating for 1 hour 30 mins at 56 ˚C in the presence of 1 M $MgSO_4$ (Binaghi & Demeulemester 1983), but an alternative treatment for 5 min at 56ºC is also successful in inactivating complement while preserving the integrity of IgE (Nakamura. Personal communication).

3.5 Materials

3.5.1 Cells

RS-ATL8 cells were kindly donated by Ryosuke Nakamura (National Institute of Health Science, Tokyo, Japan) through a material transfer agreement (MTA).

3.5.2 Human serum

Human sera were kindly donated by two individual donors who were not patients but allergic to the house dust mite. Blood was drawn into an anticoagulant collection tube (Becton Dickinson, UK) by a phlebotomist at the Queens Medical Centre, Nottingham. The blood was allowed to clot at room temperature for an hour and centrifuged at 2000 rpm at room temperature for 20 minutes to collect serum. The serum was then aliquoted and stored at -80°C until used.

3.5.3 House dust mite / *D. farinae* antigen

House dust mite powder was kindly donated by Dr Beverley Lees of Allergy Therapeutics. Purification of *D. farinae* antigen was achieved by excision of ~98 kDa band from a de-stained gel, followed by overnight incubation in a buffer (10% SDS 0.06M Tris-HCL, pH 7.0), centrifugation and re-electrophoresing of the eluate in a second SDS-PAGE as recently described (Doenhoff et al. 2016).

3.6 Methods

RS-ATL8 cells methodology is described in detail in sections 2.13 to 2.13.2.

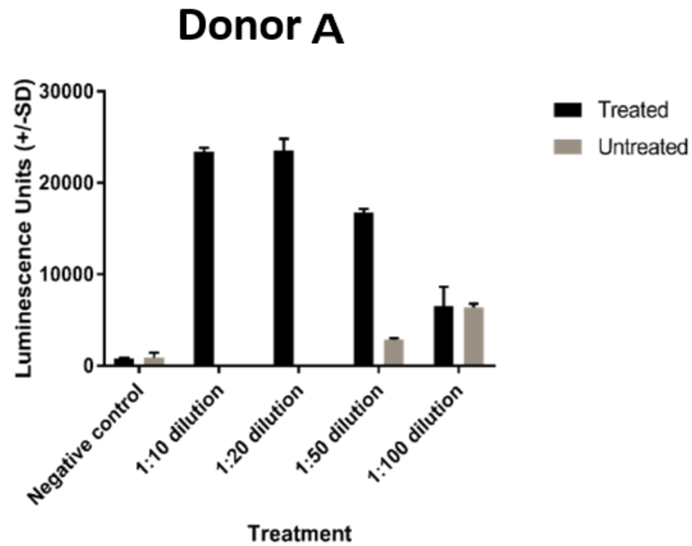
3.6.1 Serum treatment

Human serum were treated in different dilutions by heating for 5 and 30 minutes at 56ºC in a heating block in order to inactivate complement factors before sensitising the RS-ATL8 cells.

3.7 Results

3.7.1 Serum optimisation

Some human sera are toxic to the Rat Basophilic Leukaemia cells, the toxicity being attributable to complement. To inactivate complement factor, cells were treated by heating using the different donor's serum samples who both have an allergy to HDM (Figure 3.9).



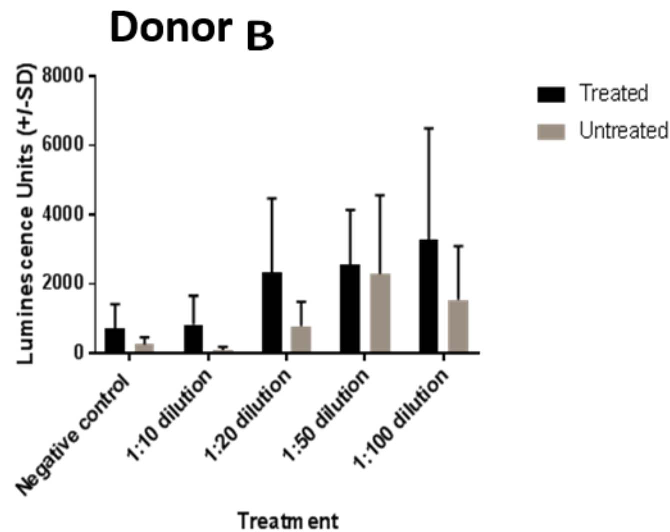
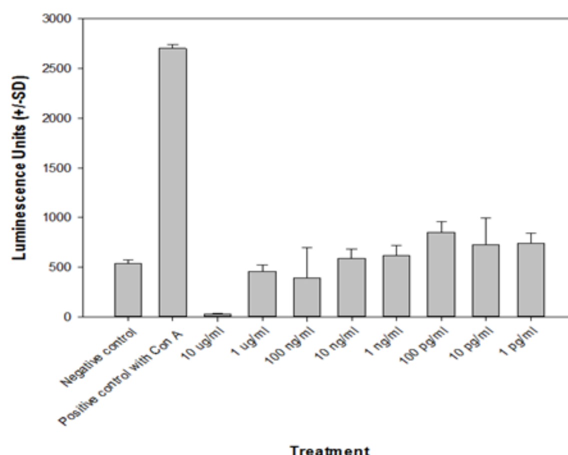


Figure 3. 9: Complement factor optimisation for two different individual donor serum samples A and B. The RS-ATL8 cells were sensitised with different dilutions (1:10, 1:20, 1:50 and 1:100) of sera of an individual with HDM allergy by adding 50 µL / well to a 96-well plate for 16 hours overnight incubation. Treated samples were heated for 5 minutes at 56°C to inactivate the complement factors before sensitisation and untreated samples were not heated. The negative control was unsensitised and unstimulated cells. The next day, the supernatants were washed off and the cells stimulated with anti-human IgE (1µg/mL) for 4 hours. After four hours of stimulation, 50 µL luciferase substrate was added to each well and luminescence was measured. Luminescence response data represent the mean (± standard deviation, SD) of one experiment performed in triplicate. The data error bar indicates SD.

3.7.2 Optimisation of purified *D. farinae* antigen

The activation of the reporter cell line RS-ATL8 was tested using different concentrations of *D. farinae* antigen to obtain the optimal concentration (Figure 3.10). In the same figure, Con A was used as a positive control which gave us a very high response with the RS-ATL8 cells. As this is expected, Con A crosslinks the alpha chain of the FcεRI directly with or without IgE.

Donor A



Donor B

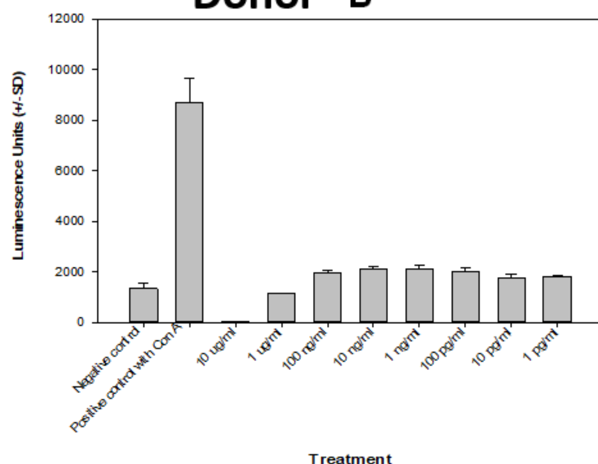


Figure 3. 10: Antigen optimisation for the two donors A and B. The Rs-ATL8 cells were sensitised using the optimal dilutions 1:20 (donor A) and 1:100 (donor B), serum were treated for 5 minutes at 56°C of donors allergic to house dust mite, adding 50 µL / well to a 96-well plate. The positive control was unsensitised cells but stimulated with Con A (1µM) and the negative control was sensitised, they were incubated for 16 hours overnight. The next day cells were stimulated in different concentrations of purified *D. farinae* antigen (Der f 15) ranging from 10µg/ml to 1pg/ml for 4 hours. Negative control was incubated with medium only. After four hours of stimulation, 50 µL luciferase substrate was added to each well and luminescence was measured. Luminescence response data represent the mean (± standard deviation, SD) of one experiment performed in triplicate. Data error bar indicates SD.

3.8 Discussion

In this chapter, firstly, we investigated serum treatment to inactivate complement and secondly optimised the concentration of purified *D. farina* antigen using the reporter system. The introduction of a nuclear factor of activated T-cells (NFAT) reporter gene into human receptor expressing RBL-SX38 cells is a suitable technique to detect IgE cross-linking induces mast cell activation with high sensitivity and low background (Nakamura et al. 2010).

In this study, we have used purified *D. farinae* antigen as the agonist in the reporter assay. However, to obtain a bell shape curve it is important to do some optimisation of the sera to find the optimal conditions for using the reporter assay which will enable us to move to the next experimental stage. Thus, concentrations of sera from two different individual donors A and B allergic to house dust mite (HDM) were optimised using the reporter assay (RS-ATL8). The RS-ATL8 cells were sensitised with the sera of these donors that putatively would have contained IgE reactive with HDM and stimulated the next day with anti-human IgE. Higher concentrations of human serum can be cytotoxic to the RBL cell line thus to avoid cytotoxicity sufficient serum dilution is necessary (Vogel et al. 2005) or by absorbing the sera to wild-type RBL-2H3 cells (Ladics et al. 2008). Removal of complement from serum maintains the viability of the reporter assay (RS-ATL8) cells as complement-mediated cytotoxicity can reduce signal strength and few viable cells will be available to produce luciferase after cell stimulation.

Therefore, in this study, we compared the effect of treated and untreated serum in which treated serum was heated at 56°C for 5 minutes to inactivate complement. Our finding shows (Figure 3. 9 A) that concentrations of donor A's serum gave the best response with the RS-ATL8 cells at a 1:10 and 1:20 dilutions. As stated by Nakamura *et al.,* (2010), human serum has a cytotoxic effect on the RS-ATL8 cells, and this effect could be reduced by more than 50% by treating the sera by heating at 56°C for 30 mins which might be due to inactivation of complement, but interestingly Takagi *et al.,* (2003) in their study found a lesser amount of β-hexosaminidase was released by stimulation with higher concentrations of some human sera. A high response was also observed at a 1: 50 dilution with the RS-ATL8, but a much lower response at a 1: 100 dilution. In comparison to untreated serum, any dilutions lower than 1:50 destroyed the RS-ATL8 cells hence no activation was observed. With regards to the negative controls as expected, they did not activate the RS-ATL8 cells because they were unsensitised and unstimulated. We concluded that heating the serum was a key step, as it enabled us to use a higher concentration of serum (1:20) in order to proceed further with the next stage of the experiment.

In a similar experiment serum from donor B who also was allergic to HDM was used. The RS-ATL8 cells were sensitised with different dilutions of serum as described above, or without serum prior to overnight incubation, stimulation with anti-human IgE (1µg/ml) and measurement of luminescence. As seen in (Figure 3. 9 B), we observed a 1: 100 dilution of donor B's serum gave the highest basophil activation with the RS-

ATL8 cells. Therefore, 1:100 dilution was the optimum in this experiment. Nakamura *et al.*, (2012) stated that 1:100 dilution was enough to sensitise the RS-ATL8 cells without cytotoxicity. In the same figure, we observe a lower level of basophil activation at a 1:20 and 1: 50 dilutions for the treated samples. For the untreated serum, we observed a 1:50 dilution gave the highest response on the RS-ATL8 cells. Therefore, there was a marked difference in the reactivity of the two donors' sera, i.e., donor B gave maximum effectiveness with a dilution of 1/100 and compared with a dilution of 1/20 for maximum effectiveness from donor A, while donor A gave a much higher level of luminescence at optimum dilution than donor B. It is not clear what the difference between the two sera is due to. Different concentrations of IgE in the two sera may be an explanation for the maximum effect being given by different serum dilutions, but this would not necessarily explain the difference in levels of luminescence.

We proceeded further with the experiment using the optimum dilutions of the two sera from donors allergic to HDM, 1:20 from donor A and 1:100 from donor B using mite allergen instead of anti-IgE as an activator in the RS-ATL8 assay. Two separate experiments were carried out, A and B as in Figure 3.10. Thus, finding the optimal concentrations of mite allergen for use in the RS-ATL8 cell assay was another important experiment in our study.

As illustrated in (Figure 3.10), the activation of the RS-ATL8 cells was very low when using the serial dilution of purified *D. farinae* antigen (Der f 15) for stimulation. There was a complete decrease with the RS-ATL8

cells at a 10 µg/mL, which could be explained using a higher concentration of the antigen for stimulation. The two donors may have had little or no IgE specific for mite allergens in their sera. As Dibbern *et al.,* (2003) stated in their study that the RBL assay is allergen-specific, as they assessed specificity by sensitising cells with sera from a patient who had peanut-specific IgE, but not anti-grass or anti-walnut IgE. When the cells were triggered with the extract of peanut, pollen or walnut they found that the peanut-induced degranulation, but no degranulation was observed in either the walnut or the grass pollen. Another possibility could be that the allergen used might be degraded, so no effect could be seen with the reporter system.

As the allergen was Der f 15 they belong to the *Dermatophagoides* species which are important sources of indoor allergens for humans and responsible for sensitization of allergic asthma (Platts-Mills et al. 1997). They are also allergens associated with atopic dermatitis in dogs (Sture et al. 1995).

The group 1 and 2 are the major mite allergens accounting for 80% in the mite allergic population (Thomas et al., 2004). About 20% of patients with HDM allergy have no IgE antibody to group 1 and 2 allergens (Thomas *et al.* 2004) but have IgE antibodies to other HDM allergens (minor allergens). Der f 15 is a minor allergen for humans but major allergen for dogs (McCall et al. 2001)

When the RS-ATL8 cells were sensitised with the two individual donors' sera a high luciferase expression was obtained when stimulated with anti-human IgE, indicating that the donor sera both contained IgE, but

when stimulated with purified mite antigen there was little response. Our findings were consistent with a study by Takagi *et al.*, (2003) who transfected the α-chain of human FcεRI using RBL-2H3 cell line and they used a dust mite allergic patient's sera to sensitise the cells and stimulated them with the mite allergen Der f 1 however, the allergen did not induce Ca^{2+} signalling in the sensitised cells, the explanation for this could be as a result of the cell surface density of human α chain is low to be cross-linked by specific allergen also the ratio of allergen-specific IgE and to the total IgE antibodies in patient's sera might be low.

In figure 3.10, a large response was observed after stimulation of the RS-ATL8 cells with Con A (1µM). Concanavalin A (Con A) is a lectin extracted from jack beans (*Canavalia ensiformis*). A previous study has reported that Con A and other lectins have the ability to cross-link IgE bound to the high-affinity receptor causing the release of histamine and triggering IL-4 and IL-13 production (Haas et al. 1999). Using Con A as a positive control was a good idea because it has the ability to crosslinks the alpha chain receptor directly and activate the cells.

To conclude, the RS-ATL8 cells expressing human FcεRI are a useful assay to explore allergen-specific IgE in human serum, but the two donor serum samples that we have used in this study did not induce a successful stimulation with the mite antigen, this could be because the mite antigen we used might not be recognized by the sera from these two donors as they might be allergic to the major allergens of mite Der f 1 and 2 rather than Der f 15 which is a minor allergen.

A more likely explanation is that the amount of IgE specific for any mite allergens may have been too small relative to the total amount of IgE in the sera. Thus, only a relatively small amount of mite-specific IgE would have been on the cells (compared with a much larger amount of IgE with no mite-specificity) and the exposure to mite antigen would have thus had little or no effect. Cross-linking a few hundred receptors is needed to get a full stimulation. If the donors have so little specific IgE, mast cells and basophils cannot be activated.

Chapter 4: Antigenic cross-reactivity between *Schistosoma mansoni* and *Periplaneta* species

Abstract

There has been an increase in diseases attributed to immunological disorders such as asthma and allergies in industrialised countries. Studies have shown that rabbit IgG antibodies raised against *Schistosoma mansoni* soluble egg antigen (SmSEA) cross-react with allergens such as grass pollen, peanut and natural rubber latex. Here, we describe antigenic cross-reactivity between SmSEA with molecules in extracts of the Australian cockroach (ACR), *Periplaneta australasiae.* A cross-reactive molecule from the ACR was identified by tandem mass spectrometry as two homologues of the *americana* cockroach allergen Cr-PI/Per a 3. Cross-reactive rabbit anti-SmSEA IgG antibodies eluted from the Australian cockroach reacted with antigens from *S. mansoni* eggs, cercariae and worms it reacted also with the cockroach extract. Treatment with sodium meta-periodate of the electroblotted allergen abrogated most of the cross-reactivity of the rabbit anti-SmSEA antibodies, indicating it was due to cross-reactive carbohydrate determinants (CCDs). Amino acid sequence analysis indicated that they had potential for N- linked, but no O-linked glycosylation. These findings could provide a possible explanation for shared antigenicity between *S. mansoni* and allergens and provide a potential starting point for allergy immunotherapy.

4.1 Introduction

The increasing incidence of allergy and autoimmune diseases in industrialised countries due to improved hygienic conditions and health care, less exposure to bacteria, fungi and parasites, has resulted in a rise in allergy. Possible underlying reasons have been proposed with the hygiene hypothesis (Okada et al. 2010).

The relationship between helminth infection and allergic disorder has given support to the hygiene hypothesis and many studies have demonstrated the concept that chronic helminth infection downregulates allergic diseases (Rujeni, Taylor & Mutapi 2012; Hermelijn H. Smits et al. 2010). The protective immune response against helminth parasite is mediated by T- helper type 2 (Th2) responses (Cooper 2009). Th2 responses are characterized by increases in interleukin-4 (IL-4) and other cytokines such as IL-14, IL-5 and IL-13 and also eosinophil-rich inflammation which infiltrates tissue (Anthony et al. 2007). Exposure to helminth infection may induce inflammatory responses to parasite antigens while little or no exposure to infection with helminths could lead to the development of clinical allergy symptoms (Nutman & Cooper 2002). In endemic areas, parasitic helminths can give rise to chronic infections because the parasite can survive in the host for many years, such situations are associated with less allergic diseases controlled by Th2 responses (Cooper 2009). Th2 immune responses are important for the survival of the parasite and prevent host tissue damage. Chronic helminth infection induces an immunomodulatory effect that can

suppress allergic inflammation through regulatory T (Treg) and B cells (Umetsu & DeKruyff 2006). Treg cells produce different immunomodulatory cytokines such as interleukin- (IL)-10 and transforming growth factor- (TGF)-β (Satitsuksanoa et al. 2018). Bystander immunoregulation by helminth infection can regulate allergic inflammatory responses directed against aeroallergens which can lower the allergic prevalence in individuals infected with helminth parasites (Hermelijn H. Smits et al. 2010). The association between chronic helminth infection with *S. mansoni* and allergic disease was observed in a study by Smits and co-authors, in their study, they found a significant reduction of airway hyperresponsiveness and ovalbumin-specific airway inflammation during the chronic infection, but increases during acute infection in mice and the suppressive mechanism was IL-10 dependent (Smits et al. 2007). Natural Treg cells such as $CD4^+$ $CD25^+$ and $FOXP3^+$ can protect against allergy by an IL-10-dependant mechanism (Umetsu & DeKruyff 2006). A study has found that individuals infected with *S. mansoni* in Kenya had higher regulatory Treg cells such as $CD4^+$ $CD25^{high}$ and these cells decreased in number on effective treatment with praziquantel (Watanabe et al. 2007). Praziquantel is an antihelmintic drug which increases the permeability of the membranes of schistosome cells towards calcium ions (Ca^{2+}) influx. Ca^{2+} induces muscle contraction of schistosome worms resulting in paralysis of the parasite (Vale et al. 2017).

B cells regulate immune responses by producing antigen-specific antibodies and aid in the induction of $CD4^+$T cell activation (Noh & Lee

2011). B regulatory (Breg) cells produce suppressor cytokines such as IL-10 that regulate or suppress T cell-mediated inflammatory responses (Rosser & Mauri 2015). A study demonstrated that infection with *S. mansoni* protected mice from anaphylaxis, the effect is dependent on the parasite inducing an IL-10 producing B cell which mediated the protection against anaphylaxis (Mangan et al. 2004).

The blocking of IgE antibody activity by IgG antibodies is another possible explanation for the negative correlation between helminth infection and allergy. Helminth infections are associated with high levels of polyclonal IgE which is non-specific for parasite antigens. This could result in saturating the high-affinity receptor FcεRI on mast cells and blood basophils and block the cross-linking of the receptor by corresponding allergens (Mitre, Norwood & Nutman 2005). Another blocking hypothesis possibility involving IgG, and in particular IgG4, is based on the fact that this IgG isotype is produced during chronic helminth infections (Adjobimey & Hoerauf 2010). Many studies have reported IgG blocking antibody in patients undergoing allergen-specific immunotherapy in peanut allergy patients (Vickery et al. 2013) and another study by Santos *et al.,* reported that IgG4 blocking antibody was able to inhibit peanut–induced mast cell and basophil activation in peanut-tolerant children sensitised to peanut allergens (Santos et al. 2015).

The N–linked carbohydrates (N-glycans) in glycoproteins of many plants and invertebrates (including helminths) can induce specific IgE antibodies, which cross-react with similar carbohydrate structures on a

variety of allergens. These have been termed cross-reactive carbohydrate determinants (CCDs) (Homann, Schramm & Jappe 2017) and they could be responsible for cross-reactivity between helminths and allergens. For example, the IgE cross-reactivity between *Ascaris* and dust mites is due to tropomyosin (Acevedo et al. 2009). The possible involvement of shared glycan epitopes in the cross-reactivity between *S. mansoni* antigen and allergens could be useful; that is IgG antibodies against *S. mansoni* egg antigen that is cross-reactive with various allergens could serve as a blocking antibodies.

For over 50 years, cockroach allergy has been accepted as a major cause of asthma (Pomés et al. 2017). In 1964, the presence of positive skin tests to cockroach extract was first reported among patients living in New York (Bernton & Brown 1964). This paved the way for many studies to study the relationship between cockroach allergy and asthma. Exposure to indoor allergens such as cockroach has been associated with allergic asthma (Eggleston et al. 1998). Cockroach allergen sensitisation has been linked to the development of asthma in low-income urban populations (Togias et al. 2010). In the United States (US) the prevalence of cockroach allergy ranges from 17% to 41% (Pomés et al. 2017). About 60 to 80% of children with asthma in an inner-city population are sensitised to cockroach allergen (Sohn & Kim 2012). Cockroach sensitisation was strongly correlated with the cockroach allergen levels measured in the homes of these inner-city children and this was supported by the National Cooperative Inner-City Asthma Study (NCICAS), which showed the relationship between asthmatic children

living in inner cities of Baltimore and exposure to cockroach allergen (Eggleston et al. 1998). Cockroach allergic patients exposed to cockroach allergens had increased asthma morbidity (Do, Zhao & Gao 2016). Approximately 25% of children with asthma are sensitised to cockroaches and a detectable level of cockroach allergens are found in their homes in Poland (Stelmach et al. 2002). In Taiwan, 57.5% of asthmatic patients had reactivity to Per a 2 cockroach allergen (Lee et al. 2012). There are two predominant species of cockroach that infest human homes and they are the main subjects of allergy research. The two species are American cockroach (*Periplaneta americana*) and German cockroach (*Blatella germanica*) which predominate in tropical and temperate regions respectively (Pomés et al. 2017). The American cockroach is common in Thailand, Brazil and Taiwan while the German cockroach can be found in the USA and Europe (Pomés & Arruda 2014). There are 9 American cockroach allergens (Per a 1-3, Per a 6-7 and Per a 9-12) with molecular weights of between 17 kDa and 72 kDa in SDS-PAGE and 9 German cockroach allergens (Bla g 1-8 and Bla g 11) with molecular weights between 21 kDa and 79 kDa in SDS-PAGE (www.allergen.org).

Antigenic cross-reactivity between *S. mansoni* and allergens has been investigated previously. Our lab has demonstrated cross-reactivity between *S. mansoni* and Ara h 1 from peanut (Igetei et al. 2017) and several plant pollen allergens (Igetei et al. 2018).

In this chapter, we have used the Australian cockroach (*Periplaneta australasiae*) and TMS analysis identified the allergen cross-reactive with

schistosomes as Per a 3/Cr-PI, indicated they both contained peptides with sequences identical to those found in *P. Americana*.

4.2 Results

4.2.1 Investigations on the cross-reactivity between *S. mansoni* and cockroach extract using rabbit anti-*S. mansoni* egg antisera

The cross-reactivity between *S. mansoni* antigens and cockroach was investigated by means of SDS-PAGE and probing electroblots of the cockroach extract with antibodies in rabbit antisera induced by *S. mansoni* egg antigens (rabbit anti-SmSEA serum). Figure 4.1 shows Australian cockroach (ACR) extract in a Coomassie blue-stained 8% polyacrylamide gel with two bands of apparent molecular sizes of 85 kDa and 88 kDa. Lane 2 is a corresponding western immunoblot showing at least four bands between 46 kDa and 100 kDa that are cross-reactive with anti-SmSEA IgG antibodies. Lane 3 is a negative control, in which no reactivity against ACR was observed with normal rabbit serum.

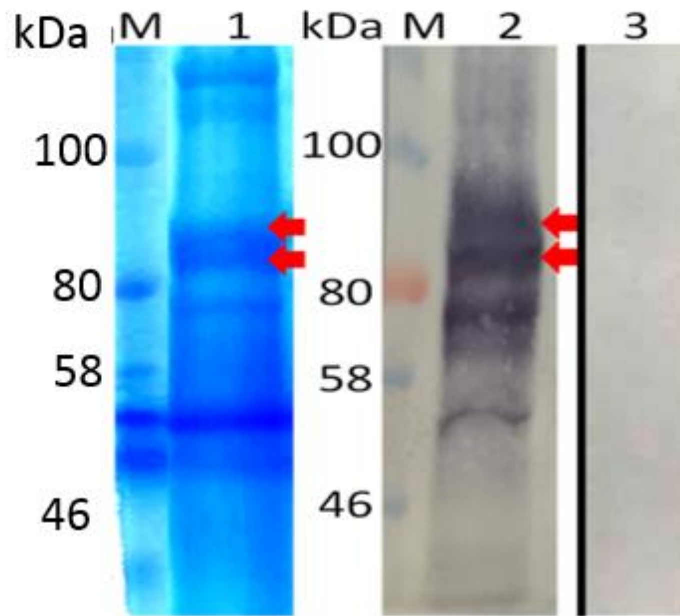


Figure 4.1: Coomassie blue-stained SDS-PAGE and Western immunoblots of ACR extract. *P. australasiae* extract (200µg/lane) Lane 1, Coomassie blue-stained gel; lane 2,3 Western immunoblots. Cockroach protein samples were boiled, with non-reduced. Blots were probed with rabbit anti-SmSEA antiserum 1025Z (2) and normal rabbit serum as a negative control (3). Arrowed bands at ~85/88 kDa (red). M: Molecular weight marker. Three independent experimental repeats were performed.

Cockroach gel slices containing two molecules estimated to be 85 kDa to 88 kDa were excised from a replicate of the gel in lane 1 and the proteins were eluted from gel slices in Tris-HCl buffer, pH7.4 by overnight incubation. The eluted proteins were re-electrophoresed and stained with Coomassie blue staining, Figure 4. 2 (lane 4 and 5) with a single band detected for each. The purified protein material from the gel was subjected to tandem mass spectrometric analysis (Table 4.1). This led to the identification of the cross-reactive cockroach molecule as the allergen Per a 3.

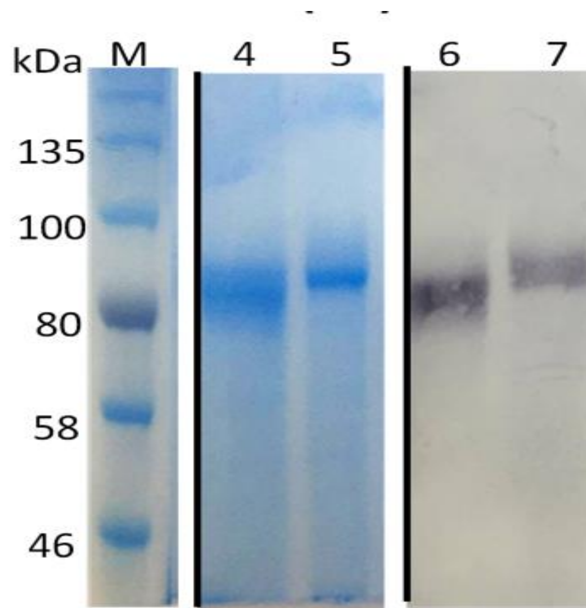


Figure 4. 2: A 8% Coomassie blue-stained SDS-PAGE of cockroach extract. *P. australasiae* purified proteins (10.6 µg/lane): Coomassie blue-stained purified *P. australasiae* protein at ~85 kDa (lane 4) and ~88 kDa (lane 5); Western immunoblot of purified cockroach proteins of ~85 kDa (lane 6) and ~88 kDa (lane 7) reacting with the rabbit anti-SmSEA 1025z antiserum. Cockroach protein samples were boiled under non-reducing conditions. M = Molecular weight marker. Three independent experimental repeats were performed.

Identification of schistosome-cross-reactive American cockroach molecules by tandem mass spectrometry (TMS)

Molecules in the cockroach extract that cross-reacted with anti-SmSEA antibodies were selected for further analysis by mass spectrometry. TMS analysis of the two purified ACR proteins of estimated size ~85 kDa and ~88 kDa, in Tables 4. 1 and 4. 2 respectively, indicated both protein bands contained peptides present in the sequence of protein Cr-PI, allergen Per a 3 (gi: 284518363) from *P. americana*.

Table 4. 1: MASCOT search output of NCBInr with the tandem MS data from the purified ~85 kDa cockroach gel band.

gi: 284518363 **Allergen Cr-PI; AltName: Allergen Per a 3 [*Periplaneta americana*]** **Mass:** 82283 Da **Score:** 884 **Matches:** 25 (25) **Sequences:** 22 (22) **emPAI[a]:** 3.01				
Peptide match	**Score**	**Expect**	**Rank**	**Unique**
R.VNEGMFMYSFSIAVFHR.D	55	3.8e-05	1	
K.NQDQLLAYFTSDVNLNAFNTYYR.Y	136	1.7e-12	1	U
R.GEQFYYTYK.Q	52	0.0048	1	
R.GEQFYYTYKQIYAR.Y	66	3e-05	1	
R.LSNDLPDVYPFYYSKPVK.S	58	0.00018	1	
R.YHNGEEMPVRPSNLYVTNFDLYYIADIK.N + oxidation	60	3.3e-06	1	U
K.MGLAPSALEHPETVLR.D	109	3.9e-09	1	U
K.MGLAPSALEHPETVLR.D + oxidation (M)	109	1.4e-08	1	U
R.DPAFYQLWK.R+ oxidation	43	9.2e-05	1	U
R.DPAFYQLWK.R	52	0.02	1	
R.YTHDELAFEGVK.V	52	0.0016	1	
K.LYTYFEQYDVSLDMSVYVNK.V	63	6.5e-06	1	U
K.VDQIPNVDVHAR.Q	44	0.015	1	U
R.LNHKPFTYNIEVSSDK.A	115	1.5e-07	1	
R.NSHDSNIVAPER.D	89	9.1e-07	1	U
R.NSHDSNIVAPERDSYR.T	59	3.7e-05	1	U
K.GHNYCGYPENLLIPK.G	53	0.0013	1	
K.GHNYCGYPENLLIPK.G	49	0.0016	1	
K.KGGQAYTFYVIVTPYVK.Q	61	0.0003	1	
K.GGQAYTFYVIVTPYVK.Q	86	4.7e-07	1	
K.QDEHDFEPYNYK.A	71	2e-05	1	
K.AFSYCGVGSNR.K	84	6.2e-06	1	U

K.AFSYCGVGSNRK.Y	37	0.028	1	U
K.IYSNDFYTPNMYFK.D	35	0.0022	1	
K.IYSNDFYTPNMYFK.D + oxidation (M)	71	4.4e-06	1	

Percentage sequence coverage: 39%. Matched peptides shown in bold and underlined.

1 MKTALVFAAV	VALVACAAFP	AHKDYKQLAD	KQFLAKQRDV	LRLFHRVHQH
51 NILNDQVEVG	NTYDIEANIG	NYKYPRVVKQ	FMAYFKKGML	PRGEPFSVYF
101 EKHREQAIML	YNLFYFANDY	DTFYKTACWA	RDR**VNEGMFM**	**YSFSIAVFHR**
151 DDMQGVMLPP	PYEVYPYLFV	DHDVIHMAQK	YWMKNAGSNE	HHSYVIPVNF
201 TLK**NQDQLLA**	**YFTSDVNLNA**	**FNTYYR**YYYP	SWYNTTLYGH	TIDRR**GEQFY**
251 **YTYKQIYAR**Y	FLER**LSNDLP**	**DVYPFYYSKP**	**VK**SAYNPNLR	**YHNGEEMPVR**
301 **PSNLYVTNFD**	**LYYIADIK**NY	EKRVEDAIDF	GYVFDEHVKP	HSLYHDVHGM
351 EYVADMIEGN	MDSPNFYFYG	SIYHMYHSMI	GHIVDPYHK**M**	**GLAPSALEHP**
401 **TVLRDPAFY**	**QLWK**RVDHLF	QKYKNRLPR**Y**	**THDELAFEGV**	**K**VENVDVGK**L**
451 **YTYFEQYDVS**	**LDMSVYVNKV**	**DQIPNVDVHA**	**R**QYR**LNHKPF**	**TYNIEVSSDK**
501 AQDVYVRVFL	GPKYDYLGRE	YDLNDRRHYF	VEMDRFPHHV	EAGKTVIER**N**
551 **SHDSNIVAPE**	**RDSYR**TFYKK	VQEAYEGKSQ	YYVDK**GHNYC**	**GYPENLLIPK**
601 GK**KGGQAYTF**	**YVIVTPYVKQ**	**DEHDFEPYNY**	**KAFSYCGVGS**	**NRK**YPDNMPL
651 GYPFDRK**IYS**	**NDFYTPNMYF**	**K**DVIIFHKKY	DEVGVQGH	

[a]emPAI, the exponentially modified protein abundance index.

Table 4. 2: MASCOT search output of NCBInr with the tandem MS data from the purified ~88 kDa cockroach gel band.

gi: 284518363 **Allergen Cr-PI; AltName: Allergen Per a 3 [*Periplaneta americana*]**

Mass: 82283 Da **Score:** 239 **Matches:** 7 (7) **Sequences:** 7 (7) **emPAI[a]:** 0.50

Peptide match	Score	Expect	Rank	Unique
K.NQDQLLAYFTSDVNLNAFNTYYR.Y	113	5.9e-10	1	U
R.GEQFYYTYK.Q	47	0.0038	1	
K.MGLAPSALEHPETVLR.D	96	3.8e-07	1	U
R.DPAFYQLWK.R	62	0.001	1	
R.YTHDELAFEGVK.V	85	3e-05	1	
K.VENVDVGK.L	57	0.043	1	
R.NSHDSNIVAPER.D	42	0.043	1	U

Percentage sequence coverage: 12%. Matched peptides shown in bold and underlined.

1 MKTALVFAAV	VALVACAAFP	AHKDYKQLAD	KQFLAKQRDV	LRLFHRVHQH
51 NILNDQVEVG	NTYDIEANIG	NYKYPRVVKQ	FMAYFKKGML	PRGEPFSVYF
101 EKHREQAIML	YNLFYFANDY	DTFYKTACWA	RDRVNEGMFM	YSFSIAVFHR
151 DDMQGVMLPP	PYEVYPYLFV	DHDVIHMAQK	YWMKNAGSNE	HHSYVIPVNF
201 TLK**NQDQLLA**	**YFTSDVNLNA**	**FNTYYR**YYYP	SWYNTTLYGH	TIDRR**GEQFY**
251 **YTYK**QIYARY	LYYIADIKNY	EKRVEDAIDF	GYVFDEHVKP	HSLYHDVHGM
301 PSNLYVTNFD	LYYIADIKNY	EKRVEDAIDF	GYVFDEHVKP	HSLYHDVHGM
351 EYVADMIEGN	MDSPNFYFYG	SIYHMYHSMI	GHIVDPYHK**M**	**GLAPSALEHP**
401 **ETVLRDPAFY**	**QLWK**RVDHLF	QKYKNRLPR**Y**	**THDELAFEGV**	**KVENVDVGK**L
451 YTYFEQYDVS	LDMSVYVNKV	DQIPNVDVHA	RQYRLNHKPF	TYNIEVSSDK
501 AQDVYVRVFL	GPKYDYLGRE	YDLNDRRHYF	VEMDRFPHHV	EAGKTVIER**N**

551 **<u>SHDSNIVAPE</u>**	**<u>R</u>**DSYRTFYKK	VQEAYEGKSQ	YYVDKGHNYC	GYPENLLIPK
601 GKKGGQAYTF	YVIVTPYVKQ	DEHDFEPYNY	KAFSYCGVGS	NRKYPDNMPL
651 GYPFDRKIYS	NDFYTPNMYF	KDVIIFHKKY	DEVGVQGH	

[a]emPAI, the exponentially modified protein abundance index.

Reactivity of acid–eluted antibodies and periodate-sensitivity

Figure 4. 3(A) shows the reactivity of 1025Z-derived antibodies, from both ~85 kDa and ~88 kDa ACR molecules, against a variety of antigens in extracts from different life cycle stages of *S. mansoni*. Pooling the eluates of both molecules was considered justifiable since the TMS results indicated that both isoforms were orthologues of the cockroach allergen Per a 3. The eluted anti-85/88 kDa ACR antibodies reacted relatively intensely against both molecules in the ACR extract (Fig. 4. 3(A), lane 1). Likewise, reactivity of anti-85/88 kDa ACR antibodies against SmSEA was also relatively intense (Fig. 4. 3(A), lane 2), as well as against *S. mansoni* worm and cercarial molecules (Figure 4. 3(A), lanes 3 and 4 respectively). Figure 4. 3(B) was treated with sodium meta-periodate treatment to de-glycosylate the proteins. The acid eluted antibodies were carried out in a similar way as the illustration seen in chapter 3 (Figure 3.2).

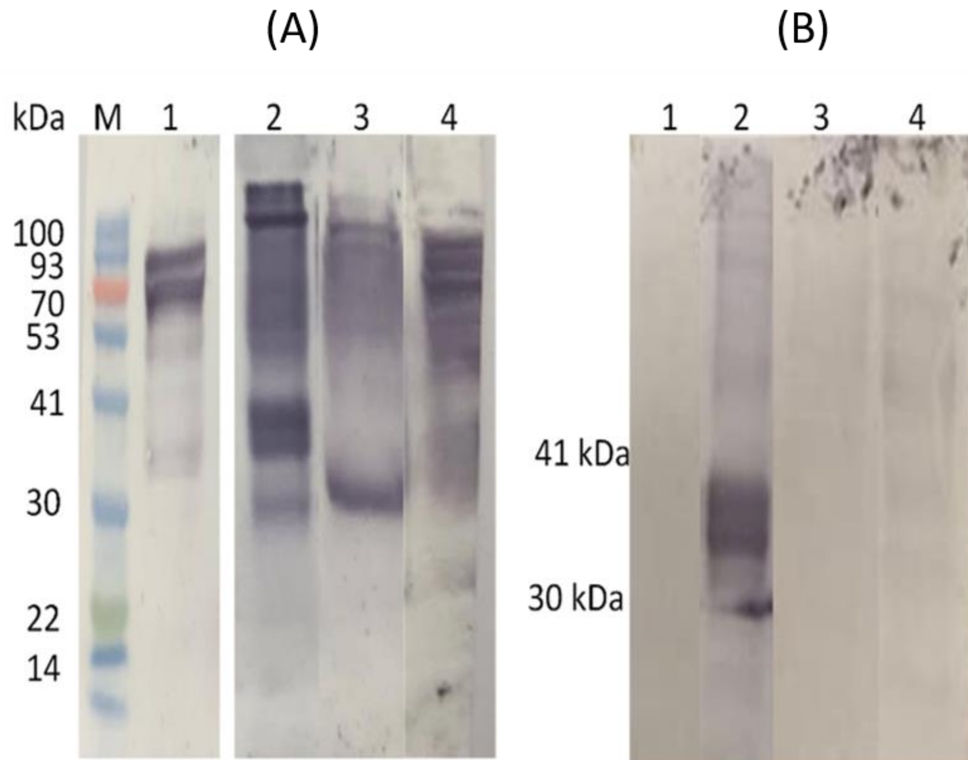


Figure 4. 3: Western immunoblots probed with rabbit anti-85/88 kDa cockroach proteins eluted from immunoblots of cockroach extract probed with anti-SEA antiserum 1025Z. Lane 1, *P australasiae* extract (200µg/lane); 2, *S. mansoni* SEA (20 µg/lane); 3, *S. mansoni* worm homogenate (20 µg/lane); 4, *S. mansoni* cercariae homogenate (20 µg/lane). (B) the same as (A) but after treatment with 10 mM Na-metaperiodate for one hour. M: Molecular weight marker. Three independent experimental repeats were performed.

The amino acid sequences of the egg antigens; IPSE/alpha-1, kappa-5 and omega-1 of *S. mansoni* were obtained from the National Centre for Bioinformatics Information (NCBI) database with accession numbers AAK26170, AAX83114 and ABB73003 respectively. To identify the level of similarity between the three egg antigens of *S. mansoni* and Per a 3/Cr-PI, an alignment was performed using the EMBOSS Needle Pairwise Sequence Alignment (European Molecular Biology Laboratory-

European Bioinformatics Institute). The results are shown in tables 4. 3, 4. 4 and 4. 5. In Tables 4.3, 4.4 and 4.5 the amino acid range (200-134), (401-182) and (351-225) is where similarities are found for Per a 3 and IPSE-alpha-1, Per a 3 and kappa-5 and Per a 3 and Omega-1 respectively.

Table 4. 3: Amino acid sequence alignment of Per a 3 and IPSE/alpha-1 using pair wise alignment tool

```
Length: 706
# Identity:      35/706 ( 5.0%)
# Similarity:    60/706 ( 8.5%)
# Gaps:         590/706 (83.6%)
# Score: 16.5


Pera3                1 MKTALVFAAVVALVACAAFPAHKDYKQLADKQFLAKQRDVLRLFHRVHQH     50
                                                                         
IPSE/alpha-1         1 --------------------------------------------------      0


Pera3               51 NILNDQVEVGNTYDIEANIGNYKYPRVVKQFMAYFKKGMLPRGEPFSVYF    100
                                                                         
IPSE/alpha-1         1 --------------------------------------------------      0


Pera3              101 EKHREQAIMLYNLFYFANDYDTFYKTACWARDRVNEGMFMYSFSIAVFHR    150
                                                                         
IPSE/alpha-1         1 --------------------------------------------------      0


Pera3              151 DDMQGVMLPPPYEVYPYLFVDHDVIHMAQKYWMKNAGSNEHHSYVIPV-N    199
                                                                 .::|.| :
IPSE/alpha-1         1 ------------------------------------------MFLIAVLS      8


Pera3              200 FTLKNQDQLLAYFTSDVNLNAFNTYYRYYYPSWYNTTLYGHTIDRRGEQF    249
                       :||.:|   |...|||              ...|...||..|.:|.    
IPSE/alpha-1         9 YTLISQ---LGITTSD--------------SCKYCLQLYDETYERG----     37


Pera3              250 YYTYKQIYARYFLERLSNDLPDVYPFYYSKPVKSAYNPNLRYHNGEEMPV    299
                         :|.::|                     |.|.| .:|          |. 
IPSE/alpha-1        38 --SYIEVY---------------------KSVGS-LSP----------PW     53


Pera3              300 RPSNLYVTNFD-------LYYIADIKNYEKRVEDAIDFGYVFDEHVKPHS    342
                       .|.::.|...:       .:|:.|..||..|: ..:..|...|:..|  |
IPSE/alpha-1        54 TPGSVCVPFVNDTKRERPYWYLFDNVNYTGRI-TGLGHGTCIDDFTK--S    100


Pera3              343 LYHDVHGMEYVADMIEGNMDSPN----------FYFYGSIYHMYHSMIGH    382
```

```
                          .:..:..::......:|.::..|          |
IPSE/alpha-1      101 GFKGISSIKRCIQTKDGKVECINQPKRRRTYCRF----------------    134

Pera3             383 IVDPYHKMGLAPSALEHPETVLRDPAFYQLWKRVDHLFQKYKNRLPRYTH    432

IPSE/alpha-1      135 --------------------------------------------------    134

Pera3             433 DELAFEGVKVENVDVGKLYTYFEQYDVSLDMSVYVNKVDQIPNVDVHARQ    482

IPSE/alpha-1      135 --------------------------------------------------    134

Pera3             483 YRLNHKPFTYNIEVSSDKAQDVYVRVFLGPKYDYLGREYDLNDRRHYFVE    532

IPSE/alpha-1      135 --------------------------------------------------    134

Pera3             533 MDRFPHHVEAGKTVIERNSHDSNIVAPERDSYRTFYKKVQEAYEGKSQYY    582

IPSE/alpha-1      135 --------------------------------------------------    134

Pera3             583 VDKGHNYCGYPENLLIPKGKKGGQAYTFYVIVTPYVKQDEHDFEPYNYKA    632

IPSE/alpha-1      135 --------------------------------------------------    134

Pera3             633 FSYCGVGSNRKYPDNMPLGYPFDRKIYSNDFYTPNMYFKDVIIFHKKYDE    682

IPSE/alpha-1      135 --------------------------------------------------    134

Pera3             683 VGVQGH    688

IPSE/alpha-1      135 ------    134
```

http://www.ebi.ac.uk/Tools/services/web/toolresult.ebi

Key

(I) Conserved sequences

(:) Semi-conserved mutations

(.) Non-conservative mutations

Table 4. 4: Amino acid sequence alignment of Per a 3 and Kappa- 5 using pair wise alignment tool

```
Length: 748
# Identity:      57/748 (7.6%)
# Similarity:    83/748 (11.1%)
# Gaps:         578/748 (77.3%)
# Score: 27.5

Pera3              1 MKTALVFAAVVALVACAAFPAHKDYKQLADKQFLAKQRDVLRLFHRVHQH     50

Kappa5             1 --------------------------------------------------      0

Pera3             51 NILNDQVEVGNTYDIEANIGNYKYPRVVKQFMAYFKKGMLPRGEPFSVYF    100

Kappa5             1 --------------------------------------------------      0

Pera3            101 EKHREQAIMLYNLFYFANDYDTFYKTACWARDRVNEGMFMYSFSIAVFHR    150

Kappa5             1 --------------------------------------------------      0

Pera3            151 DDMQGVMLPPPYEVYPYLFVDHDVIHMAQKYWMKNAGSNEHHSYVIPVNF    200

Kappa5             1 --------------------------------------------------      0

Pera3            201 TLKNQDQLLAYFTSDVNLNAFNTYYRYYYPSWYNTTLYGHTIDRRGEQFY    250

Kappa5             1 --------------------------------------------------      0

Pera3            251 YTYKQIYARYFLERLSNDLPDVYPFYYSKPVKSAYNPNLRYHNGEEMPVR    300

Kappa5             1 --------------------------------------------------      0

Pera3            301 PSNLYVTNFDLYYIADIKNYEKRVEDAIDFGYVFDEHVKPHSLYHDVHGM    350

Kappa5             1 --------------------------------------------------      0

Pera3            351 EYVADMIEGNMDSPNFYFYGSIYHMYHSMIGHIVDPYHKMGLAPSALEHP    400

Kappa5             1 --------------------------------------------------      0
```

```
Pera3            401 ETVLRDPAFYQLWKRVDHLFQKYKNRLPRYTHD--------ELAFEGVKV    442
                                     |::..|.:..     |.        ..|....|:
Kappa5             1 ----------------DNILHKSEEE-----HQVKWALNYLNAARSTWKL     29

Pera3            443 ENVDVGKLYTYFEQYDVSLDMSVY--VNKVDQIPNV------DVHARQYR    484
                     ||.|:.|           ..||.|  |||. .:||.      ...||.:.
Kappa5            30 ENEDMFK-----------KTMSSYVGVNKT-IVPNFCMTMLQQSSARNWD     67

Pera3            485 LN-HKPFTYNIEVSSDKAQDVYVRVFLGPKYDYL----GREYDLNDRRHY    529
                     .| .|..||..||..              ||..|    .::.||
Kappa5            68 TNIQKDITYGCEVLK--------------KYSELKWGARKKLDL------     97

Pera3            530 FVEMDRFPHHVEAGKTVIERNSHDSNIVAPERDSYRTFYKKVQEAYEGKS    579
                                   .|:...|..|.|                     |:|
Kappa5            98 --------------MTIRWLNGSDEN---------------------GQS    112

Pera3            580 QYYVDKGHNYCGYPENLLIPKGKKGGQAYTFYVIVTPYVKQDEHDFEPYN    629
                     |:..|||.||....|                      |::..:...:.:.
Kappa5           113 QHISDKGFNYHSKKE----------------------YLECAQSVMKIHR    140

Pera3            630 YKAFSYC--GVGSNRKY------PDNMPLGYPFDRKIYSNDFYTPNMYFK    671
                     .||...|  .||...|.      |.:......|| ||..|       :.|
Kappa5           141 TKAEVDCRSTVGEFLKLQQVKDTPSSKTQAIQFD-KINEN-------FNK    182

Pera3            672 DVIIFHKKYDEVGVQGH-------------------------------    688
                     .::...::.:.:.:...
```

Table 4. 5: Amino acid sequence alignment of Per a 3 and Omega- 1 using pair wise alignment tool

```
# Length: 755
# Identity:      43/755 (5.7%)
# Similarity:    78/755 (10.3%)
# Gaps:         597/755 (79.1%)
# Score: 25.5


Pera3                1 MKTALVFAAVVALVACAAFPAHKDYKQLADKQFLAKQRDVLRLFHRVHQH     50


Omega-1              1 --------------------------------------------------      0


Pera3               51 NILNDQVEVGNTYDIEANIGNYKYPRVVKQFMAYFKKGMLPRGEPFSVYF    100


Omega-1              1 --------------------------------------------------      0


Pera3              101 EKHREQAIMLYNLFYFANDYDTFYKTACWARDRVNEGMFMYSFSIAVFHR    150


Omega-1              1 --------------------------------------------------      0


Pera3              151 DDMQGVMLPPPYEVYPYLFVDHDVIHMAQKYWMKNAGSNEHHSYVIPVNF    200


Omega-1              1 --------------------------------------------------      0


Pera3              201 TLKNQDQLLAYFTSDVNLNAFNTYYRYYYPSWYNTTLYGHTIDRRGEQFY    250


Omega-1              1 --------------------------------------------------      0


Pera3              251 YTYKQIYARYFLERLSNDLPDVYPFYYSKPVKSAYNPNLRYHNGEEMPVR    300


Omega-1              1 --------------------------------------------------      0


Pera3              301 PSNLYVTNFDLYYIADIKNYEKRVEDAIDFGYVFDEHVKPHSLYHDVHGM    350


Omega-1              1 --------------------------------------------------      0


Pera3              351 EYVADMIEGNMDSPNFYFYGSIYHMYHSMIGHIVDPYHKMGLAPSALEH-    399
                                .|.:.:..|..||          :|...| :|.:.::.::
Omega-1              1 ---------MMRAQSLCFLVSI----------LVTILH-VGYSQNSWDYY     30
```

```
Pera3            400 ------PETV-----------LRDPAFYQLWKRVDHLFQKYKNRLPRYT-    431
                           |.|.           |||...:.||..:      :.||.|..|
Omega-1           31 VFSVTWPPTYCESIQCRLPRGLRDFTIHGLWPTI------FPNRQPNCTG     74


Pera3            432 ---HDELAFEGVK-------------------------------VENVDV    447
                        .|....:|::                               ||:..|
Omega-1           75 SLRFDIRRLQGIRNELDLMWPHLKNYRESPSFWKHEFEKHGLCAVEDPQV    124


Pera3            448 GKLYTYFEQYDVSLDMSVYVNKVDQIPNVDVH-ARQY------RLNHKPF    490
                     ...|.|| ::.:.|...:.:.|......:..| :|||      .:..:.|
Omega-1          125 FNQYGYF-KFGIQLMQKLNLLKTLMKYRISPHDSRQYDTINLMNVLEREF    173


Pera3            491 TYNIE---VSSDKAQDVY----VRVFLGPKYDYLGREYDLNDRRHYFVEM    533
                     .||..   :.....:.:|    |.|.|..|::::...:..|..:.:.
Omega-1          174 GYNGSANCIRKPGRRGMYHLEEVHVCLNRKHEFMNCPFLGNCPKKFI---    220


Pera3            534 DRFPHHVEAGKTVIERNSHDSNIVAPERDSYRTFYKKVQEAYEGKSQYYV    583
                       ||...
Omega-1          221 --FPPFQ-------------------------------------------    225


Pera3            584 DKGHNYCGYPENLLIPKGKKGGQAYTFYVIVTPYVKQDEHDFEPYNYKAF    633


Omega-1          226 --------------------------------------------------    225


Pera3            634 SYCGVGSNRKYPDNMPLGYPFDRKIYSNDFYTPNMYFKDVIIFHKKYDEV    683


Omega-1          226 --------------------------------------------------    225


Pera3            684 GVQGH    688


Omega-1          226 -----    225
```

Identification of potential N- and O- glycosylation sites in the amino acid sequence of the cockroach allergen

Analysis of the possible involvement of CCDs in the cross-reactivity of *S. mansoni* antigen and the cockroach allergen was done by identifying the potential glycosylation sites in the amino acid sequence of Per a 3 (gi 284518363). The amino acid sequence was screened using the CBS software prediction servers NetNGlyc and NetOGlyc of potential N and O-linked glycosylation sites respectively. Prediction of amino acid sequences suggested that ACR Per a 3 has two N-linked sites (Table 4. 6) and no O-linked sites. Asparagine predicted to be N -glycosylated highlighted in bold on the sequence.

Table 4. 6: Potential N-linked glycosylation sites for Cr-PI/allergen Per a 3.

>Per a3 sequence Length = 688

MKTALVFAAVVALVACAAFPAHKDYKQLADKQFLAKQRDVLRLFHRVHQHNI
LNDQVEVGNTYDIEANIGNYKYPRVVKQFMAYFKKGMLPRGEPFSVYFEKHR
EQAIMLYNLFYFANDYDTFYKTACWARDRVNEGMFMYSFSIAVFHRDDMQGV
MLPPPYEVYPYLFVDHDVIHMAQKYWMKNAGSNEHHSYVIPV**N**FTLKNQDQL
LAYFTSDVNLNAFNTYYRYYYPSWY**N**TTLYGHTIDRRGEQFYYTYKQIYARY
FLERLSNDLPDVYPFYYSKPVKSAYNPNLRYHNGEEMPVRPSNLYVTNFDLY
YIADIKNYEKRVEDAIDFGYVFDEHVKPHSLYHDVHGMEYVADMIEGNMDSP
NFYFYGSIYHMYHSMIGHIVDPYHKMGLAPSALEHPETVLRDPAFYQLWKRV
DHLFQKYKNRLPRYTHDELAFEGVKVENVDVGKLYTYFEQYDVSLDMSVYVN
KVDQIPNVDVHARQYRLNHKPFTYNIEVSSDKAQDVYVRVFLGPKYDYLGRE
YDLNDRRHYFVEMDRFPHHVEAGKTVIERNSHDSNIVAPERDSYRTFYKKVQ
EAYEGKSQYYVDKGHNYCGYPENLLIPKGKKGGQAYTFYVIVTPYVKQDEHD
FEPYNYKAFSYCGVGSNRKYPDNMPLGYPFDRKIYSNDFYTPNMYFKDVIIF
HKKYDEVGVQGH

Position	Residue	Score*	N-Glyc result$
199	**N**FT	0.6672	+
234	**N**TT	0.6939	++

4.3 Discussion

The antigenic cross-reactivity between *S. mansoni* and cockroach was investigated to assess whether there is an immunological relationship shared between the two organisms. Cockroaches have been identified as an important source of indoor allergens worldwide and as the cause of allergies (Do, Zhao & Gao 2016). Cockroach sensitisation has been linked to increase asthma morbidity and about 60 to 80% of inner-city children with asthma are sensitised with cockroach allergens (Sohn & Kim 2012; Togias et al. 2010).

The present work showed cross-reactivity between schistosome antigens and cockroach antigens (Figure 4.1), with four bands between 46 kDa and 100 kDa in the cockroach extract reacting with rabbit anti-SmSEA antibodies. Negative control using serum from rabbits injected with complete Freund's adjuvant showed no reactivity against cockroach (Figure 4.1 lane 3). TMS identified two SmSEA cross-reactive molecules of ~85 kDa and ~88 kDa, the amino acid sequence of both proteins were similar to those found in *P. americana* Cr-PI, Per a 3 allergen (gi: 284518363). The Per a 3 is an allergen of the American cockroach that is the target of IgE antibody response in 26.3% to 94.7% of cockroach allergic patients and belongs to the hemocyanin protein family (Wu et al. 1997).

The American and German cockroaches *(P. americana* and *Blatella germanica*) respectively are the predominant species responsible to cause allergy in humans with many known allergens in *P. americana*

(www.allergen.org) (Do, Zhao & Gao 2016). Cr-PI/Per a 3 is an important allergen in humans, previous studies have demonstrated that partially purified fractions of Cr-PI in which 73% of patients who were sensitive to the crude *P. americana* extracts elicited a skin reaction (Wu & Lan 1988). Based on its amino acid sequence, Per a 3 has four isomers with molecular sizes ranging from 46 to 79 kDa (Wu et al. 1997). There are two major proteins in Cr-PI of the American cockroach with molecular sizes 72 kDa and 78 kDa (Wu & Lan 1988). The amino acid sequence of two recombinant clones encoding the Per a 3 allergen had a molecular weight of 75.5 kDa and 79.3 kDa and each has two potential N-linked glycosylation sites (Wu, Lee & Liao 1996). Likewise, the two allergens in the *P. australian* that we found in our study have an estimated molecular weight of 85 kDa and 88 kDa, which is higher than the one described by Wu *et al* 1988. in *P. americana*. This could be due to differences between the two cockroach species. Interestingly, the number of potential N-linked glycosylation sites (Table 4. 6) that we found concord with the Wu *et al* 1996 study. Although there was no O-linked glycosylation site for Per a 3, according to the TMS the sequence calculated mass was 82.3 kDa. We observed a molecular mass of 85-88 kDa by electrophoresis: the mass difference between our and previous results are therefore not great.

Results revealed that the amino acid sequences of Per a 3 and IPSE/alpha-1 have only 5.0% identity and 8.5% similarity (Table 4. 3), Per a 3 and kappa-5 show 7.6% identity and 11.1% similarity (Table 4. 4) while Per a 3 and omega-1 share 5.7% identity and 10.3% similarity

(Table 4. 5). This suggests that the antigenic cross-reactivity is unlikely to be due to shared peptide sequences.

After treatment with sodium meta-periodate, neither the 85 kDa nor the 88 kDa ACR molecules reacted with the acid-eluted anti-ACR 85/88 kDa antibodies (Fig .4. 3(B), lane 1). Cross-reactivity of these acid-eluted antibodies against adult *S. mansoni* worm and cercarial antibodies was destroyed by periodate treatment (Fig. 4. 3(B), lanes 3 and 4), except for a 30-35 kDa band in SmSEA which remained reactive with these antibodies (Fig. 4. 3(B), lane 2), which is not fully understood, as seen in previous studies (Doenhoff et al. 2016).

The observation of IgG cross-reactivity of *S. mansoni* with the cockroach allergen, Per a 3 in this chapter was significant as the IgG antibodies involved could serve as allergen immunotherapy for IgE reactivity to Per a 3 or other cockroach allergens. Immunotherapy is associated with an increase in allergen-specific IgG antibodies, these antibodies block the binding of the allergen to crosslink the IgE receptor and reduce the allergen-specific IgE antibodies (Flicker et al. 2013; Freidl et al. 2017). The advantages of such immunotherapy is that it will modulate the immune system, reduce IgE-mediated allergy and have a long-lasting effect on the control of allergies.

Chapter 5: Investigation of Recombinant IgE and Phl p 7 allergen with Recombinant IgG (blocking antibody)

Abstract

Our main purpose for the second part of chapter 3 was to use anti-schistosome IgG antibodies that are cross-reactive with *D. farinae* allergen can block IgE mediated allergic reaction using the reporter system. However, this was not carried out so we thought that we could use the reporter system to demonstrate that recombinant IgG antibodies can block IgE that cause allergic reaction.

In this work, we developed an *in vitro* system to show that our approach can be a useful tool to detect allergen-specific immunoglobulin E. Using this system, we have shown that recombinant IgG antibody can block IgE-mediated activation. This system expressed the human FcεRI αγ subunit, transfected stably with nuclear factor activated T cells (NFAT) reporter gene. It uses a highly sensitive detection method to detect allergen-specific IgE. In this work, we have used plasmids that encode human IgE and IgG that are specific for Phl p 7 allergens. The two proteins of IgE and IgG were successfully expressed in mammalian HEK 293 cell line and the harvested IgG protein was purified using Affinity Chromatography ÄKTAstart system. First, we optimised the recombinant IgE using the RS-ATL8 system and we found that 1: 100 was the optimal dilution. Next, we used purified Phl p 7 allergen and anti-Phl p 7 IgE

antibody (1:100) with the recombinant IgG. We observed the strongest decrease in the activation of the basophils at A+ 100 μg/mL and A + 10 μg/mL which was statistically significant. This data is proof that our system can be a useful tool to be able to demonstrate the effect of IgG antibody can block IgE-mediated activation.

5.1 Introduction

Allergen-specific immunotherapy has been used for many years as a desensitizing therapy for allergic diseases and provides a curative and specific treatment method. Allergen-specific immunotherapy induces allergen-specific IgG antibodies. One suggested mechanism for these antibodies in modulating allergic reactions is that it will compete with IgE for allergen binding thus preventing allergic reactions.

Our intention was to establish an *in vitro* system in which these interactions could be studied. To accomplish this, a few important steps are required, such as the choice of a suitable allergen: and a suitable protocol for quantification and blocking analysis *in vitro*. In this chapter, these steps were performed. Firstly, by using recombinant Phl p 7-specific human IgE and IgG antibodies expressed in mammalian HEK293-6E cell line. Secondly, we used Affinity Chromatography (AC) ÄKTAstart system to purify the recombinant IgG protein. Lastly, the novel RS-ATL8 reporter system was used to assess the blocking effect of IgG antibodies.

Mammalian expression systems use well-established cell lines such as HEK293 cells. In mammalian (like any other) cell expression system, four main aspects need to be considered to produce sufficient yields of recombinant proteins. First of all, the mammalian host cell line, secondly the expression vector, thirdly the transfection vehicle and lastly the culture medium (Durocher 2002).

Human embryonic kidney 293 cells, also known as HEK293 cells, are cell lines derived originally from human embryonic kidney tissue cultures, the number 293 came from Graham numbering his experiments. The cells were transformed with adenovirus 5 DNA (Graham et al. 1977). The Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA1) is one of the genetic variant made on HEK293 cells (Durocher 2002). EBNA1 plays an important role during latent viral infection through replication and partitioning of viral genomic DNA thus, EBNA1 binds to specific DNA sites in the viral EBV latent origin of replication (oriP) (Frappier 2012). EBNA1 increases the level of recombinant protein expression by allowing more plasmid copies to persist in the transfected cells (Van Craenenbroeck, Vanhoenacker & Haegeman 2000).

The human cytomegalovirus (CMV) promoter, which is highly active in the host cell line, is an important vector for high yield recombinant protein expression (Foecking & Hofstetter 1986). The promoter is very important in HEK293 cells, where it has been reported that expressed adenovirus E1a can activate CMV promoter (Gorman et al. 1989). When using this, promoter adenovirus expression cassette allows the expression level of recombinant proteins to a level of up to 20% of total cell protein (Massie et al. 1998).

The third aspect is related to the efficacy of the transfection vehicle or reagent. Many effective transfection reagents are available commercially, however, cost-effective reagents are preferable for use in a large-scale expression. The transfection reagent should be effective to use in suspension-growing cells, simple to use and possess minimal

cytotoxic effects in a large-scale transient transfection. Polyethyleneimine (PEI) happens to be one of the transfection reagents that meet all these criteria due to its high gene transfer activity in many cell lines paired with low cytotoxicity (Boussif et al. 1995). HEK293-EBNA1 suspension can be efficiently transfected with PEI also due to its cost-effectiveness (Schlaeger & Christensen 1999). There are two isoforms for this polymer (linear and branched) with a broad range of polydispersities and molecular weights; these properties are important for efficient transfection activity (Durocher 2002).

The last aspect for efficient protein expression is the culture medium. Using a serum-free medium can enhance transfection yield (Hacker, De Jesus & Wurm 2009) and some gene transfer reagents work only with them (Durocher 2002).

5.1.1 Allergen Specific Immunotherapy (IgG blocking antibodies)

Allergen-specific immunotherapy (SIT, also known as allergen immunotherapy, AIT) plays a major role in the treatment of patients with allergies triggered by exposure to environmental allergens such as pollen, mite and food allergens (Orengo et al. 2018). Allergen immunotherapy is administered as a subcutaneous injection (SCIT), starting with a smaller dose of the allergen extracts until a maintenance dose is reached effectively in inducing immunological tolerance to the allergens (Cady et al. 2010). Alternatively, there is also sublingual

immunotherapy (SLIT) which involves an aqueous solution or tablet of the extract containing the allergen. This kind of immunotherapy is safe, simple and convenient in comparison to subcutaneous immunotherapy (SCIT) (Lin et al. 2018). The aim of SIT is to reduce the symptoms caused by allergens and prevent the recurrence of the allergy. However, there are risks associated with SIT, and it can take a longer duration to induce tolerance and allergen administration can cause adverse reactions in patients (Orengo et al. 2018) usually several years. Allergen-specific immunotherapy (SIT) induce allergen-specific regulatory T cells (Treg). Treg cells play a key role in regulating immune responses to allergens through different mechanisms (Hussey Freeland et al. 2016). Treg cells produce the different types of inhibitory cytokines such as Interleukin 10 (IL-10) and Transforming Growth Factor Beta (TGF-β). A study by Tordesillas and co-authors reported that Treg TGF-β production was responsible for protection against anaphylaxis in mice undergoing epicutaneous immunotherapy by suppressing the activation of mast cell (Tordesillas et al. 2017). B regulatory cells also play a role in regulating immune responses by suppressing effector T cells; through the production of anti-inflammatory cytokines such as IL-10, they support the development of immunological tolerance (Rosser & Mauri 2015). Patients allergic to bee venom receiving allergen-specific immunotherapy show an increase in allergen-specific IL-10 producing B cells and IgG4 antibodies (Van De Veen et al. 2013). Successful immunotherapy is associated with an increase in allergen-specific IgG antibodies, particularly IgG4 and a decrease in allergen-specific IgE

antibodies. As stated above, the IgG4 can act as a blocking antibody thereby blocking the interaction of IgE with allergen (Cady et al. 2010). The IgG antibody will be competing with IgE for allergen binding thereby intercepting the allergic response (Orengo et al. 2018).

5.1.2 Immunoglobulin G (IgG)

Immunoglobulin G is the most abundant protein and represents about 10-20% of plasma protein in human serum (Vidarsson, Dekkers & Rispens 2014) and the most common type of antibody found in the circulation. Immunoglobulin G provides protection against viral and bacterial infections and can cross the placenta. IgG antibodies induce responses such as complement-mediated cytotoxicity, antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis (Gudelj, Lauc & Pezer 2018). Immunoglobulin G is divided into four subclasses (IgG1, IgG2, IgG3 and IgG4) based on their abundance in serum (Roy et al. 2018).

5.1.3. The structure of IgG

Immunoglobulin G antibodies are heterotetrameric molecules with an approximate molecular weight of 150 kDa (Table 5.1) and a tetrameric quaternary structure. The immunoglobulin G molecules consist of two identical heavy (H) chains gamma (γ) of 50 kDa and two identical light (L) chain Kappa (k) or Lambda (λ) with a size of 25 kDa, which are linked

together by disulphide bonds (Vidarsson, Dekkers & Rispens 2014). The light chains consist of a variable (VL) and a constant domain (CL). The heavy chain consists of one variable domain (VH) and three constant domains (Cγ1-3), with a hinge region linking (Cγ1) and (Cγ2). The light chain domains together with the VH and Cγ1 domains formed the F_{ab} (fragment antigen binding) see figure 5.1, the lower part of the antibody consisting of the hinge region and the Cγ2/Cγ3 domains is called the F_c (fragment crystalline) (Vidarsson, Dekkers & Rispens 2014). The F_c regions of IgG contain a highly conserved N-glycosylation site at asparagine 297 in the heavy chain constant region (Cobb 2019).

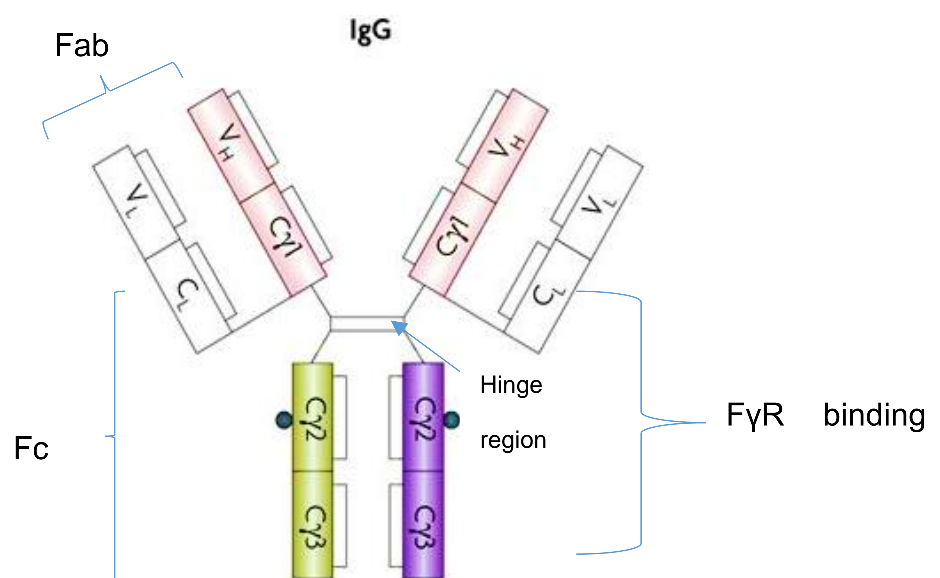


Figure 5. 1: Diagram representation of IgG structure. Diagram from Gould & Sutton (2008).

Immunoglobulin G subclasses have more than 90% amino acid sequence homology. They are differentiated based on the size of the

hinge region, their molecular weight and the number of interchain disulfide bonds. The classes differ also in their ability to activate complement.

Table 5. 1: Representation of IgG structure. Adapted from (Vidarsson, Dekkers & Rispens 2014)

Properties	IgG1	IgG2	IgG3	IgG4
Molecular weight (kDa)	146	146	170	146
Amino acids in hinge region	15	12	62	12
Inter-heavy chain disulphide bonds	2	4	11	2
Mean level in adult serum (g/L)	6.98	3.8	0.51	0.56
Relative abundance (%)	60	32	4	4

5.2 Materials and Methods

5.2.1 Mammalian cell culture

Cell culture work was carried out in a microbiological cabinet class II under sterile conditions.

5.2.2 Maintenance and freezing of HEK293-6E cells

The HEK293-6E cells (National Research Council, Canada) were cultured in 16 mL of Freestyle F17 Expression Medium (ThermoFisher Scientific, UK) supplemented with 2% L-glutamine and 0.1% kolliphor P-

188 (Merck, UK) without G418 (Invitrogen, USA) to a disposable 125 mL Corning® Erlenmeyer flask. Suspension HEK293-6E cells were incubated in a humidified incubator at 37˚C with 5% CO_2 on an orbital shaker plate set at 120 rpm (MaxQ CO_2 Plus Shaker) from Fisher Scientific. The cells were diluted when they reached 1x10^6 cells/mL with 16 mL F17 medium, as the cells established a doubling time, G418 (Invitrogen USA) 25 µg/mL was added. Cells were maintained by diluting them every three days to a density of 0.25 x 10^6.

For freezing of HEK293-6E cells, 10% DMSO in F17 medium as the freezing mixture was added to the harvested cells obtaining a density of 5-50 x 10^6 cells/mL. Aliquots were prepared in cryogenic storage vials to be stored in a Mr Frosty Freezing Container at -80 overnight, the next day aliquots were transferred to liquid nitrogen for long term storage.

5.2.3 Phl p 7 allergen

The Phl p 7 allergen of timothy grass pollen *Phleum pratense* was bought from Alpha Diagnostic International. It was a recombinant protein supplied in a powder form. The protein was reconstituted in Dulbecco's phosphate-buffered saline (DPBS) without Ca^{2+} or Mg^{2+} (Merck, UK) to make a stock solution of 100 µg/mL. The stock solution was then aliquoted into smaller vials and stored at -40˚C until use.

5.2.4 DNA Preparation

pVITRO1-102.1F10-IgE/λ and pVITRO1-102.1F10-IgG1/λ Plasmids encoding the sequence of Phl p 7-specific human IgE and IgG1 antibodies were obtained from AddGene, catalogue numbers 50365 and 50366, respectively. Next, the DNA constructs were amplified in *E. coli* strain XL-10 grown in LB medium + Hygromycin (100 µg/mL). PureYield™ Plasmid Midiprep System (Promega, UK) was used to isolate and purify the plasmids according to the manufacturer's instruction. The purified plasmids were quantified using NanoDrop (ThermoFisher Scientific, UK).

5.2.5 Preparation of Polyethyleneimine (PEI)

A stock solution of 1 mg/mL linear PEI with MW≈25,000 (Polysciences, Inc., USA) was prepared in sterile water, followed by adding HCl to adjust the pH <2.0. A few minutes later, more HCl was added in order to readjust the pH to <2.0, stirred for 2-3 hours at the same pH <2.0 until PEI fully dissolved. NaOH was added to adjust the pH 7.0. The solution was sterile-filtered using a 0.22µm membrane, aliquoted and stored at -20°C.

5.2.6 Mammalian cell expression of IgE and IgG

5.2.6.1 Small scale transfection of HEK 293-6E cells

A small scale transfection of HEK293-6E cell was performed at a density of 1.5 -2.0x10^6 cells/mL at viability of >80%. A cell suspension was distributed in a 6 well-plate (1.8 mL/well). The plate was transferred to an orbital shaker containing 5% CO_2 in a humidified incubator at 37˚C. Prior to transfection, the DNA-PEI complex was prepared at a ratio of (1:2) (w/w). Preparation of DNA solution was made for each well, by adding 100 µL transfection medium to 6 x 1.5 mL Eppendorf tubes, 2 µg of DNA was added onto each tube. A solution of PEI was prepared for the entire 6 well-plate, adding 672 µL of transfection medium to 1.5 mL tube following the addition of 28 µL PEI solution with vortexing. This was followed by adding 100 µL PEI solution to each DNA solution with vortexing immediately, incubated for 3 minutes at room temperature. The culture was removed from the incubator and the DNA-PEI complex mixture was added and swirled. This procedure was carried out for the remaining 5 wells. The plate was returned to the orbital shaker in the incubator. For the secreted proteins, 20% w/v of Tryptone N1 (Tekniscience Inc., Canada) in transfection medium without G418 was added to a final concentration of 0.5% within 24 to 48 hours post-transfection. After 120 hours, the supernatant of secreted proteins was collected and analysed by SDS-PAGE and western blotting.

In this study, we have used two plasmids pVITRO1-102.1F10-IgEλ and pVITRO1-102.1F10-IgG1λ, their features are explained, and plasmid maps shown in Figure 5.2 and 5.4 respectively.

The plasmid encodes a human elongation factor which functions as an enzyme which catalyzes the binding of aminoacyl tRNAs to the ribosome and enhances the level of gene expression in mammalian cell lines. The plasmid encodes a Simian Vacuolating virus 40 (SV40), the origin of replication (Ori) a sequence of DNA enabling the plasmid to reproduce itself. The human cytomegalovirus (CMV) enhancer containing promoter determines the efficiency of viral replication and regulates the level of gene expression. The EM 7 promoter helps in the expression of the antibiotic resistance gene in bacteria and the plasmid contains a gene conferring resistance to hygromycin.

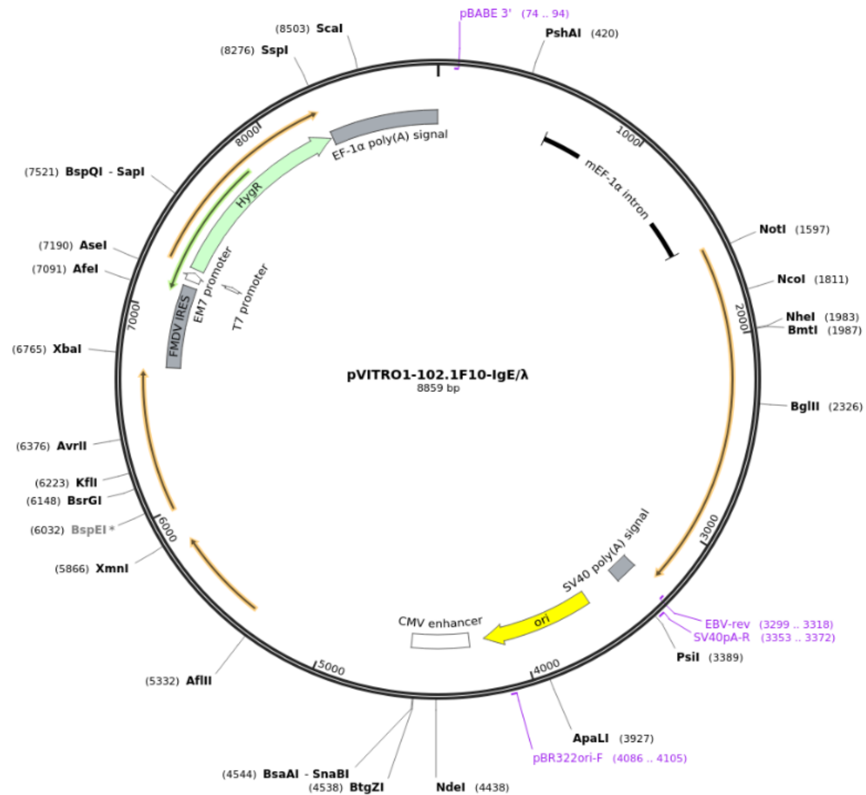


Figure 5. 2: Plasmid map of PVITRO1-102.1F10-IgE/λ

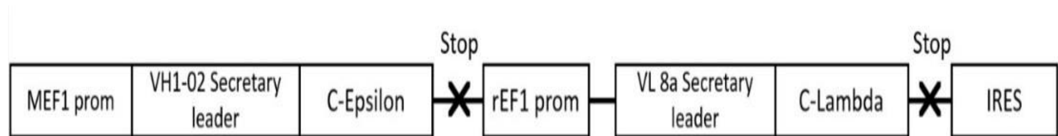


Figure 5. 3: Schematic representation of the expression cassette. The following abbreviations represent: mouse elongation factor 1α promoter (mEF1 prom); rat elongation factor 1α promoter (rEF1 prom); Immunoglobulin variable heavy chain secretary leader (VH1-02 Secretary leader); Immunoglobulin variable light chain secretary leader (VL 8a Secretary leader); stop codon (Stop x); Th constant region of IgE (C-Epsilon).

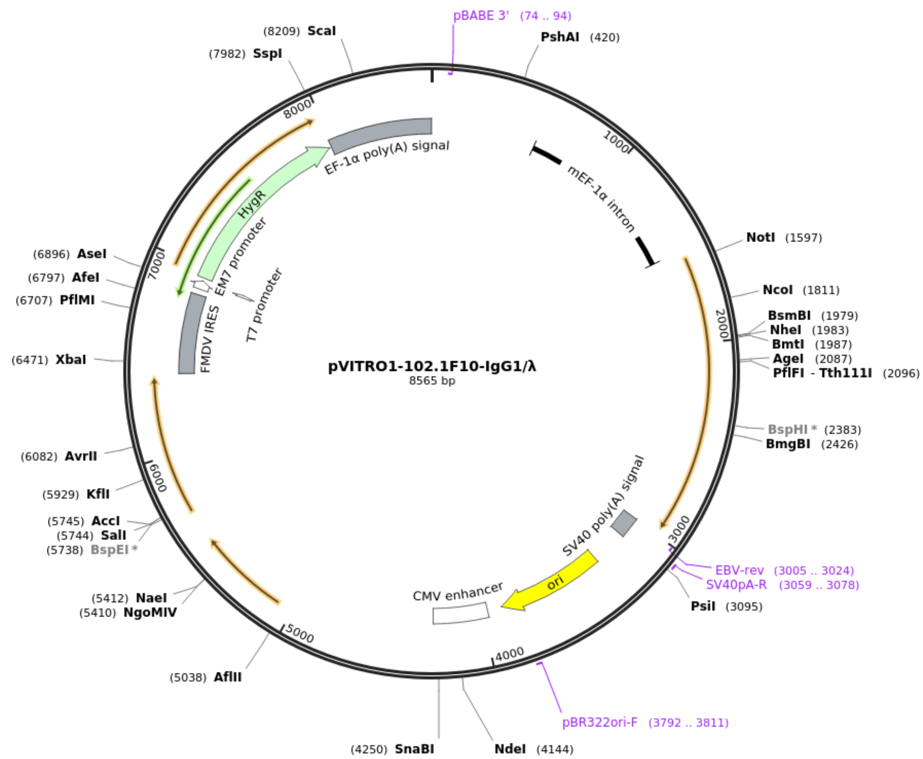


Figure 5. 4: Plasmid map of pVITRO1-102.1F10-IgG1/λ

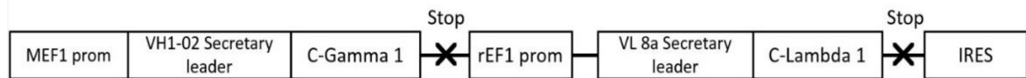


Figure 5. 5: Schematic representation of the expression cassette. The following abbreviations represent: mouse elongation factor 1α promoter (mEF1 prom); rat elongation factor 1α promoter (rEF1 prom); Immunoglobulin variable heavy chain secretary leader (VH1-02 Secretary leader); Immunoglobulin variable light chain secretary leader (VL 8a Secretary leader); stop codon (Stop x); The constant region of IgG (C-Gamma 1).

5.2.6.2 Large-scale transfection of HEK 293-6E cells

Large-scale transfection of HEK293-6E cells suspended in 125 mL F17 medium at a density of 2 x 10^6 cells/mL in a disposable 2 L Corning® Erlenmeyer flask (Merck, USA) on transfection day and cell density was determined using a haemocytometer. The DNA-PEI complex with a ratio of 1:2 (w/w) for 400 mL of the growth culture was prepared as follows: 400 µg of each construct was added to 12.5 mL of pre-warmed F17 medium without G418 in a 15 mL Falcon Conical Centrifuge Tube (Fisher Scientific, USA). 1 µg of each construct was used to transfect 1 mL of HEK293-6E cells. 800 µg PEI were added to 12.5 mL F17 medium in another 15 mL Falcon tube with vortexing. Condensation of the negatively charged DNA with the positively charged polymer was formed, PEI solution was transferred to the DNA solution with vortexing and incubated for 3 minutes at room temperature, the DNA-PEI mixture was added to the cell culture directly and the flask was swirled. The flask was incubated on an orbital shaker set at 120 rpm at 37˚C in a humidified incubator containing 5% CO_2. The supernatant containing secreted proteins after 48 hours was then harvested and centrifuged at 2000 x g for 15 minutes at 4^oC, the supernatant was then filtered using 0.22 µm syringe filters and one protease inhibitor cocktail tablet (Roche, USA) was added to the supernatant in order to prevent protein degradation, and stored at 4^oC.

5.2.7. Affinity Chromatography (AC) using ÄKTAstart system

The ÄKTAstart system (GE Healthcare, USA) was used for the purification of the harvested IgG protein. The supernatant containing recombinant IgG (rIgG) protein was mixed with a 2x binding buffer (40 mM sodium phosphate pH 7.0) at a ratio of 1:1, then loaded onto a 1mL HiTrap protein A sepharose column (GE Healthcare, USA). The flow rate was 1 mL/min. The column was washed with 50 mL wash buffer consisting of 20 mM sodium phosphate at a pH of 7.0. The protein was eluted using an elution buffer containing 0.1 M glycine with pH 2.7. A buffer containing 1M Tris/HCl pH 9.0 was prepared, in which 100 µL was added to the elution fractions so that the final pH of the protein will be neutral. The elution fractions were analysed by SDS-PAGE gel using Coomassie staining.

5.2.8. Protein quantification

After the purification, all elution fractions containing IgG protein were collected and loaded into Amicon® Ultra Centrifugal Filters (MWCO 30 kDa, Merck Millipore, UK) to concentrate the protein samples. Pierce™ BCA protein assay Kit (Thermo Fisher Scientific, UK) was used according to the manufacturer's instruction to quantify the protein concentration.

5.3 Results

5.3.1 Recombinant IgE and IgG protein expression in HEK293-6E cells grown in suspension

The suspension of HEK293-6E cells was used to express the two proteins of IgE and IgG. A pTTo/GFPq vector (National Research Council, Canada) encoding a green fluorescent protein was used to transfect the cells in order to evaluate the transfection efficiency of PEI transfection reagent at a ratio of 1:2 (DNA to PEI). Fluorescence microscopy was used to evaluate the success of transfection after 48 hours. Approximately 70 to 80% of the cells were successfully transfected with the PEI reagent which was a high transfection efficiency (Figure 5.6).

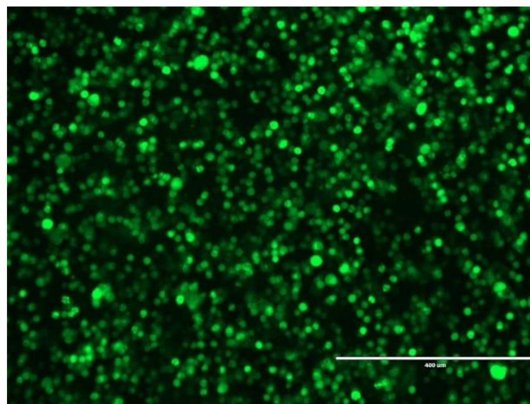


Figure 5. 6: Fluorescence microscopy image was taken 48 hours post-transfection of 293-6E HEK cells with pTTo vector using PEI transfection reagent at a ratio of 1:2 (w/w) (pTTo/GFPq vector to PEI). Straight line is the scale bar (400 um).

For expression of IgE and IgG recombinant proteins, plasmids were transfected into HEK293-6E cells. Five days post-transfection, the harvested proteins were analysed by western blotting using anti-IgE Fc region and anti-IgG Fc region antibodies, respectively. Figure 5.7 shows the successful expression of both IgE (A) and IgG (B), as protein bands of the expected MW were observed. The apparent molecular size of ~150 kDa under non-reducing condition and a ~75 kDa band under reducing condition was seen in Figure 5. 7 (B). This observation is compatible with the molecule of IgG antibodies which has an approximate molecular weight of 150 kDa, as discussed previously.

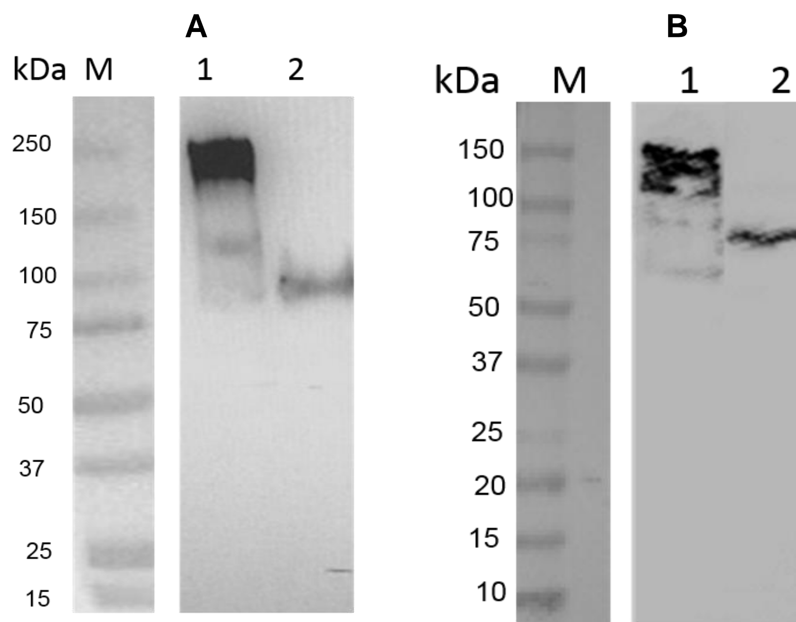
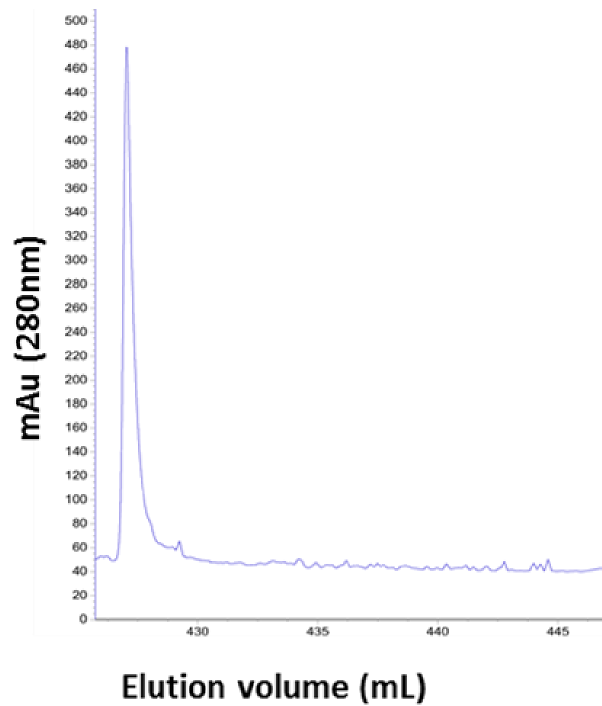


Figure 5. 7: Western blot analysis of expression recombinant IgE and IgG. Expression of recombinant IgE and IgG from transient transfection of 293-6E HEK cells A) M: Molecular weight marker, 1: IgE with ~200 kDa under non-reducing conditions 2: IgE with reducing buffer ~100 kDa. B) M: Molecular weight marker, 1: IgG with non-reducing ~150 kDa, 2: IgG with reducing buffer ~75 kDa.

5.3.2 Large-scale purification of recombinant IgG Protein using AC ÄKTA System

The supernatant containing the harvested IgG protein filtered was with 0.22 µm filter and purified by ÄKTA start using 1ml Histrap protein A column, as indicated by the two graphs in Figure 5.8 which show the peaks of the IgG protein. However, IgE protein was not purify this is due to the expenditure of the column.



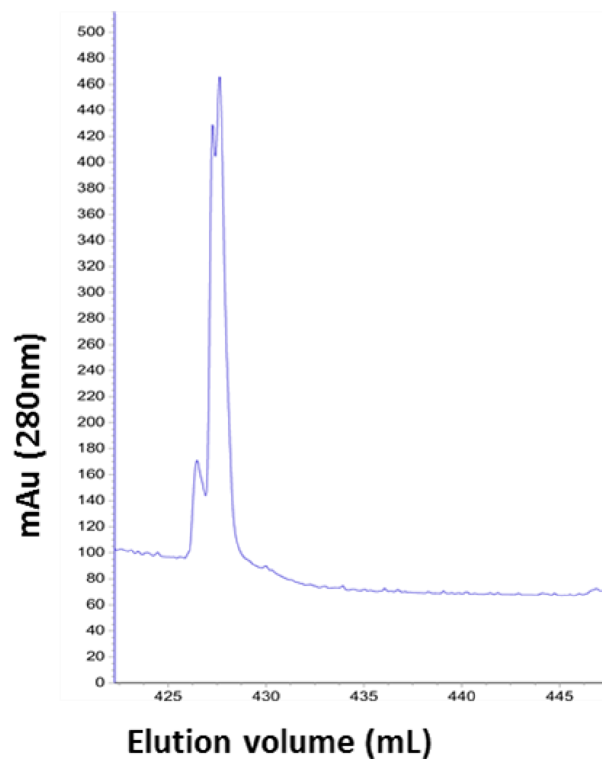


Figure 5. 8: AC purification of recombinant IgG protein. Traces of the IgG protein, consisting of the main peaks between 425-430 mL elution volume.

The present of IgG proteins in the fractions were assessed using absorbance at 280 nm (Figure 5.8). The fractions were collected and

analysed by SDS-PAGE gel stained with Coomassie (Figure 5. 9). The purification was performed in two consecutive days (day 1 and 2) as can be seen in the two gels (Absorbance was read at 280 nm and mAU is the milli Arbitrary Units.

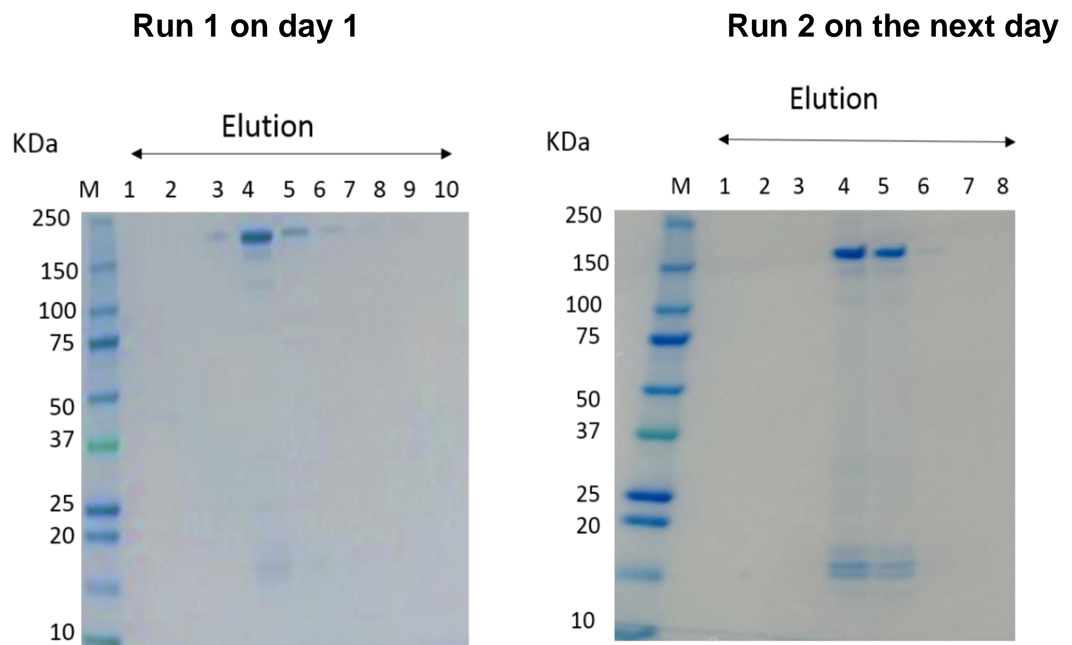


Figure 5. 9: SDS PAGE analysis of the IgG protein eluted fractions. Lane 4 and 5 on the figures correspond to the samples from the main peak of the AC and reveal a pure IgG protein. Samples run on a non-reducing buffer. M: Molecular weight marker.

The elution and wash fractions of IgG protein were collected and analysed by SDS-PAGE gel stained with Coomassie. The result clearly shows a successful purification of IgG protein in the elution fraction

confirmed by the presence of a band at a molecular weight of 150 kDa under non-reducing conditions (Figure 5.10). The eluted fractions of purified recombinant IgG protein were collected and quantified using BCA assay kit. The IgG concentration was 1.88 mg per 1L culture.

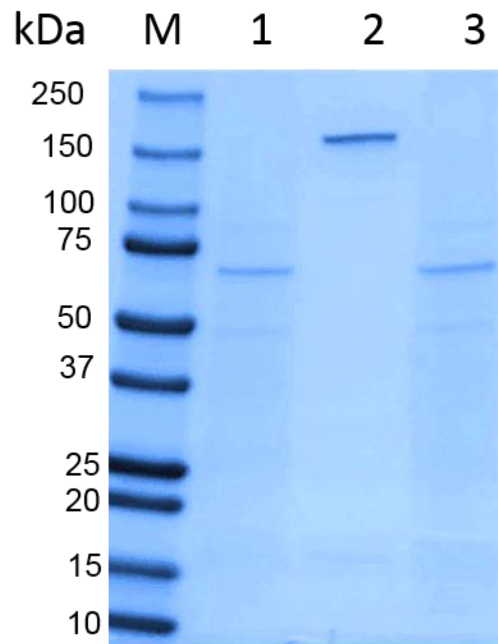


Figure 5. 10: AC Purification of IgG protein. SDS-PAGE gel in Coomassie-stained of AC fractions from the purification of the IgG protein. M: Molecular weight marker 1: Concentrated Supernatant 2: Pooled eluted fractions 3: Wash fraction

5.3.3 Proof-of-principle of Recombinant IgG (rIgG) blocking experiment using the RS-ATL8 cell line

5.3.3.1 Recombinant IgE (rIgE) optimisation

The activation of the reporter cell line RS-ATL8 was tested using different dilutions of recombinant IgE expressed in mammalian HEK293-6E cell line to obtain an optimum condition a bell-shaped curve for IgE mediated activation as in Figure 5.11. One-way ANOVA was used to analysed the data and significance values.

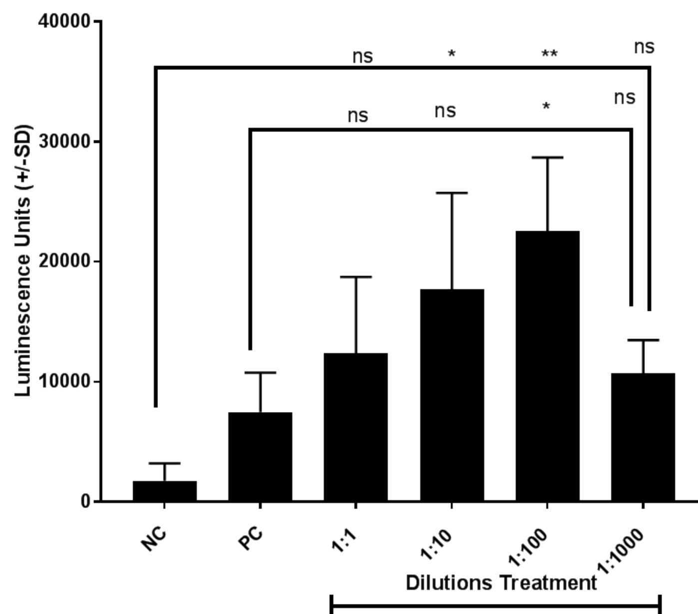


Figure 5. 11: Recombinant IgE optimisation. The RS-ATL8 cells were sensitised with different dilutions of recombinant IgE (1:1 dilution: 1:10 dilution; 1:100 dilution and 1:1000 dilution) for 16 hours overnight incubation, the negative control was cells only and the positive control was recombinant human IgE LAMBDA. The cells were stimulated with Anti- IgE (1μg/mL) for 4 hours. Data represent the mean (± standard deviation SD) of three biological replicates, presented relative to control. Data error bar indicated SD. Abbreviations: (NC) Negative control, (PC) Positive control, (ns) not significant and (*) = 0.0268, (**) = 0.0040, significant $p < 0.05$ was considered statistically significant.

5.3.3.2 Recombinant IgG antibody blocking experiment

The blocking effect of rIgG antibody was tested using the RS-ATL8 reporter assay with the previously optimised rIgE optimum dilution and purified Phl p 7 antigens (Figure 5.12).

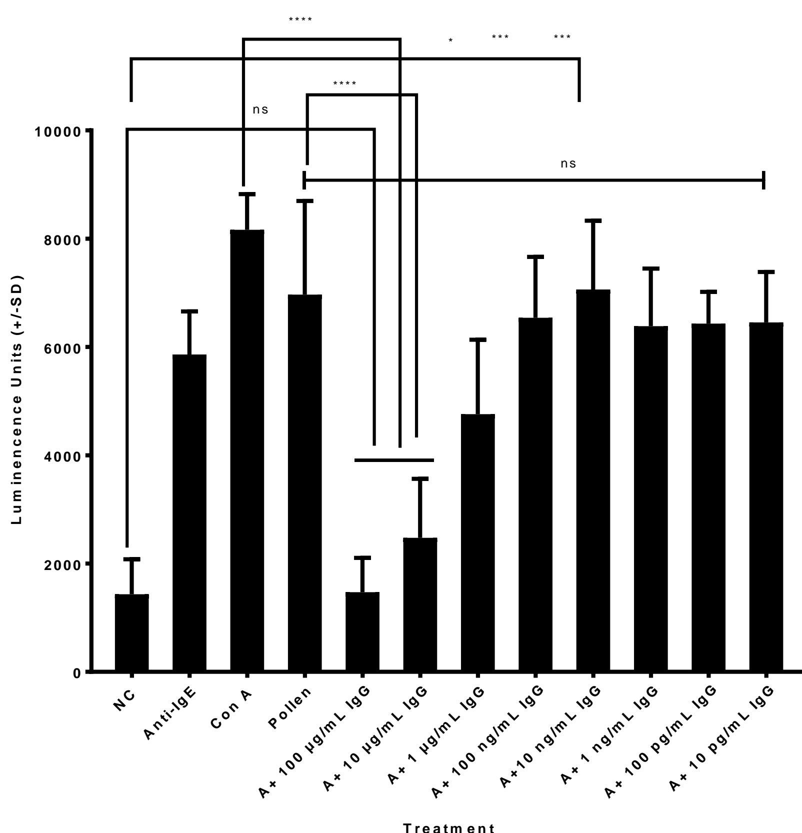


Figure 5. 12: Recombinant IgG and Phl p 7 allergen. The cells were sensitised with the recombinant IgE using the optimal dilution (1/100) for 16 hours overnight in a CO_2 incubator. The negative control and Con A were cells only. Positive control with pollen was sensitised with the recombinant IgE (1/100 dilution). The next day the cells were stimulated with Phl p 7 allergen (2 ug/mL) mixed with Recombinant IgG (200ug/mL), the mixture was 1:1 using the mixer for 35 minutes incubation before adding it to the cells. Positive control with pollen was stimulated with 2 µg/mL of Phl p 7 allergen. Negative and positive control were stimulated with anti-IgE (1 µg/mL) and Con A respectively, all were incubated for 4 hours. The data represent the mean (± standard deviation SD) of three biological replicates, using one-way ANOVA for the analysis of the data and significance values. Abbreviations: (NC) Negative control, (ns) not significant and (*) = 0.0267, (***) = 0.002, (****) = < 0.000, (A) Allergen (2 µg/mL).

In Figure 5.12, we observed a strong decrease in the activation of the basophil when using the highest concentration of the antibody (100 µg/mL to 10 µg/mL). The basophil activation is fully restored as the concentration of the blocking antibody started to decrease from 100 ng/mL to 10 pg /mL was observed. There were three positive controls used, Anti-IgE, Con A and Pollen. There was a high dose response with pollen (Phl p 7 allergen) without the addition of the antibody. Con A gave us the highest dose respond with the RS-ATL8 cells, as this is expected because it binds to the receptor directly. The negative control did not activate the reporter cell line.

5.4 Discussion

The key aim of allergen-specific immunotherapy is to modulate the immunological responses against allergens (Burks et al. 2013). Previous studies have found that immunotherapy may be mediated by allergen-specific IgG, especially IgG4 associated with blocking antibodies that prevent IgE binding to the allergen, inhibiting the activation of mast cells and basophils and thereby reducing the allergic response (Cady et al. 2010; Möbs et al. 2012). IgG1 and IgG4 are among the subclasses of human IgG antibodies. IgG4 antibodies has an average serum concentration of 0.4mg/ml compare to IgG1 which has an average concentration of 8mg/ml (James & Till 2016). IgG4 is the only subtype that does not bind complement and therefore does not lead to inflammation likewise IgG1 activates complement and triggers inflammation (Collins & Jackson 2013). In addition, for IgG4 the cysteine residues in the hinge region formed an intra-heavy chain disulphide bonds as opposed to IgG1 in which inter-heavy chain bonds are present (James & Till 2016), thus IgG4 are dynamic molecules by exchanging half molecule Fragment antigen-binding (Fab) arms with other IgG4 molecules which results in a bispecific antibodies having two different antigen-binding sites, this lead to its anti-inflammatory activity (Rispens et al. 2011). Such antibodies (IgG4) therefore, cannot form immune complexes because they cannot cross-link antigen (Rispens et al. 2011). The Fcγ receptors are protein expressed on the surface of immune cells, they bind to the Fc region of IgG antibodies (Bruhns & Jönsson 2015).

Thus, the activating receptor such as FcγRI has the highest affinity binding for IgG antibodies. However, the inhibitory receptor FcγRIIB is of low-affinity binding and bearing an immunoreceptor tyrosine-based inhibition motif (ITIM) in the intracellular domain, thus IgG4 antibodies are the only subtype that can bind to both FcγRIIB and the activating receptor compared to IgG1 (Nimmerjahn & Ravetch 2008). A study found that cross-linking of FcγRIIb and Fcε receptor 1 blocks IgE mediated basophil activation (Zhu et al. 2002).

However, IgG4 represents up to 4% of the total IgG in healthy individuals, thus it can represent about 75% of total IgG in people undergoing allergen immunotherapy (Eckl-Dorna et al. 2019). The aim of this chapter was to show that recombinant IgG antibodies can block IgE-mediated activation using the humanized Rat Basophilic Leukemia RS-ATL8 reporter system. The RS-ATL8 system is derived from RBL-SX-38 (Wiegand et al. 1996). The RS-ATL8 expressed the human FcεRI αγ subunit (Ali et al. 2019), transfected stably with nuclear factor activated T cells (NFAT) reporter gene such as luciferase for detection of allergen-specific Immunoglobulin (IgE) (Nakamura et al. 2010). The advantages of such a reporter system is that it uses a sensitive detection method in comparison with the traditional biochemical assay, and does not require any sensitivity enhancing agents such as D_2O (Falcone et al. 2015).

In order to do this, we have used plasmids that encode human IgE and IgG that are specific for Phl p 7 allergens. Phl p 7 is a minor allergen found in pollen and is highly cross-reactive. Structurally Phl p 7 is a calcium-binding protein with a molecular mass of 8.6 kDa. IgE antibodies

to Phl p 7 are found in 5 to 10% of individuals with pollen allergy (Sekerkova, Polackova & Striz 2012). Secondly, we expressed IgE and IgG proteins from mammalian HEK293-6E cell lines to produce recombinant proteins that are specific for Phl p 7. Finally, we used Affinity Chromatography (AC) ÄKTAstart system to purify the recombinant IgG protein using 1 ml Hitrap protein A column, doing all of this will enable us to show that the recombinant IgG antibody can block IgE mediated activation using the novel reporter assay.

A 25 kDa linear PEI was used in this study which has a high transfection efficiency in cell suspension and a low cytotoxicity effect (Bragonzi et al. 1999; Tom, Bisson & Durocher 2008). According to many studies, the DNA construct to PEI (1:2) w/w ratio is used as an optimal ratio for transient transfection in suspension growing HEK293-6E cell line (Durocher 2002; Pham et al. 2003; Tom, Bisson & Durocher 2008). We maintained the same ratio of DNA and PEI for both our small and large scales expression. Transfection of HEK293-6E cells with pTTo vector using PEI transfection reagent (pTTo-GFPq vector to PEI) shows a high gene transfer efficacy, approximately 70 to 80% transfected growing suspension cells have shown a green fluorescent protein evaluated with fluorescence microscopy (Figure 5.6). After assessing the transfection efficiency using pTTo-GFPq vector with HEK293-6E cells, a small scale expression screening was performed for both IgG and IgE protein as observed in Figure 5.7 which have shown a successful expression of both proteins. A large-scale transfection of the IgG protein was performed after successfully expressing the protein on a small scale. The IgG

protein were purified using protein A column by affinity chromatography, the graphs in Figure 5.8 shows the peak of the elution fractions of the recombinant IgG (rIgG) protein. The elution fractions of the IgG proteins were collected and analysed by SDS-PAGE gel as in Figure 5.9. This clearly showed a strongly stained bands with the predicted molecular size under non-reducing buffer. The purification was successfully achieved, as the column used contained protein A sepharose which is designed for purification of IgG antibodies from cell supernatant. The protein A has six regions in which five of its regions specifically binds to the Fc region of the IgG.

We proceeded further with the experimental work and the next step was to use the reporter assay and test the blocking effect of the rIgG after a successful purification with Phl p 7 allergen which is the centre of this work. In order to do this first, we needed to do some optimisation condition with the rIgE to obtain the optimal condition for the next stage of the experiment, the reporter cell line RS-ATL8 was sensitised with different dilutions of rIgE overnight incubation and stimulated the next day with anti-human IgE as seen in Figure 5.11. At a 1:1000 dilution there was a strong decrease of the basophil activation but at a 1: 100 dilution a high basophil activation with the reporter assay was observed and was statistically significant. Our result was consistent with previous finding showing that the activation of basophil upon stimulation with anti-IgE antibodies or antigens follows a bell-shaped dose response curve or the cross-linking of Fcε receptor bound IgE on basophil is a bell-shaped curve for the release of cytokines such as IL4 and IL 13 (Gibbs et al.

2006). At a 1:10 dilution, versus the negative control, stimulation was also significant. As expected, the negative control were cells only during sensitisation and stimulated with the medium, so they did not activate the reporter cell line. The positive control was sensitised with recombinant IgE LAMDA and stimulated with anti-IgE, it gave a low respond with the cells, this could probably be that the anti-IgE antibodies that we used to stimulate the cells degraded.

Moreover, we proceeded further to investigate the key aim of this work using the optimum dilution of anti-Phl p 7 IgE antibody (1:100 dilution) and purified Phl p 7 allergen. The cells were sensitised with the rIgE for overnight incubation and stimulated the cells with Phl p 7 allergen mixed with the rIgG together, as illustrated in Figure 5.12 there was a strong decrease in the basophil activation at a A+100 µg/mL IgG and A+10 µg/mL. The explanation for this could be there was blocking of the IgG antibody that took place. As the IgG antibody increased the basophil activation decreases. The antibody blocks the binding of the allergen to crosslink the IgE receptor. This is in agreement with a study by Orengo *et al.,* who used two human monoclonal IgG antibodies (REGN1908-1909) specific for Fel d 1, a major cat allergen to block the allergen Fel d 1 binding to cat allergen-specific polyclonal human IgE, a basophil activation test which uses flow cytometry to measure the expression CD203c on the surface of basophils, one of the activation markers that are upregulated following the cross-linking of IgE antibodies bound to the FcεRI. In this study, REGN1908-1909 antibodies were able to block the allergen-induced basophil activation (Orengo et al. 2018). In the same

study, the same two monoclonal antibodies inhibit Fel d 1–induced mast cell degranulation *in vivo* using passive cutaneous anaphylaxis with either cat hair extract or Fel d 1 in a mouse model. Another study by Flicker and co-authors investigates the passive immunization of mice with allergen-specific IgG antibodies to block allergic reactions in sensitised mice using basophil cell degranulation assay. In this study, mice passively immunized sera were incubated with the different doses of allergens exposed to RBL-2H3 cells and measured the release of β-hexosaminidase. Sera from Phl p 5 sensitised mice before the administration of Phl p 5 specific IgG antibodies were taken. The study found that for a concentration of 0.02 μg/mL and 0.1 μg/mL Phl p 5 allergen there was a median β-hexosaminidase release of 45% and 78% respectively. After a successful passive immunization with the allergen-specific IgG antibodies there was a massive reduction of the RBL degranulation. For a concentration of 0.02 μg/ml of the allergen, a 6% median degranulation was observed and for a concentration of 0.1 μg/ml allergen a 20% median degranulation was found (Flicker et al. 2013).

In this study, the Phl p 7 allergen concentration (2 μg/mL) was maintained throughout the experimental work but the concentration of the IgG was decreasing as can be seen in Figure 5.12. Interestingly we observed an increase in the basophil activation as the concentration of the IgG antibody started to decrease from 1 μg/mL IgG to 10 ng/mL IgG. This is expected as Phl p 7 allergen are highly cross-reactive, and the IgG antibodies used in this study are specific for this allergen. For blocking to occur there must be a shared carbohydrate epitope and then the IgG

antibodies will recognise and bind to the allergen and block the IgE mediated activation. In this case, in which the IgG antibodies decrease, it could be that less likely for sharing of the same epitopes to occur and then the IgG antibodies will not recognise any allergen to bind to and no blocking will take place which increases the activation of the basophils.

The positive control with Con A gave the highest basophil activation in the reporter assay as this is expected, Concanavalin A (Con A) is a lectin extracted from jack beans (*Canavalia ensiformis*). Con A binds to the receptors directly and activates the cells. Con A has been reported to release histamine from human basophil and cross-link IgE bound to high-affinity receptor FcεRI (Siraganian & Siragania 1975).

In this chapter, using such a novel reporter system to show that recombinant IgG antibodies can block IgE mediated activation was significant. The use of such a system that uses a very sensitive detection technique, secondly the robustness of the system which is unaffected by temperature and with low background compared to beta hexosaminidase assay.

Chapter 6: Overall conclusion and future work

The goal of this research can be categorized into two parts; the first part is based on an investigation of the cross-reactivity between *S. mansoni* antigens and various molecules that are known to be allergenic in humans. Chapters three and four fulfill that aim by identifying the cross-reactivity between *S. mansoni* antigens and the allergens. In the second part, we established an *in vitro* system to show that IgG antibodies can inhibit IgE mediated activation using the RS-ATL8 reporter system. This was achieved by the expression of recombinant IgG and IgE proteins, then purification of a recombinant IgG protein and tests of the blocking effect of the recombinant IgG antibody in the reporter assay. This is reported in chapter five.

6.1 Cross-reactivity between *S. mansoni* and allergens

Cross-reactivity between *S. mansoni* antigens and molecules from different allergen extracts which included house dust mite and cockroach was investigated using antibodies in sera from rabbits immunized against different life stages of *S. mansoni.* The antibodies in rabbit anti-*S. mansoni* egg antisera showed reactivity with the molecules in the different allergen extracts. In addition, the rabbit anti-*S. mansoni* soluble egg antigen IgG antibodies that were cross-reactive with the different allergenic extracts were purified by acid-elution from immunoblots and they reacted with immunodominant *S. mansoni* egg antigens, namely IPSE/alpha-1, kappa-5 and omega-1. Sequence alignment of the

allergen cross-reactive molecules and the egg antigens of *S. mansoni* revealed only a low level of peptide similarity/identity, suggesting that cross-reactivity might not be due to peptide sequences common to both *S. mansoni* egg antigens and allergens. However, another option was to investigate whether cross-reactive carbohydrate determinants (CCDs) were responsible for cross-reactivity. With the use of sodium meta-periodate treatment, it was found that the reactivity of the IgG-binding epitopes present on *S. mansoni* antigens and the allergen molecules were destroyed, thus indicating CCD involvement.

CCDs are carbohydrate structures present in many plants, parasites such as helminths and invertebrates (Homann et al., 2017). CCDs generally contain core α1,3 fucose and xylose, structures that constitute common carbohydrate epitopes in plants and invertebrate and which have been involved in IgE cross-reactivity between these organisms (Van Ree 2002; Tretter et al. 1993).

In *S. mansoni,* the egg has been reported to contain more core xylose and core fucose epitopes than extracts from other life stages of the parasite (Faveeuw et al. 2003; Meevissen et al. 2010, 2011). Faveeuw and co-authors revealed that the core xylose and fucose epitopes present in *S. mansoni* egg glycoproteins induce T helper -2 immune responses during infection with the parasites (Faveeuw et al. 2003). Thus, the shared glycan-epitopes that are present in both *S. mansoni* egg antigens and invertebrate allergens could be a factor for their observed antigenic cross-reactivity in this present study.

In this study, two allergens Der f 15 and Per a 3/Cr-PI were identified from house dust mite and cockroach respectively, and identification of potential glycosylation sites in their primary amino acid sequences using software analysis was carried out. The results revealed that Der f 15 has 3 N- and 54 O-linked potential glycosylation sites, while Per a 3 has 3 N- and no O-linked potential glycosylation sites.

The glyco-epitopes involved in the cross-reactivity between invertebrate allergens and *S. mansoni* antigens, particularly the eggs of the latter, may provide an alternative explanation for the hygiene hypothesis; that is that IgG antibodies raised against *S. mansoni* egg antigens that are cross-reactive with allergens could act as blocking antibodies for anti-allergen IgE reactivity, thereby inhibiting allergic sensitisation in allergic individuals. If correct, this may serve as a basis for the development of allergen-specific immunotherapy (SIT). SIT involves administration of initially small doses of allergen extracts until a maintenance dose is reached for the treatment of allergic diseases (Fujita et al. 2012; Orengo et al. 2018). SIT reduces allergy symptoms and prevents allergy recurrence, but frequent administrations of injections to patients can take many years and may cause adverse reactions in some (Valenta et al. 2016). The mechanism by which SIT believed to be effective is that it induces allergen-specific T regulatory cells (Treg), Treg cells regulate immune responses to allergens. They produce anti-inflammatory cytokines such as TGF-β and IL-10 which can suppress mast cell activation (Hussey Freeland et al. 2016; Tordesillas et al. 2017). Allergen immunotherapy is also associated with an increase in allergen-specific

IgG4 antibodies, which play a major role in promoting immune tolerance. Santos and co-authors demonstrated that the ratio of peanut-specific IgG4 to IgE directed peanut allergen was greater in intolerant patients compared to peanut allergy patients (Santos et al. 2015). IgG4 antibodies in sera from birch pollen allergic subjects undergoing birch pollen immunotherapy have been reported to compete with IgE antibodies for pollen allergen-specific epitopes (Groh et al. 2013). This observation was also seen in a peanut-allergic subject undergoing peanut oral immunotherapy, where an increase in IgG4 antibodies and a reduction in IgE binding was described (Vickery et al. 2013). IgG4 can act as a blocking antibody and compete with IgE for allergen binding and thus block the allergen-induced, basophil activation (Orengo et al. 2018).

IgG4 responses are not immune-restricted to peptide epitopes but can also be against carbohydrate epitopes, as demonstrated in a study in which carbohydrate-specific IgG4 antibodies were found in sera from allergic patients undergoing grass pollen immunotherapy (Van Ree & Aalberse 1995). Therefore, infection with schistosomes could possibly protect against allergy, due to *S. mansoni* egg antigens that are antigenically cross-reactive with allergens inducing IgG antibodies that can block the interaction of allergens with specific IgE on the surface of basophils and mast cells. A study has reported that chronic infection with *S. mansoni* can protect mice from allergic airway inflammation (Obieglo et al. 2018). Another study also demonstrated that infection with schistosomes can lower the prevalence and intensity of allergic reactivity to common house dust allergens compared to non-infected individuals

(Resende et al. 2019). However, studies have shown that antihelminthic treatment can increase the risk of atopy (van den Biggelaar et al. 2004; Wammes et al. 2014).

6.2 Observation of complement factor optimisation and mite allergen on the RS-ATL8 reporter system

The aim of work in this chapter was to use anti-*S. mansoni* IgG antibodies that are cross-reactive with mite allergen to block reactivity of allergen-specific IgE using the reporter system. Unfortunately, however, this aim was not achieved. The reason behind this could be that the sera from the two donor's serum used in these experiments might not have had anti-mite IgE specific for Der f 15.

Complement factor optimisation using two different individual donors' sera were assessed in the reporter assay as in the second part of chapter 3. We also assessed the ability of mite antigen to activate the RS-ATL-8 system. Complement removal from serum firstly was essential to maintain the RS-ATL8 cells' viability, and secondly, inactivation of complement proteins can also increase the activation of the cells upon allergen stimulation. As stated by Dibbern and co-authors, complement proteins from human serum used for sensitising the Rat Basophilic Leukaemia were the main toxic factor for the cells (Dibbern et al. 2003). However, to avoid such toxicity there was a need to assess these complement proteins in the two sera; this was performed by heating for 5 mins at 56˚C in a heating block and to compare the product with

unheated samples. The findings revealed that in serum from donor 1(A) after heat treatment dilutions of 1:10 and 1:20 gave us the highest activation of the RS-ATL8 cells, and no activation was observed for untreated serum at the same concentration. This result was significant as it enabled us to know the optimum dilution of serum. In serum from donor 2 (B), a similar experiment was performed, and the optimum dilution was 1:100. The results revealed that the two sera might have little or no IgE specific for mite allergen and thus they were unable to activate the cells.

6.3 Observation on the expression of recombinant IgE and IgG proteins

We have successfully achieved the expression of recombinant IgE and IgG proteins as shown in chapter 5. The two plasmids of IgG and IgE were transfected into embryonic kidney HEK-293-6E cells to get a high yield of recombinant proteins (Thomas & Smart 2005). The IgE and IgG DNA constructs were transfected into kidney cell lines using a cationic polymer polyethyleneimine (PEI) which facilitates the introduction of DNA into cell lines (Longo et al. 2013). The proteins were analysed by western blotting. The findings revealed that the two proteins were successfully expressed as the expected molecular weight was observed.

Purification of recombinant IgG protein using AC ÄKTA system was carried out using Hitrap protein A column. The elution fractions of the purified IgG protein were analyses by SDS-PAGE. The result revealed

a successful purification of the IgG protein with the expected molecular size.

6.4 Observations of the effect of recombinant IgG blocking antibodies using the reporter system (RS-ATL8)

In chapter 5 we demonstrate that IgG antibodies can block IgE-mediated activation using the reporter system. Firstly, optimisation of the recombinant IgE was necessary to enable us to know the optimum dilution. A bell-shaped curve indicated that a 1:100 dilution of IgE was the optimum. We proceeded using this optimum dilution of IgE and Phl p 7 as the allergen and tested the blocking effect of recombinant IgG antibodies. The findings revealed that upon cell stimulation with the allergen and the IgG antibodies, there was a strong decrease with the reporter system at a A+100 µg/mL and A+10 µg/mL this was statistically significant as A (allergen). The effectiveness of IgG as a blocking antibody was thus observed in this work. The blocking ability of IgG antibodies as immunotherapy for allergies has been observed in many studies especially IgG4 antibodies. However, there are two possible mechanisms by which immediate hypersensitivity reactions can be inhibited: firstly by sequestering antigen, thus preventing the cross-linking of receptor-bound IgE, and secondly, as the low-affinity receptor for IgG (FcγRIIB) contains an immunoreceptor tyrosine-based inhibition motif (ITIM) on the surface of allergic cells blocking antibodies may inhibit mast cell and basophil activation *in vitro* (James & Till 2016; Malbec et

al. 1998). Orengo and co-authors have thus shown that monoclonal IgG antibodies were able to block allergen-induced basophil activation (Orengo et al. 2018).

6.5 Future work

This research work has achieved aims expressed in the first parts of chapter 3, 4 and 5, though not in the second part of chapter 3 in which we were not able to demonstrate that anti-schistosome IgG antibodies which were cross-reactive with the mite allergen could block anti-allergen IgE reactivity using the reporter assay. Further work is thus needed to test if the acid-eluted anti-*S. mansoni* IgG antibodies which are cross-reactive with allergens can block allergen-induced IgE antibodies. Secondly, screen lots of sera from patients allergic to HDM (Der f 15) in hospital setting. Thirdly, to investigate whether IgG antibodies from individuals who have undergone successful immunotherapy to prevent allergy have antibodies that react against schistosome antigens. If so egg or other antigens from *S. mansoni* might be useful for allergen-specific immunotherapy.

In chapter 5, further work is necessary to purify the recombinant IgE antibody and measure the concentration of the antibody. It will also be ideal to used pollen extract and patient's sera allergic to pollen using the reporter system.

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Appendices

Table 1: volumes of reagent required for 2 x 0.8mm thick 12% resolving gels in BIORAD mini system

Stock solution/Regeants	Volume (mL)
1.5m Tris HCL, pH 8.8	2.5
10% SDS	0.10
30% Acrylamide	3.9
Water	3.5
10%APS	0.05
TEMED	0.005

Table 2: volumes of reagent required for 2 x 0.8mm thick stacking gels in BIORAD mini system

	Volume (mL)
Stock solution	4.50%
0.5m Tris pH 6.8	1.25
10% SDS	0.050
30% Acrylamide	0.75
Water	2.95
10%APS	0.025
TEMED	0.0025

10x Running Buffer recipe for 1 litre

1. 30 g Tris (Tris Ultrapure, Invitrogen, # 15504-020, Lot 1222263).
2. 144 g Glycine (#G8898-1kg, Lot: 055K0188).
3. 10 g SDS (Lauryl Sulfate, # L-4509, Lot: 102K0203).
4. Distilled water.

a. Ingredients dissolved in ~700 mL distilled water

b. Stir to dissolve

c. Add distilled water to final volume of 1 litre

d. Store at room temperature

e. Dilution used 1 in 10

1.5 M Tris HCl pH 8.8 @ 25°C, 100ml

 Tris Ultrapure (Invitrogen, # 15504-020, Lot 1222263).

1. Dissolve 18.17 g in 80 mL distilled water.
2. Cool to RT for adjusting pH (pH of Tris is temperature dependent).
3. Use specific pH electrode for use with Tris.
4. Adjust to pH 8.8 with concentrated HCl.
5. Adjust final volume to 100 mL with distilled water.
6. Autoclave, store at room temperature.

0.5 M Tris HCl, pH 6.8 @ 25°C

 Tris Ultrapure (Invitrogen, # 15504-020, Lot 1222263).

1. Dissolve 3.0 g in 80 mL distilled water.
2. Put in oven to room temperature for adjusting pH (pH of Tris is temperature dependent).
3. Use specific pH electrode for use with Tris.
4. Adjust pH 6.8 with concentrated HCl.
5. Adjust final volume to 100 mL with distilled water.
6. Autoclave, store at room temperature.

10% SDS, 50 mL (Lauryl Sulfate, Sigma, # L-4509, Lot: 102K0203)

1. Dissolve 5 g SDS (wear face mask) in ~40 mL distilled water.

2. Stir to dissolve, takes a long time (best overnight or heat to around 68°C).

3. Add distilled water to final volume of 50 mL.

4. Don’t autoclave, store at room temperature.

10% Ammonium persulphate (APS)

- BDH Chemicals Ltd Poole, UK, #27195, Lot 5797970B (general purpose reagent).
- Catalyses polymerisation of acrylamide gels.
- Prepare 1 mL.
- Aliquot in 100 μL and store at -20°C.

10x Transfer buffer for1 litre

250 mM Tris base 30 g

1.92 mM glycine 144 g

Top up with ddH_2O to 1 litre

1x Transfer buffer for 1 litre

100 mL from 10x transfer buffer

100 ml methanol 10%

Top up with ddH_2O to 1 litre

Adjust pH to 8.3

5 mL 20% SDS

10x TBS for 1 litre

500 mM Tris base 60.00 g

1.5 M NaCl 87.66 g

Top up with ddH2O to 1l

Adjust pH to 7.5

TBST – 1 litre

100 mL from 10 x TBS

500 µL of 0.5% Tween20

Top up with ddH_2O to 1litre

Blocking buffer – 50 mL

2.5 g skimmed milk powder

50 mL TBST

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