PHAGE DISPLAY SELECTION OF RECOMBINANT ANTIBODIES DERIVED FROM A CHICKEN IMMUNE LIBRARY AGAINST CRYOPRESERVED *EIMERIA TENELLA* SPOROZOITES

A Dissertation

by

DAAD ALI ABI GHANEM

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2006

Major Subject: Poultry Science

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ABSTRACT

Phage Display Selection of Recombinant Antibodies Derived from a Chicken Immune Library against Cryopreserved *Eimeria tenella* Sporozoites. (August 2006)

Daad Ali Abi Ghanem, B.S.; M.S., American University of Beirut, Lebanon

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An antibody library against *Eimeria tenella* sporozoites was constructed by phage display. Total RNA was isolated from the spleen, bone marrow, and ceca of immune chickens, and was used to reverse-transcribe cDNA. Heavy and light antibody variable genes were amplified from cDNA by the Polymerase Chain Reaction (PCR), using primer pairs that contain complementary sequences encoding a short linker sequence. The single-chain antibody fragment (scFv) was obtained by a secondary overlap PCR with primers that incorporate *Sfi*I restriction sites, thus allowing for subsequent cloning into the phagemid vector pComb3X. Vector and scFv insert were digested with *Sfi*I, ligated, and transformed into competent XL1-Blue *Escherichia coli* cells by electroporation, yielding a library with 7.4×10^7 total transformants. The culture was grown under carbenicillin selective pressure, rescued with helper phage, and the antibody-displaying phage was precipitated by PEG/NaCl, and subsequently used for panning. Five panning rounds were performed using cryopreserved *E. tenella* sporozoites, with a gradual increase of washing stringency to select for specific, high-affinity binders. A 1000-fold increase in phage output was obtained after 3 rounds of

panning. There was clear enrichment of the positive clones over the panning rounds, with the 3rd round resulting in a 3,000-fold enrichment over the first one, as the binding clones became the dominant population in the library. Selected antibodies from the last round of panning were sequenced and characterized by immunoblotting. Soluble antibody fragments were produced in a non-suppressor *E. coli* strain, and recognized a 66-KDa sporozoite antigen on a Western blot.

Primary cultures of chicken enterocytes were prepared in the hope of serving for invasion assays with *E. tenella* sporozoites. The isolation procedure, however, proved to be cumbersome and time-consuming.

Future investigations will focus on purification and further characterization of antibodies selected from the constructed library. Such antibodies can be tested, alone or in combination, for their ability to block in vitro the invasion mechanism of *E. tenella*.

DEDICATION

To my mom, Najah Barakat, for her grace, courage, and passion;

To my dad, Ali Abi Ghanem, for his generous heart, and for believing in his little girl;

To my sister, Roula Abi Ghanem, for being my best friend, my staunchest ally and the wind beneath my wings.

With love.

ACKNOWLEDGMENTS

> We pass through the present with our eyes blindfolded. We are permitted merely to sense and guess at what we are actually experiencing. Only later when the cloth is untied can we glance at the past and find out what we have experienced and what meaning it has.
>
> —— Milan Kundera, Laughable Loves

When I look back at the past five years, I feel that I have indeed been blindfolded for a good part of the journey. Now that the "cloth is untied", I can say: Wow, I did it! Not on my own, however. So many things happened, countless laughs, countless tears, all dissolved in a blur. Many people embarked with me on this journey, some abandoned the ship at some time, but many stuck with this sailor until we hit land, and to those I am eternally grateful.

First and foremost, I want to thank Dr. Luc R. Berghman for simply being - and this is not just another cliché - the best advisor any student could hope for; for being my mentor and my friend; for his amazing patience and constant encouragement and support; for generously sharing his knowledge and his ideas; for believing without any doubt that I could do this.

I owe a lot to Dr. Suryakant Waghela who gave me my first crash course in molecular biology, and showed me how to learn from my mistakes. Dr. David J. Caldwell was always ready to help. He generously provided the *Eimeria tenella* stocks used in this study, as well as his lab resources to carry out the immunization and oocyst

production protocols. I am also thankful to Dr. Guan Zhu for his valuable comments and insights.

My gratitude goes to Dr. Nancy Ing and Dr. Suresh Pillai, who kindly allowed me access to their respective labs at all times; Dr. Jo-Ann Fleming who was always available to answer technical questions; Dana Dean and Roula Mouneimne at the Image Analysis Lab for their expert help in microscopy. Many thanks are also due to the members of Dr. Caldwell's lab, current and past: Sid Anderson, Keith Ameiss, Scott Stevens, Phelue Anderson, and Neal Eckert, for their instrumental help with the immunization and oocyst production protocols.

I am thankful to Cindy Balog, who graciously put up with my endless questions and requests, and was always next door to lend an ear and a shoulder. In the past year, I had the good fortune of having Freddy Koenig around, not only for his excellent technical skills, but also for his moral support, and for laughing at and with me.

I am forever indebted to Carmen Aroztegui, Azza Al-Zein, and Archana Thiagarajan, my sisters in combat, for helping me keep my sanity; for being my safety net, my home away from home; for putting up with my -ok I admit it- crazy moods, and for always being "just a teardrop away". Heartfelt thanks are also due to Nahum Puebla for being a great colleague and friend; Michela Gentile, for being the voice of reason; Amitava Sinharay for being such a gentleman; Marcelo Aguiar for all the delicious pizzas and the Harry Potter discussions and insights; Ziad, George and Kamal Naufal for being the funniest band of brothers; Bassel Timani for being such a good listener; and Jamie Haseley for her encouragement and support at a most difficult time.

I am extremely blessed for being able to share my life with Nadine Kabengi, Tania Ghandour, and Samer El-Kadi, and I want to thank them for a decade of friendship, for knowing the ebbs and flows of my soul, and for not being bored with me after all this time.

My eternal gratitude goes to Rami Zurayk for his ever-strong friendship, for being so supportive, and so close no matter how far; Maya Abou Jaoude for her amazing spirit, and for bringing out the best in me; Farshad Tami for constantly renewing my faith in humanity; and Jens Niemann for being my friend all those years, and for believing in me.

"Gratitude is the memory of the heart", said Jean-Baptiste Massieu. And my heart is filled of memories of my incredible, loving family, who is my rock and the backbone of my life. I thank them for their inexhaustible, unconditional support; for believing I could fly on my own; for being with me every step of the way. My mom and dad are my heroes, and I am humbled by their love, a love beyond any measure or comprehension. One of the hardest things I had to cope with was being away from my sister and brothers and missing out on their lives. I now realize this has only brought us closer, and no words can describe how proud I am of them, and how much I owe them: Roula for being no less than an angel; Firas for his eternal optimism, his belief that change is always possible, and for constantly challenging me to be the person I could be; and Rami for his kindness, wit, intellect, and for an incredible, always much needed sense of humor.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

The advent of phage display technology has revolutionized the world of monoclonal antibody production and paved the way for innovations hitherto unheard of. The strength of the technology lies in the simple, elegant fact that a peptide displayed on the surface of a filamentous page is physically linked to the very DNA that encodes it. This linkage provides for replicability and mutability of the peptide in question. Since the early 1990's, phage display has been harnessed to mimic the B-cell selection and maturation strategies, i.e. to carry out the immune response in vitro (Winter et al., 1994). Recombinant antibodies could be produced in a test tube, so to speak, by making use of the *Escherichia coli* machinery for protein expression. Huge libraries (up to 10^{12} total independent transformants), naïve, immune, synthetic, or semi-synthetic could be constructed and used to select antibodies against almost any fathomable antigen, including non immunogenic molecules, toxins, and antigens conserved between species (Hoogenboom and Chames, 2000).

Eimeria tenella is one of the most pathogenic species causing coccidiosis, a devastating disease that inflicts huge economic losses on the worldwide poultry industry. *Eimeria* species invade and multiply in the avian intestinal tract, by means of a mechanism that is yet to be completely elucidated, but that is known to imvolve the sophisticated apical complex located at the anterior end of invasive life stages.

This dissertation follows the style of Immunity.

The objective of this project was to construct an antibody library against *E. tenella* sporozoites, and to select specific antibodies that potentially could be tested for inhibition of invasion of intestinal cells by *E. tenella* sporozoites. The chicken B-cell immune system, with its single heavy and light variable functional domains, makes it possible to amplify the whole B-cell repertoire using a single pair of primers designed around conserved regions flanking these variable segments. The library described in our study was constructed using tissues from immune animals, and selected against cryopreserved *E. tenella* sporozoites. Panning on whole cells offered the advantage of isolating antibodies against native epitopes, but required readily available sporozoites for every round of selection. To that end, we explored the possibility of cryopreservation of *E. tenella* sporozoites, the production of which on a daily basis is otherwise practically impossible. Enrichment of the library with specific clones was evident after 3 rounds of panning, and antibodies recognizing a 66-KDa sporozoite antigen were isolated.

To provide for an "ideal" invasion assay for *E. tenella* sporozoites, we hypothesized that the use of the natural host cells would be a logical choice. We then resorted to isolating primary chicken enterocytes, a process, although eventually successful, proved to be cumbersome, time-consuming, and less user-friendly than invasion assays already in place, which use established cell lines.

Future investigations will focus on purification and further characterization of antibodies selected from the constructed library. Such antibodies can be tested, alone or in combination, for their ability to block in vitro the invasion mechanism of *E. tenella.*

Development of the chicken B-cell immune system

The bursa of Fabricius provides an ideal microenvironment for the development and differentiation of the chicken humoral immune system. It arises around day 4 of embryogenesis as an invagination of the cloaca (Moore and Owen, 1966). By 4 weeks posthatch, the bursa contains 10,000 lymphoid follicles, each of which harbors 10^5 lymphocytes (Olah and Glick, 1978). The bursa thus represents the main site for B-cell proliferation and repertoire formation. Prebursal stem cells are derived from hematopoietic precursors in the dorsal aorta (Arakawa and Buerstedde, 2004). On day 5 or 6 of embryogenesis, the first Diversity-Joining (DJ) segments in the heavy chain locus can be detected in the yolk sac. Unlike mice and humans, chickens possess one functional V_H gene and one functional V_L gene, and chicken immunoglobulin gene rearrangement occurs only during a brief period of embryonic development (McCormack et al., 1991). Recombination of variable (V) segments with (D)J segments at either the heavy or the light chain locus is essentially complete by embryonic day 15 (Reynaud et al., 1994). Cells that have already completed V(D)J recombination at both loci can be detected in the blood, spleen, and thymus. These progenitor populations only expand in the bursa and decline with time at the other sites (Arakawa and Buerstedde, 2004). Immunogobulin gene remodeling in the bursa occurs mainly through gene conversion, and, at a low frequency, through somatic hypermutation (Arakawa and Buerstedde, 2004). Even in the absence of environmental antigen stimulation, gene conversion in bursal B-cells is initiated around embryonic day 15 (Mansikka and Toivanen, 1991), and most likely continues until the bursa involutes 4–6 months after

hatching. Repeated rounds of such gene conversion events lead to the development of a highly diverse repertoire in the bursal follicles (Arakawa et al., 2002). These events involve recombination with a pool of donor non-functional pseudogenes that are clustered upstream of the functional gene (Yamanaka et al., 1996). Twenty-five pseudo V_L genes and 80 pseudo V_H genes have been identified. These contain sequences similar to the functional V_H or V_L gene, but lack other functional elements, including the promoter, leader exon, and the V(D)J recombination sequences (Arakawa and Buerstedde, 2004). Not only avian species, but also rabbits, cattle, swine, and horses use gene conversion for B-cell repertoire formation (Butler, 1998). In the adult bird, antigen stimulation results in the generation of splenic germinal centers. In the early germinal center stage, immunoglobulin genes of B-cells are diversified both by gene conversion and somatic hypermutation. In the later stage, however, gene conversion events are down-regulated and the immune repertoire is mostly generated through somatic hypermutation (Arakawa et al., 1998). Conserved nucleotide regions flanking the functional V_H or V_L genes make it possible to amplify by PCR the complete spectrum of rearranged variable gene fragments, using a single pair of primers designed around these conserved regions (Sapats et al., 2003). Immunization of chickens may be a useful way of producing antibodies against conserved mammalian epitopes that cannot be obtained by immunizing rabbits or mice due to immune tolerance (Andris-Widhopf et al., 2000).

Eimeria tenella

Introduction

Coccidiosis, the most economically important poultry disease, is caused by members of the protozoan *Eimeria* species. Protozoa are made up of a single eukaryotic cell, have a complex life cycle and a sophisticated mechanism of invasion and maintenance in host cells. Coccidia belong to the family of Eimeriidiae, and the phylum Apicomplexa (Levine, 1982). All apicomplexan members are obligate intracellular parasites, and are responsible for serious human and animal diseases such as malaria, toxoplasmosis, and cryptosporidiosis (Augustine, 2001). The name of the phylum derives from the presence of an apical complex, a sophisticated apparatus located at the anterior end of the invasive life-cycle stages (Levine, 1970). This complex includes an actin-myosin contractile system and membrane-bound organelles (Augustine, 2001), and facilitates the entry of the parasites into their host cells (Soldati et al., 2001). The largest group of apicomplexan organisms is the coccidia, which contain some of the most advanced sporozoa (Cox, 1994). An Eimerian species (*Eimeria stiedai*) was one of the first protists ever visualized by Antoni van Leeuwenhoek in rabbit bile in 1674 (Levine, 1988). *Eimeria* parasites have a homoxenous life cycle. They invade and multiply in the avian intestinal tract, causing tissue damage that results in blood loss, dehydration, interruption of nutrient absorption, and increased susceptibility to other pathogens, such as *Clostridium perfringens* and *Salmonella Typhimurium* species (McDougald and Reid, 1991). Coccidia are the most important parasites of poultry, in terms of distribution, frequency, and economic losses (McDougald and Reid, 1997), and coccidia-free

commercial chicken flocks are extremely rare (Williams, 1999). The main reason for this widespread occurrence of coccidia is their extreme fecundity or reproductive ability. Within 7–12 days of ingestion, each oocyst may give rise to hundreds of thousands of infective oocysts in the feces (Ruff, 1999). Movement of personnel and equipment facilitate transmission of parasites from one farm to another, and even new farms will have the parasite present within a few weeks after poultry are introduced (McDougald and Reid, 1997). Although mortality from coccidiosis is under control by anticoccidial medications, coccidiosis is expected to cost the worldwide poultry industry around USD 2400 million annually (Shirley et al., 2005). These costs are mainly attributed to decreased weight gain, poor feed conversion, and to the cost of prophylaxis and treatment (Vermeulen et al., 2001).

Species and pathogenicity

Seven *Eimeria* species have been described in avian species: *E. tenella*, *E. acervulina, E. maxima, E. brunetti, E. necatrix, E. mitis, and E. praecox* (Allen and Fetterer, 2002). Little or no cross-protection is observed against heterologous species, and even against different strains of the same species (Martin et al., 1997). Species can be differentiated based on oocyst size, site of infection, prepatent period, clinical signs, and morphology of intracellular stages (Allen and Fetterer, 2002). All species produce weight loss, increased feed conversion ratios, loss of skin pigment, and decreased egg production (Ruff, 1999). In intensively reared poultry, infections with *E. tenella, E. acervulina, and E. maxima* are frequent (McDougald et al., 1997). Pathogenicity varies considerably between the different *Eimeria* species, and is assessed by the negative

effects of the infection on the birds' performance. Accordingly, *E. tenella and E. necatrix* are the most pathogenic, and ingestion of a large number of oocysts from either species is devastating. A number of factors determine the severity of a coccidial infection: pathogenicity and fecundity of the species, age and number of ingested oocysts, and age, susceptibility, and genetic background of the host. Young birds are less susceptible, and if infected, the damage caused is limited (Voeten et al., 1988). The genetic background of the host has a direct effect on the proliferation of parasites, severity of lesions, and effects on weight gain (Long, 1970). No specific resistance genes have been identified. It is more likely that resistance is acquired by inheritance of traits from diverse breeds (Lillehoj and Ruff, 1987). The course and outcome of infection also depends on the presence of concurrent infections with pathogens such as Newcastle Disease Virus, Reovirus, Marek's Disease Virus, Infectious Bronchitis Virus, *Clostridium perfringens*, and *Escherichia coli*. In such cases, symptoms are aggravated (Ruff, 1993), especially when combined with mediocre management practices such as poor hygiene, high stocking density, and high litter moisture (Graat et al., 1998).

Host and site specificity

Eimeria species exhibit stringent host specificity for development, but not for invasion. As such, the ability of *Eimeria* parasites to develop and cause infection (as measured by their ability to complete their life cycle) is almost solely dependent on the host species (Augustine, 2000). On the other hand, *Eimeria* parasites can invade intestinal cells in a number of avian and mammalian hosts, as well as a variety of cells in culture (Doran, 1982). Most *Eimeria* species are site-specific, and invade narrowly

defined areas within the host intestine (Joyner, 1982). Furthermore, *Eimeria* species have a predilection for specific cell types. For example, *E. brunetti* and *E. praecox* develop at the site of invasion (the villus tip), whereas others such as *E. tenella* are transported via intestinal intraepithelial lymphocytes to the crypt epithelium where they undergo development (Allen and Fetterer, 2002; Jeurissen et al., 1996). This site specificity for invasion occurs not only in the natural host but also in foreign hosts (Augustine and Danforth, 1990). The mechanism underlying this site selection for invasion is not well understood, and it is not clear whether it is determined primarily by the sporozoite, the host cell, or both (Augustine, 2001). Farr and Doran (1962) attributed it to the length of time required for the sporozoite, the invasive life stage of the parasite, to emerge from the oocyst, with species that require the longest time to excyst (such as *E. tenella*) invading the lowest sites in the intestine. Thus, site specificity for invasion by each species would be determined before invasion takes place (Shiotani et al., 1992). Properties of the invasion site itself play a role in the development of site specificity (Jeurissen et al., 1996). Unique conditions in the intestinal lumen such as pH, enzyme makeup, mucus, cell metabolites may also determine site specificity for invasion by each species (Augustine, 2001). Other authors have attributed site specificity to common epitopes between sporozoites and host cells. Vervelde et al. (1993) described the detection of common molecules on both the cecal epithelium and sporozoites by a monoclonal antibody directed against *E. tenella* sporozoites. The authors proposed that these epitopes might serve as recognition molecules for invasion. In apicomplexa-host cell interactions, it has been proposed that attachment is mediated by lectin-ligand

binding, and that these interactions may play a role in site specificity for invasion by *Eimeria* sporozoites (Crane and Dvorak, 1982). Host cell membrane glycoconjugates have been identified as possible host receptors for invasion, and could also be potential receptors for parasitic molecules other than lectins (Augustine, 2001). Microneme proteins of *Eimeria* and other apicomplexan parasites contain conserved regions that may act as attachment points between the parasite and the host cell (Tomley et al., 1997). Host cell receptor molecules may be only one of many forces in the attachment and internalization of *Eimeria* parasites (Augustine, 2001). Soluble products of intestinal cells may act upon the host cell, sporozoite, or both, thereby increasing the host-parasite attraction and/or the permissiveness of host cells for invasion (Augustine and Jenkins, 1998).

Life cycle

Eimeria parasites must complete their development within their host cells before these are sloughed off. Hence, the length of the life cycle of all *Eimeria* parasites (7-10 days) appears to run in parallel with the time needed for intestinal cell regeneration (5-7 days) (Vermeulen, 2004). The life cycle of *Eimeria tenella* is short (7 days) and self-limiting (Figure 1.1). It is divided into an exogenous (sporogony) phase and an endogenous phase. The former occurs in the litter or feces, while the latter takes place in the epithelial intestinal cells and is divided into merogony (two discrete asexual cycles), and gamogony (one sexual cycle). Infection begins when oocysts are ingested by a susceptible bird (Augustine, 2000). An oocyst is the resistant ingested form of the parasite and is surrounded by a thick wall that encloses four sporocysts, each of which

harbors two sporozoites (the initial invasive stages). The oocyst wall is crushed in the gizzard, and sporozoites are released by the action of bile salts and chymotrypsin in the intestine (Day 1 of infection). On day 2, sporozoites rapidly migrate from the intestinal lumen, make contact with and propel themselves into their specific epithelial host cells, where they undergo multiple fission events and mature into schizonts I, thus completing the first asexual cycle. Schizonts I rupture the intestinal cells and release Merozoites I, which in turn invade intestinal cells and mature into second-generation schizonts (Day 4). The maximum damage in *E. tenella* infection occurs when schizonts II rupture to release Merozoites II (Day 5). Merozoites II differentiate into either large macrogametes or small motile microgametes. On day 6 (sexual cycle), microgametes seek out and fertilize mature macrogametes, resulting in the formation of a diploid zygote. Soon after fertilization, a membrane forms around the zygote. Three cytoplasmic membrane-forming bodies then migrate toward and fuse with the cell membrane, forming the oocyst wall. The oocyst is released from the intestinal mucosa and is shed in the feces. With suitable environmental conditions (oxygen level, moisture level, and temperature), the life cycle ends on Day 7 with sporulation: the diploid zygote undergoes one round of meiosis, and two rounds of mitosis, resulting in the formation of 4 sporocysts, each containing 2 genetically identical haploid sporozoites. The sporulated oocsyt is then ready to start another infective cycle (McDougald and Reid, 1991; Shirley et al., 2005).

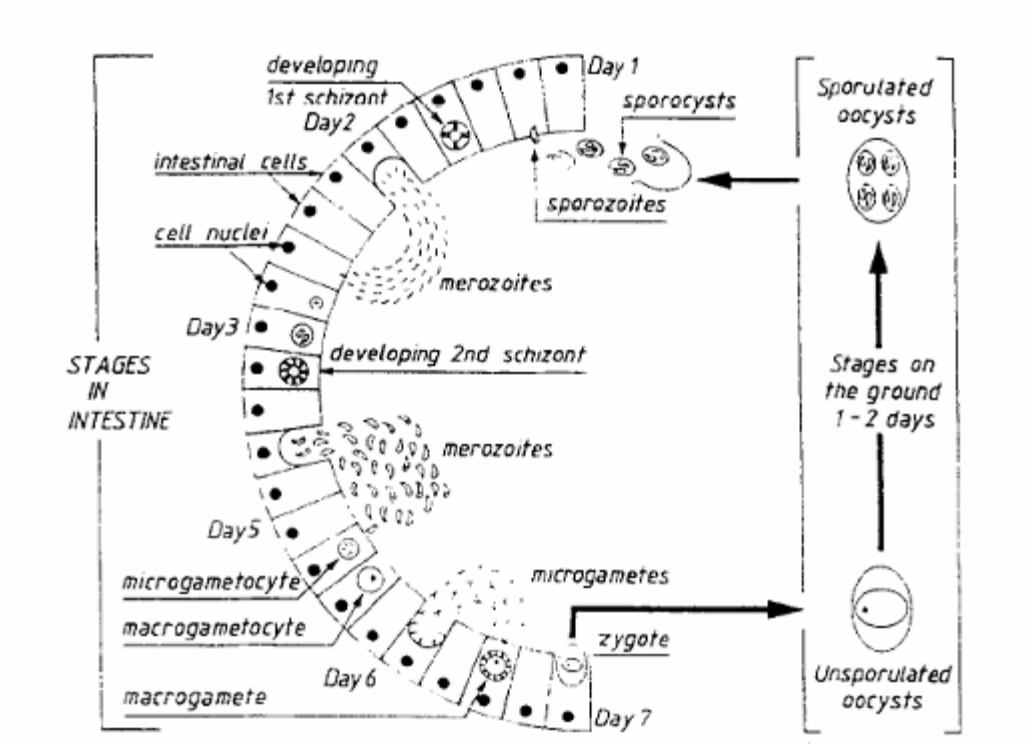

Figure 1.1. Life cycle of *Eimeria tenella* (Jeurissen et al., 1996).

Genome

The nuclear genome of *E. tenella* is comprised of 14 chromosomes, ranging in size from 1 to more than 7 Mbp. The total size of the genome is estimated to be 60 Mbp (just 1/20th the size of the chicken genome), with a GC content of around 53% (Shirley, 2000). Repeats of the trinucleotide sequence GCA and its complement TGC are abundant within all chromosomes. Repeats of the telomeric-like sequence TTTAGGG are also abundant and present within each chromosome (Shirley, 1994). Extra-chromosomal DNA is present within the mitochondrial and the plastid genomes. The latter is an AT-rich, 35 Kbp molecule, of a similar size to its counterparts in *Toxoplasma gondii* and *Plasmodium falciparum* (Cai et al., 2003). Double-stranded RNA segments

have been identified in *E. maxima*, *E. necatrix*, and laboratory strains of *E. acervulina*, *E. brunetti*, *E. maxima*, and *E. necatrix*, but not *E. tenella* (Ellis and Revets, 1990; Lee et al., 1996). The role of these RNA segments is still unknown. Within an *E. tenella* sporocyst, the two sporozoites are identical (Shirley and Harvey, 1996). Similar to other apicomplexans, meiosis in *E. tenella* provides a means to achieve individual variation through crossing over and randomization of the parental contributions to the diploid nucleus (Shirley, 2000).

Mechanism of invasion

Like other apicomplexan species, *Eimeria* parasites must encounter, attach to, and rapidly invade their host cells (Bumstead and Tomley, 2000). During invasion, cellular internalization of sporozoites occurs within an hour of inoculation, and requires active participation on the part of the parasites (Russell and Sinden, 1981). Invasion is a multi-step process and is known to occur via the parasite's apical complex. Characterization of invasion has proved to be a colossal undertaking partly due to (1) the obligate intracellular nature of *Eimeria* parasites, (2) the common biochemical processes between sporozoites and host cells, and (3) the fact that invasion is a quick process that occurs in the complex environment of the host's intestine (Augustine, 2001). The invasion process involves complex interactions between parasite and host, and is mediated by products released from the parasite when it comes in contact with the host cell, and by components of the parasite's apical complex. The initial contact between the parasite and the host (Augustine, 2001) cell appears to trigger a recognition signal that launches the whole process (Dubremetz et al., 1998). Like with other

apicomplexans, invasion is fully driven by the parasite, and hence wholly relies on the gliding ability of the parasite. Motility, and therefore invasion is very limited if a suitable substrate with which the sporozoite can interact is not available (Doran, 1982). Host cell entry usually takes about 5 to 10 seconds, and is initiated by contact between the apex of the parasite and the cell surface. This is immediately followed by progressive internalization at the site of apical contact and formation of a parasitophorous vacuole. Proceeding from anterior to posterior, the parasite is then translocated into the vacuole, which eventually closes behind the parasite (Dubremetz et al., 1998; Morrissette and Sibley, 2002). During invasion, an invagination in the host cell forms at the zone of attachment of the parasite. This highly specialized parasite-host cell interface is called a moving junction and is characterized by a thickened host cell membrane and a constriction in the parasite body (Aikawa et al., 1978). The parasite enters the nascent vacuole by capping the moving junction down its body (Figure 1.2).

Host cell invasion by *Eimeria* motile stages has been attributed to the successive exocytosis of three of the apical organelles: micronemes, rhoptries, and dense granules (Dubremetz et al., 1998). Micronemes are involved in host cell recognition, binding, and motility, while rhoptries are needed for the formation of the intracellular vacuole in which the parasite moves. Dense granules function in remodeling the vacuole into a metabolically active compartment suitable for further parasite development (Dubremetz et al., 1998). Micronemes (a name meaning small and thread-like) appear as oblong structures that cluster in the anterior region of the parasite. Their abundance is correlated with how far and how rapidly parasites glide over culture cells or glass slides

(Tomley and Soldati, 2001). The first evidence that microneme proteins are crucial ligands for binding to host cells came from studies on *Plasmodium* merozoites which were recognized by antibodies that interfered with parasite adhesion to erythrocytes (Miller et al., 1988). Micronemes are conserved in all apicomplexans (Bumstead and Tomley, 2000), and several micronemal genes have been identified by sequence homology (Tomley and Soldati, 2001). Many of these proteins contain adhesive domains that are homologous to ones from higher eukaryotes. Adhesins from the micronemes are translocated along the parasite and are shed at the moving junction. Members of the thrombospondin-related adhesive protein (TRAP) family are localized to micronemes, are apically secreted, and are capped backwards during invasion (Carruthers et al. 1999; Morrissette and Sibley, 2002). Seven micronemal proteins (EtMIC1-7) have been identified in *Eimeria* species. Ryan et al. (2000) have shown that expression of five of these proteins (EtMIC1-5) is regulated both at the transcriptional and translational level. Once a sporozoite makes contact with a host cell, a signal transduced from the surface causes microneme exocytosis, apical adherence to the host cell, and formation of a moving junction. Rhoptries are then exocytosed, while the moving junction glides backwards, and the forming vacuole starts expanding (Dubremetz et al., 1998). Micronemal proteins expand on the sporozoite surface, bind to host cell ligands through their extracellular adhesive domain, while their short cytoplasmic tail establishes a linkage to the parasite's actinomyosin motor (Naitza et al., 1998). Microneme proteins are capped behind the moving junction, and are eventually pinched off at the posterior end of the parasite, thus sealing the vacuole (Dubremetz et

al., 1998). This movement is powered by the parasite's contractile actin-myosin system, as actin-disrupting drugs and myosin inhibitors disrupt motility and invasion (Morrissette and Sibley, 2002).

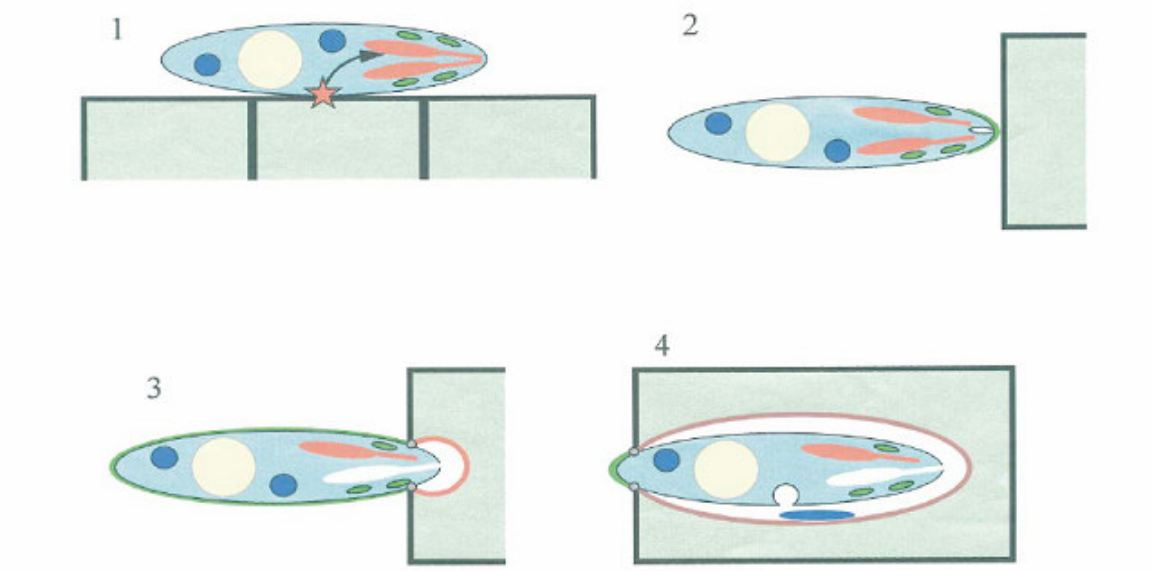

Figure 1.2. Invasion mechanism of *Eimeria tenella* (Dubremetz et al.,1998).

Immunity

Immunity to coccidial infection ranges from innate to solid active immunity acquired as a result of infection (Augustine and Danforth, 1990). Sporozoites are the coccidial life stage that is most amenable to control by the host's immune system. Immunity hampers sporozoite development: sporozoites are unable to reach the crypt epithelium, and those that do are unable to complete their development (Vervelde et al.,

1996). There are three stages where the host immune system can inhibit the development of *Eimeria* sporozoites: (1) When the sporozoite invades and binds to the intestinal epithelium, (2) inside the epithelium amongst the intra-epithelial lymphocytes, and (3) during its passage through the lamina propria to the crypt epithelium. Afterwards, immune cells will have no chance of encountering the sporozoites (Jeurissen et al., 1996). Passive protection of chicks by maternally transmitted antibodies (Wallach et al., 1992) and monoclonal antibodies (Crane et al., 1988; Wallach et al., 1990) has been reported. Antibodies have also been shown to prevent cell invasion by sporozoites in vitro (Larsen et al., 1991).

Much of the knowledge on the immunobiology of avian *Eimeria* species has been acquired from work on *E. vermiformis* infections in mice with targeted gene disruptions at different immune loci (Allen and Fetterer, 2002). In mice, IFN-producing, TCR$\alpha\beta^+$ $CD4^+$ MHC-II-restricted T-cells are essential components of the primary immune response. Minor but essential roles are played by B-cells and interleukin-6. Requirements for a successful immune response to a secondary infection are less stringent, but still absolutely depend on TCR$\alpha\beta^+$ T-cells (Smith and Hayday, 1998). In avian species, immune responses to *Eimeria* are very complex and involve both humoral and cellular compartments (Lillehoj and Lillehoj, 2000). Ingested *E. tenella* oocysts excyst in the bird's intestine and release invasive sporozoites. The gut-associated lymphoid tissues (GALT) constitute the primary line of defense against infection (Lillehoj and Lillehoj, 2000). Barriers to infection in the GALT include non-specific (gastric secretions, competitive exclusion by the normal flora) and specific (lymphocytes

and antibodies) components (Kruzel et al., 1998). In the GALT, antigens are transported into the M cells, where they encounter subepithelial macrophages present within the M cell pocket (Ermak et al., 1990). Macrophages process and present the antigens to the T-cells (mostly $CD4^+$) present as well in the M cell pocket (Yun et al., 2000). Although parasite-specific IgM, IgY, and IgA antibodies are produced (Girard et al., 1997), their ability to limit infection is thought to be minimal, since agammaglobulinemic chickens are resistant to reinfection (Lillehoj, 1987). Secretory IgA (sIgA) in the gut may attach to the sporozoite surface and prevent its binding to the epithelial cell (Yun et al., 2000). Shortly after invasion, sporozoites are seen within intestinal intraepithelial lymphocytes, primarily $CD8^+$ T-cells and macrophages (Trout and Lillehoj, 1996). During primary infections with *E. tenella*, these cells are believed to be the ones that transport sporozoites (Lawn and Rose, 1982) through the lamina propria to the crypt epithelium, where they exit and invade the crypt cells (Allen and Fetterer, 2002). Depletion studies, in which chickens are treated with T-cell suppressors, have shown that $CD4^+$ T-cells of the GALT are the important effectors in primary *E. tenella* infections (Trout and Lillehoj, 1996). *E. tenella* merozoites have also been found in goblet cells and mast cells (Daszak et al., 1993). Populations of cells with natural killer cell activity increase early during primary infection, and may thus be involved in immune surveillance (Chai and Lillehoj, 1988). Metabolites of nitric oxide, a product of activated macrophages which is known to be toxic to bacteria and some parasites, have significantly elevated levels during primary infection with *E. tenella* (Allen, 1997). Depletion studies also showed that secondary infection with *E. tenella* elicits a $CD8^+$ T-cell response.

Following challenge, these cells are frequently seen in close contact with infected cells, where they theoretically function as cytotoxic cells (Lillehoj and Trout, 1994). Macrophages are also activated during challenge responses, and, with T-lymphocytes, are the source of various cytokines (Allen and Fetterer, 2002). The main cytokines produced during primary and secondary infections are IFN-γ and TNF-α. IFN-γ is produced at the site of infection (Martin et al., 1994). Treatment of *Eimeria*-infected chickens with recombinant IFN-γ significantly hindered intracellular parasite development and improved weight gains (Lillehoj and Choi, 1998). TNF-α is an inflammatory cytokine produced by activated macrophages, and may play a role in the pathophysiology of coccidial infections (Allen and Fetterer, 2002). *Eimeria* species differ in their immunogenicity, with *E. tenella* and *E. necatrix* being the least immunogenic. Although it has been shown that the earlier asexual life cycle stages are the most important for eliciting a protective immune response, the identification of protective *Eimeria* antigens has proven to be an elusive task (Shirley et al., 2005). This search is made even more difficult by the complexity of the chicken immune system (Williams, 2002).

Control

The ubiquity and reproductive potential of coccidian parasites, along with intensive rearing conditions make it very difficult to keep chickens free of coccidia (Allen and Fetterer, 2002; Williams, 2002). Given the fact that a natural infection with *Eimeria* confers protection against a homologous challenge, vaccination with live

oocysts has become common practice in the poultry industry. Live, wild-type vaccines have been available for 50 years, and continue to be used extensively. Live attenuated vaccines contain precocious parasites, so called because of their abbreviated endogenous life cycles (Shirley et al., 2005). The loss of one or two generations of schizogony reduces the parasites' prepatent period as well as their reproductive potential, and hence makes them less virulent (Williams, 2002). The immunogenicity of these precocious lines, however, remains similar to their wild-type parents. Precocious lines of all fowl *Eimeria* species are nowadays available, and are present in most of the live attenuated vaccines such as Livacox® and Paracox®. All live vaccines include oocysts of *E. acervulina*, *E. maxima*, and *E. tenella*. In addition, vaccines intended for use in layer birds have to include *E. necatrix* as well, which can be a major cause of coccidiosis at the onset of egg production (Shirley et al., 2005). Live vaccines administered to chickens during their first week of life produce a low-level infection. Subsequent cycling of the parasites through the litter confers immunity against future field challenges (Vermeulen et al., 2001).

Prophylactic control of *Eimeria* infections relies on anticoccidial drugs that have been in use since the 1940s. These drugs are divided into two groups: (1) chemicals with specific mode of action against parasite metabolism, such as amprolium, a thiamine antagonist, and (2) ionophores such as monensin, which alter cellular ion transport and disrupt osmotic balance (Allen and Fetterer, 2002). Extensive and prolonged use of anticoccidial drugs has led to the emergence of *Eimeria* strains that are resistant to the drugs, despite the use of shuttle and rotation programs (Jeurissen et al., 1996). In

addition, concerns about the presence of drug residues in poultry products, increased consumer awareness and desire to ban drugs from animal feeds, have pushed for a move from chemotherapy to vaccination (Williams, 2002). However, the costs of vaccination and its possible adverse effects on performance, as well as the need for timely onset of immunity in short-lived birds, have left broiler growers reluctant to use anticoccidial vaccines (Belli et al., 2004). A sub-unit vaccine, CoxAbic ®, is now available in several countries, and consists of a purified protein isolated from gametocytes of *E. maxima*. After administration of the vaccine to the laying hens, produced antibodies are passed into the egg yolk and provide maternal immunity to broiler hatchlings (Wallach, 1997). There have also been several attempts to produce recombinant vaccines, with little success to date, the major hurdle being the identification of protective antigens (Allen and Fetterer, 2002).

Phage display

Introduction

Phage display is a powerful technology that provides a convenient format for the production of large numbers of peptides and proteins, including antibodies (Azzazy and Highsmith, 2002). It was born at the hands of George P. Smith in the mid-1980s, while he was working on filamentous bacteriophage. He realized that the location, domain structure, and flexibility of the pIII bacteriophage minor coat protein might allow insertion of foreign polypeptides as fusion proteins to pIII. The "display" of the protein on the phage surface would make it accessible to antibodies, thus allowing the screening

of a large library of pIII fusions against a specific antibody (Smith, 1996). To test his theory, Smith fused a fragment of the gene encoding the restriction endonuclease *Eco*RI between the amino and carboxy termini of the pIII protein of an M13 bacteriophage. Using affinity chromatography with an immobilized polyclonal antibody, he proved that phages containing the fusion gene could be enriched a 1000-fold over wild-type phage (Smith, 1985). Using recombinant DNA technology, large peptide libraries could thus be built, with every phage displaying a unique random peptide. More importantly, a direct physical linkage is created between the displayed protein and its encoding gene. This linkage endows the protein with the two key characteristics of molecular evolution: replicability and mutability (Smith and Petrenko, 1997). Specific clones can be selected and amplified from pools of billions, simply by allowing the phage to infect male *Escherichia coli* cells. Many applications were borne out of George Smith's ground-breaking work, including the construction of antibody libraries.

Biology of filamentous bacteriophage

Filamentous bacteriophages are viruses capable of infecting a variety of gram-negative bacteria. The most characterized class of bacteriophages is the Ff class which includes M13, f1, and fd strains. These infect *E. coli* cells through the bacterial F conjugative pilus. A filamentous bacteriophage particle consists of a single-stranded, closed DNA genome. The entire genome includes 11 genes, and is encased in a long capsid protein cylinder with a diameter of 7 nm and a length of 900-2000 nm. This cylinder is composed of 2,700 copies of the major capsid protein, pVIII. Five molecules each of pVII and pIX are present at one end of the particle, while the other end harbors 5

molecules each of pIII (minor coat protein) and pVI (Webster, 2001). The minor coat consists of 3 domains; the N1 domain is required during infection, the N2 domain is responsible for binding to the F pilus, and the CT domain is essential for forming a stable phage particle. A filamentous phage does not kill its host bacterium, but rather slows its growth by about half. Infection is initiated by attachment of the phage through the N terminal of the pIII end to the F pilus of a male *E. coli* cell (Figure 1.3). The coat proteins are then disbanded on the cytoplamic membrane, and the viral single-stranded circular DNA is translocated into the cytoplasm, where it is replicated by bacterial enzymes, and converted into a double-stranded replicative form molecule. This molecule serves as a template for synthesis of all the phage proteins. When the concentration of pV protein reaches a certain level, it sequesters single stranded DNA, and prevents its conversion into the replicative form (Pini and Bracci, 2000). The viral particles are then assembled and extruded through the bacterial envelope at several hundred per cell per division cycle (Smith and Petrenko, 1997).

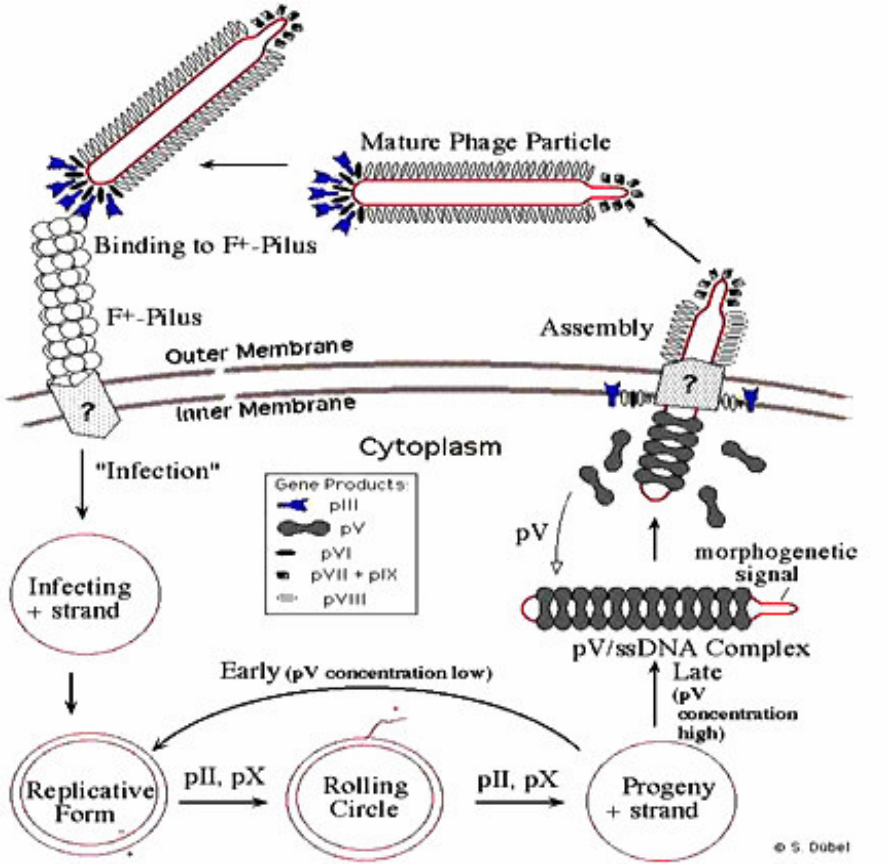

Figure 1.3. Life cycle of filamentous bacteriophages (Breitling and Dübel, 1999).

Phage display vectors

Gene sequences of interest can be inserted into the gene for one of the coat proteins, usually gIII or gVIII. As the phage replicates its DNA inside its host, so does the foreign DNA (Smith and Petrenko, 1997). Phage display vectors (Figure 1.4) are classified according to (1) the coat protein (pIII or pVIII) used for display, (2) the number of copies of coat protein on which the foreign protein is displayed, and (3) the genome (phage or phagemid) on which the fusion protein is encoded (Scott and Barbas, 2001).

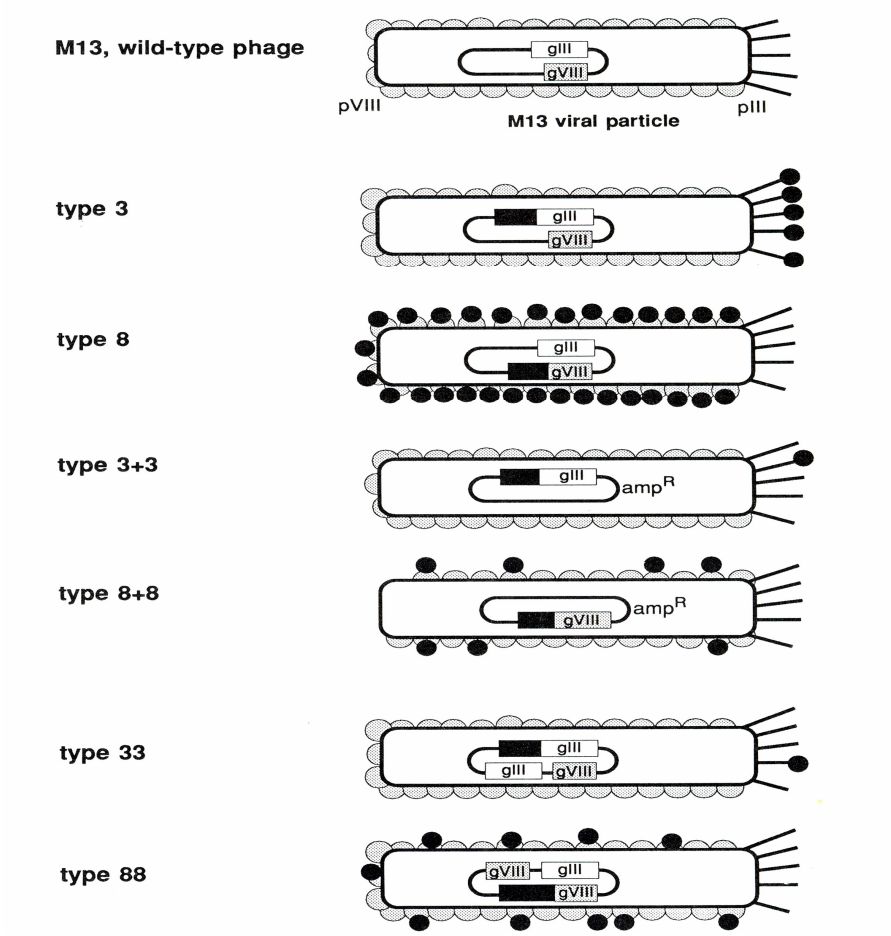

Figure 1.4. Phage display vectors. Foreign inserts and the corresponding peptides are shown as black boxes and spheres, respectively. Types 3 and 8 carry single copies of the fusion gene, while types 33 and 88 carry two copies. Types 3+3 and 8+8 are phagemid-based and require helper phage rescue (Armstrong et al., 1996).

Phagemid vectors

Phagemids, plasmids that carry the recombinant coat protein gene as well as a phage origin of replication, can direct the expression of the desired protein in bacteria, and thus serve as excellent cloning vehicles (Scott and Barbas, 2001). Subsequent

infection of the phagemid-transformed bacterial cells with a "helper" phage activates the phage replication origin (Pini and Bracci, 2000). The helper phage also provides all the proteins and enzymes needed for phage replication. The proteins act in trans on the phage origins of replication, thus resulting in packaging of both the phagemid and the helper phage DNA into phage particles. Packaging of the phagemid genome, however, is favored, as the helper phage genome bears a defective origin of replication. The helper phage genome also carries the kanaymcin resistance gene, which in addition to an ampicillin resistance gene on the phagemid, ensures the selection of only those cells that are transformed by both genomes (Scott and Barbas, 2001). The phagemid vector pComb3X was constructed as a derivation of pComb3, itself derived from the pBluescript vector (Stratagene, CA) by Barbas et al. (1991). In addition to plasmid and phage replication origins, pComb3X includes amino acid residues 230-406 of the gene III fragment, two *Sfi* I restriction enzyme sites that delineate the "stuffer" fragment, a histidine tag, and a hemagglutinin tag (Figure 1.5). The two asymmetric *Sfi* I sites allow for single-step directional cloning of the gene of interest at the C-terminal of the truncated gene III fragment. Sites for this rare-cutting enzyme are almost never found in immunoglobulin sequences (Scott and Barbas, 2001). The presence of a hemagglutinin tag and a histidine tail permit detection and purification of the antibody fragments, respectively.

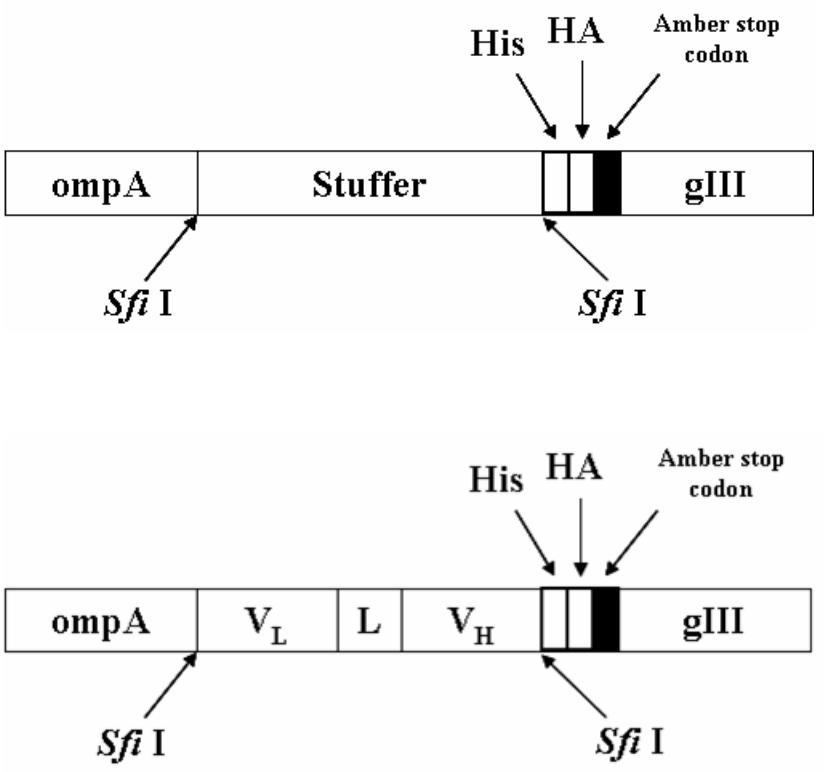

Figure 1.5. Cloning into the pComb3X vector. The antibody fragment is cloned between *Sfi*I sites, with His and HA tags inserted at the C-terminal of the V_H fragment. An amber stop codon just upstream of gIII permits expression of soluble antibodies (Andris-Widhopf et al., 2000).

Construction of antibody libraries

Antibody fragments can be displayed on the surface of a phage as Fab fragments, single-chain variable region fragments (scFvs), or dimeric scFvs (diabodies) (Rader and Barbas, 1997). Following the cloning of the genes encoding the antibody heavy and light gene fragments, a large antibody repertoire can be constructed. Because heavy and light chains are combined randomly, each phage has the potential to display on its surface a unique antibody with a specific antigen-binding site (Rader and Barbas, 1997;

Pini and Bracci, 2000). The genetic information encoding the displayed molecule is contained within the phage coat, thus providing a direct physical link between genotype and phenotype (Rader and Barbas, 1997). This linkage allows the selection and amplification of a specific clone from pools of millions. Moreover, the amino acid sequence of a selected phage can be deduced by deciphering the DNA sequence within (Barbas and Wagner, 1995).

To construct an scFv library, V_H and V_L genes are amplified by the polymerase chain reaction (PCR), and assembled using a peptide linker fragment (Azzazy and Highsmith, 2002). The length of the linker dictates the association state of the antibody molecule (Burton, 2001). Long linkers (15-20 amino acids) favor the formation of monomeric scFvs (Andris-Widhopf et al., 2000). If the linker is shortened to 5-10 amino acids, only intermolecular pairing of V_H and V_L fragments can occur, whereas intramolecular associations become impossible (Pini and Bracci, 2000). This results in the formation of diabodies, where the V_H of one scFv molecule is paired with the V_L of the other, and vice versa (Burton, 2001). The assembled scFv fragment is then ligated into a phagemid vector, and the recombinant phagemid is introduced into competent *E. coli* cells. The bacterial cells are then infected with a helper phage, thus yielding recombinant phages that display the scFv fragment fused to one of the phage coat proteins (Azzazy and Highsmith, 2002).

Phage display of antibody fragments eliminates the need to immortalize the immune B-cells, as the V-genes from those cells are rescued and immortalized instead (Winter and Milstein, 1991). Naïve antibody libraries, constructed from V-gene pools of

non-immune B-cells, sidestep the use of immunization and animals altogether. Such libraries include huge collections of binding sites with every thinkable specificity, and can thus be propagated and repeatedly used to select antibodies against most antigens. By optimizing the selection procedure (antigen concentration, binding and washing times), antibodies with the highest affinity, increased stability, and exquisite binding features can be selected from the population. This process is strikingly similar to the B-cell strategies for selecting and evolving antibodies (Barbas and Wagner, 1995). In vivo, the display of antibody on the B-cell surface allows for proliferative stimulation upon binding to the antigen (Rosenblum and Barbas, 1995). This linkage of recognition and replication is mimicked by phage display, which similarly allows for selection and clonal amplification (Barbas and Wagner, 1995). In fact, phage antibodies with 10^{11} M^{-1} affinities have been obtained by in vitro selection procedures, a figure ten-fold higher than the B-cell affinity ceiling in vivo (Schier et al., 1996). Recombinant antibodies that recognize conformational and cell surface epitopes can be isolated by in vivo selection (Pasqualini and Ruoslahti, 1996). Antibodies selected from phage display libraries can be further fine-tuned by procedures such as error-prone PCR, CDR mutagenesis, and V-gene chain shuffling. In addition, high-throughput screening of libraries is possible, the selection procedure being amenable to automation (Hoogenboom and Chames, 2000).

Single-chain antibody fragments (scFvs) are quite stable and relatively small, with a molecular weight of less than one fifth of a complete antibody. This enables them to penetrate more rapidly and deeper in a tumor tissue (Yokota et al., 1992). Antibody fragments can also be tailored for diagnostic assays by fusing them to proteins and

peptides (Little et al., 2000). In fact, scFvs have been expressed as fusion products to alkaline phospatase, green fluorescent proteins, and lipids (Azzazy and Highsmith, 2002). To circumvent the human anti-murine antibody (HAMA) response elicited by murine antibodies, a variety of "humanization" techniques have been devised and applied to recombinant antibody fragments. Such antibodies can then be used for diagnostic and therapeutic purposes (Maynard and Georgiou, 2000).

Bivalent and bispecific antibodies are another outgrowth of recombinant antibody technology. The former are generated by crossover pairing of two V_H-V_L chains, and exhibit an increased functional affinity (FitzGerald et al., 1997). Bispecific antibodies recognize two different antigens, and have been successful in therapeutic approaches through the recruitment of effector molecules (such as immunoglobulins and complement factors) and cytotoxic T-cells to tumor cells (Kontermann et al., 1997).

Selection of antibody libraries

Panning, where phage particles expressing the antibody fragment are selected against an appropriate antigen, is used to screen and enrich antibody libraries for antigen-specific clones (McCafferty and Johnson, 1996; Nissim et al., 1994). Unbound phages are removed by washing, whereas antibody-displaying phages are eluted and re-amplified in *E. coli* (Azzazy and Highsmith, 2002). Culling the initial population of phage particles through several rounds of selection gives rise to a subpopulation with increased fitness (Smith and Petrenko, 1997). In vitro, selection can be performed on either a solid phase (Clackson et al., 1991), or on an antigen in solution (Hawkins et al., 1992). Alternatively, antibody-displaying phage particles can be directly selected

against markers on cell surfaces (Azzazy and Highsmith, 2002). Such cell panning selects for antibodies that are more likely to recognize epitopes accessible in vivo, and is important for selection of therapeutic antibodies (Rader and Barbas, 1997). In vivo panning, where phage repertoires are directly injected into animals, allows the selection of peptides that home to target organs (Pasqualini and Ruoslahti, 1996).

Epithelial cell culture

Introduction

Epithelium refers to the various cell layers that cover the surfaces or line the internal body cavities (Freshney, 2002). Epithelial tissue consists of tightly bound polyhedral cells that are supported on a basement membrane made up of collagen, laminin, fibronectin, and proteoglycans. The cellular apical surfaces are either exposed to the environment (e.g. epidermis, bronchial epithelium) or to a fluid-filled space such as intestinal and kidney epithelia (Shaw, 1996). Cell polarity is very important for epithelial cell functioning, as transport of fluid, ions, oxygen, and nutrients across these cells occur along the basal-apical axis in a polarized fashion (Freshney, 2002). Enterocytes forming the simple columnar epithelium of the intestine provide a clear example of cell polarity. The apical surface of these cells is exposed to the intestinal lumen, while their basal surface is firmly tethered to the basement membrane. Apical and basolateral membrane proteins must remain separate for these cells to function properly (Shaw, 1996). The integrity of epithelial cells in vivo is maintained by the presence of intercellular junctions which firmly link adjacent cells and regulate

permeability and transport functions. The basement membrane regulates the passage of macromolecules across the epithelial cells, provides a matrix for cell attachment, and ensures cell polarity through interactions with basal receptors. Terminally differentiated epithelial cells are readily replaced. In the intestine and skin, where cell trauma is high, this regeneration process is quite rapid, whereas it is quite slow in the liver and pancreas. In the intestinal crypts, undifferentiated stem cells produce daughter cells committed to a specific cell lineage. As these cells mature and differentiate, they develop specific phenotypic properties, and progressively lose their capacity to proliferate (Shaw, 1996).

Isolation and culture

Epithelial cells, closely associated in vivo, survive better in vitro when they are isolated as clusters or sheets of cells. Thus, dissociation techniques (such as trypsinization and some filtration methods) that reduce the cell population to a single cell suspension are not usually successful, and have been largely replaced by methods that use gentle mechanical disruption or collagenase digestion combined with filtration (Freshney, 2002). Fibroblasts are frequently co-isolated with epithelial cells, and if not removed, will quickly overgrow the culture. For short-term cultures, this phenomenon can be avoided by selective attachment. When seeded in a cell culture flask, fibroblasts tend to stick down before epithelial cells do. Transferring the non-attached - primarily epithelial - cells to a fresh flask will hence suppress fibroblastic overgrowth. A more successful method for inhibiting fibroblast overgrowth is to use of a monolayer of irradiated feeder cells to which epithelial cells, but not fibroblasts, can attach. Selective media have also been developed to promote epithelial cell growth. They are serum-free,

and often include growth factors such as hydrocortisone, insulin, and epidermal growth factor. Reconstruction of the basement membrane that maintains cell polarity is an essential requisite for growing functional epithelium in vitro. Epithelial cells are hence commonly cultured on porous surfaces such as polycarbonate filters, which provide epithelial cells with a flexible support, and supply them with their nutrients (Shaw, 1996). It has been shown that the longevity of an epithelial cell culture is dependent on the longevity of the stem cell population (Freshney, 2002). The presence of these immature, highly proliferative cells is hence another critical requirement for successful epithelial cell culture (Freshney, 2002). In the small intestine, epithelial cells derive from stem cells located at the base of the crypt (Grossmann et al., 1998a). As they differentiate, they migrate along the crypt-villus axis, until they reach the luminal surface after 3 to 5 days, where they detach and are shed (Potten and Allen, 1977). Death of epithelial cells is thought to occur through a form of apoptosis known as detachment-induced cell death, or anoikis, a Greek term meaning state of homelessness (Frisch and Francis, 1994). Anoikis is mediated by the expression of pro- and anti-apoptotic proteins such as bax, bak, and bcl-2, and eventual activation of caspases (Grossmann, 2002). Caspases are a family of intracellular cysteine aspartate proteases situated at the center of the molecular machinery responsible for apoptosis (Heussler et al., 2001). Caspases exist within the cell as inactive zymogens and are organized in cascades, where an apoptotic signal activates an upstream caspase by proteolytic cleavage. The activated caspase in turn cleaves and activates downstream caspases at an aspartate (Budihardjo et al., 1999; Heussler et al., 2001), ultimately leading to activation

of endonucleases, DNA fragmentation, and cell death (Grossmann, 2002). Morphological and physiological changes associated with apoptosis are directly or indirectly the result of the caspase cascade (Thornberry and Lazebnik, 1998). Epithelial cells that are no longer anchored to their extracellular matrix and/or have lost cell to cell contact quickly undergo apoptosis (within 3 hours), making the in vitro culture of epithelial cells a real challenge (Grossmann et al., 1998b). Moreover, the culture environment lacks inputs from the nervous and endocrine systems, which are involved in homeostasis in vivo (Kaeffer, 2002). Traditionally, attempts at producing primary cultures of intestinal cells have relied on combinations of enzymatic and mechanical methods, with varying degrees of success. Isolation of viable proliferative cells seems to be highly correlated with the number of stem cells present, and with the maintenance of anchorage, a key concern for prevention of caspase activation, and hence of apoptosis.

CHAPTER II

PRODUCTION OF *EIMERIA TENELLA* SPOROZOITES

Introduction

A coccidial infection begins when *Eimeria* oocysts are ingested by a susceptible bird (Augustine, 2000). An oocyst is the resistant infective form of the parasite and is surrounded by a thick wall that encloses four sporocysts, each of which harbors two sporozoites. The oocyst wall is crushed in the gizzard, and sporozoites, the initial invasive stages, are released by the action of bile salts and chymotrypsin in the intestine (Shirley et al., 2005). Sporocyst and sporozoite release can be simulated in vitro, the former by mechanically breaking the oocysts, and the latter with the use of bile salts and pancreatic enzymes (Dulski, 1990). The viability of sporozoites decreases drastically after excystation (Tomley, 1997), impeding assays that require whole sporozoites, as these would have to be produced on the same day of the assay. The objective of this study was to devise a feasible method for efficient production and long-term storage of sporozoites. The effects of oocyst age and different enzyme formulations on sporozoite yields were also investigated.

Materials and methods

Recovery and sporulation of E. tenella *oocysts*

One hundred broiler chicks were obtained from a local commercial hatchery (Hyline International, Bryan, TX) on the day of hatch. All chicks were randomized and

placed on the floor of an isolation unit. Chicks were maintained at age-appropriate temperatures and given ad libitum access to water and a complete chick starter ration formulated to meet or exceed current NRC recommendations for poultry (National Research Council, 1994). The feed also contained Coban® (Elanco, Greenfield, IN), an ionophore coccidiostat, at 0.7 g/kg of feed. At two weeks of age, the coccidiostat was withdrawn from the feed. Three days later, each bird was orally infected with $7.5x10^4$ *E. tenella* sporulated oocysts (Strain WLR-1, kindly provided by Dr. Harry Danforth, United States Department of Agriculture, Beltsville, MD). A week later, the birds were euthanized by CO_2 asphyxiation, and their ceca were collected. Ceca were cut into pieces and were blended with water into thin slurry. Trypsin (Sigma, St Louis, MO) was added to 1.5% (w/v), the mixture was stirred for one hour at 30-35ºC, and was then sieved to eliminate large pieces of tissue. To achieve sporulation of the oocysts, potassium dichromate (Sigma, St Louis, MO) was added to 2.5% (w/v), and the mixture was incubated for 72 hours at 25-30ºC with forced aeration. The culture was centrifuged at 1000*g* for 15 minutes, and then washed with tap water to remove the potassium dichromate. The pellet was resuspended in 1M sucrose, centrifuged as above, and the supernatant was filtered through sterile 100-µm Nitex gauze (Sefar America, Buffalo, NY), and diluted at least 4 times with tap water. Sporulated oocyts were pelleted as above, counted and stored with 2% potassium dichromate (w/v) at 4ºC.

In preparation for excystation, sporulated oocysts ($3x10^7$) were repeatedly washed with distilled water and pelleted at 1,600*g* for 10 min. Washes were initially performed in 250-ml centrifuge bottles, and gradually moved to 50-ml centrifuge tubes.

At least five washes were needed to remove the potassium dichromate. The oocysts were then resupended in PBS, and Clorox bleach was added to 10% (v/v), followed by incubation on ice for 10 min. Oocysts were pelleted as above, resupsended in 40 ml saturated sodium chloride solution, and carefully overlayed with 3 ml of distilled water. After centrifugation as above, clean sterile oocysts were collected from the salt-water interface, washed repeatedly in water, and counted.

Excystation and storage of sporozoites

Clean oocysts were pelleted in a round-bottom centrifuge tube, resuspended in a minimal amount of PBS, and vortexed with an equal volume of glass beads (0.5 cm diameter, Sigma, St Louis, MO) for 2 min. Breakage was monitored by microscopy at regular intervals to ensure that sporocysts were not damaged. Sporocysts were recovered by additions of PBS, pelleted, resuspended in 50% Percoll (density 1.13 g/ml, GE Healthcare, Piscataway, NJ), and centrifuged in a swinging-bucket rotor at 3,000*g* for one hour. Sporocysts were washed twice with PBS, resuspended at 10^6/ml in excystation medium, and incubated at 41ºC for 90 min with end over end mixing. The excystation medium consisted of PBS with 4% (w/v) taurodeoxycholic acid (EMD Biosciences, San Diego, CA), 1 mg/ml TPCK (tosyl-L-phenylalanine chloromethyl ketone) -treated trypsin, 0.1 mg/ml α-chymotrypsin (Sigma, St Louis, MO), and 10mM $MgCl_2$. Sporozoites were pelleted at 700*g* for 20 min, washed in PBS, and counted. Freshly prepared sporocysts or freshly excysted sporozoites were resuspended in cell freezing medium, i.e. fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) containing 10% (v/v) DMSO (Sigma, St Louis, MO), divided in 1-ml aliquots, gradually

frozen to -80ºC at the rate of 1º/min, and stored in liquid nitrogen. To evaluate any detrimental effects of cell freezing, an aliquot of frozen sporocysts or sporozoites was thawed out and washed with PBS. Sporocysts were resuspended in excystation medium, and excystation was carried out as described above. Viability and morphology of frozen sporozoites was assessed by trypan blue staining. Sporozoites were observed with an Olympus microscope and pictures were taken using a SPOT camera. To assess the effect of oocyst age on excystation efficiency, the same protocol was repeated with one-month old oocysts.

Results

Recovery and sporulation of E. tenella *oocysts*

Oocyst propagation in chicks did not cause any mortality, and mild cecal lesions were observed. The experiment generated 10^9 sporulated oocysts, and these accounted for more than 99% of the obtained oocysts. Oocysts were surface-sterilized with bleach, and most of the debris was removed when the salt floatation step was repeated twice, yielding a very clean oocyst preparation. Breakage of the oocysts was achieved by vortexing with glass beads for 2 min. At that point, about 60% of the oocysts were broken, and a few sporozoites were observed. Breakage was hence stopped to prevent further damage to the sporocysts.

Excystation and storage of sporozoites

Sporocysts were purified on a 50% Percoll gradient. With this density gradient, unbroken oocysts and oocyst shells floated, while sporocysts remained in the bottom

third of the gradient. Sporocysts were then washed in PBS to eliminate the Percoll, and resupsended in excystation medium, yielding about 90% excystation after incubation at 41ºC for 90 min (Figure 2.1). Almost all sporocysts were excysted and there was little contamination with whole oocysts and oocyst walls. Other recipes including taurocholic acid (Sigma, St Louis, MO), another bile salt, and a different enzyme formula (2.5 or 4 mg/ml chymotrypsin-containing trypsin) gave less satisfactory results (less than 20% excystation) when used to excyst sporozoites from 3-month old oocysts.

Sporocysts appeared transparent after they had undergone the freezing process. Freezing also prevented sporozoite excystation from sporocysts. Frozen sporozoites, however, retained normal morphology and more than 50% viability (Figure 2.2). Older oocysts (1 month) were hard to break, but sporocyst and sporozoite release was still adequate. However, with oocysts older than 3 months, excystation efficiency was useless for all practical purposes.

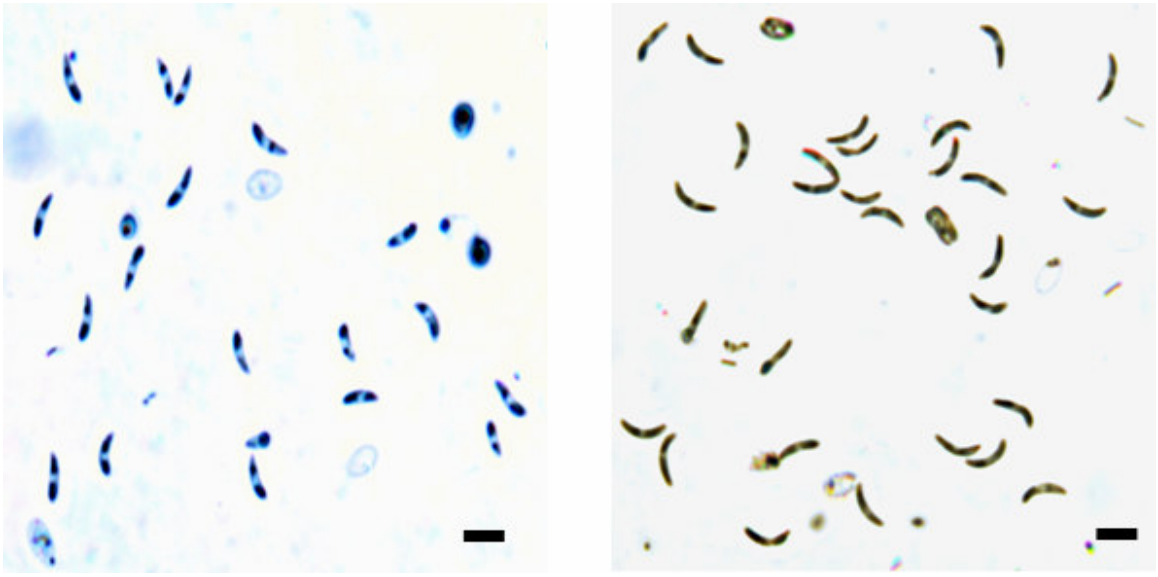

Figure 2.1. Freshly excysted *E. tenella* sporozoites. Scale bar: 25 µm.

Figure 2.2. *E. tenella* sporozoites after freezing and thawing. Scale bar: 25 µm.

Discussion

Eimeria tenella oocysts were successfully propagated by passage through 2-week old birds. Oocysts were isolated from ceca, sporulated, and cleaned from debris, essentially according to established protocols (Tomley, 1997). As *E. tenella* oocysts can be harvested directly from the ceca, recovery is simpler than with other *Eimeria* species (Tomley, 1997), which must be isolated from fecal material. Sporocysts were obtained by mechanical breakage of the oocysts, which mimics the action of the chicken gizzard. Recovery of sporocysts was not optimal but could be improved by recycling the unbroken oocysts through more breakage rounds. It has to be noted that parasite losses (about 30 %) invariably happened through oocyst breakage and the Percoll purification steps. For the purpose of this experiment, the number of sporocysts and sporozoites obtained was more than sufficient.

Addition of bile salts and pancreatic enzymes dissolves the sporocyst plug (Stieda body) and allows expulsion of sporozoites (Dulski, 1990). Taurodeoxycholic acid has been reported to be a more effective bile salt for excystation than taurocholic acid (Patton and Brigman, 1979). Chymotrypsin has been described as the essential enzyme for excystation (Wang and Stotish, 1975), and researchers have traditionally used chymotrypsin-containing crude trypsin for excystation. The addition of $MgCl_2$, which appears to be necessary for expulsion of the sporozoite after the removal of the Stieda body, has been shown to improve excystation yield (Dulski and Turner, 1988). In this experiment, the use of excystation medium containing taurodeoxycholic acid, TPCK-treated, essentially chymotrypsin-free trypsin, pure α-chymotrypsin, and $MgCl_2$

resulted in a very efficient release of sporozoites. Trials with different formulas gave very low sporozoite yields. However, the oocysts used in those trials had been stored at 4ºC for 3 months. It has been observed that sporozoite lysis occurs in oocysts older than 1 month (Dulski and Turner, 1988). Since the trypsin used in those trials had not been TPCK-treated, and hence contained chymotrypsin, it is most probable that the excystation outcome was largely influenced by the oocyst age. This conclusion is further corroborated by the fact that oocysts stored at 4ºC for as little as a month were much harder to break. Repeated breakage could have damaged the sporocysts. A marked decrease in the number of released sporocysts had been reported after grinding 6 to 8 months old oocysts, possibly explaining the drop in the excystation rate (Ruff et al., 1981). Moreover, the same authors observed a correlation between oocyst aging and the decreased survival of excysted sporozoites, and a decreased invasive potential by sporozoites prepared from cultures older than 6 months.

Ready-to-use sporozoites were needed for panning and selection of the antibody library on a daily basis, as well as for the analysis of selected antibodies. Production of sporozoites and panning proved to be tedious, time-consuming processes, and practically impossible to run in parallel. Hence, cell freezing was resorted to as a means of providing a readily available source of sporozoites. Frozen sporozoites could be thawed and used immediately. More than 50 % of the frozen sporozoites remained viable, and even dead ones remained intact, and thus were still useful for panning. Thawed sporozoites have been reported to develop into mature schizonts when inoculated into bovine embryonic kidney cells (Doran and Vetterling, 1968). Frozen sporocysts failed

to excyst, which is in contrast to previous reports (Norton and Joyner, 1968). Other reports have shown that thawed sporocysts fed to birds were able to cause infection, albeit with a lower intensity than freshly released sporocysts (Doran and Vetterling, 1968).

CHAPTER III

LIBRARY CONSTRUCTION AND SELECTION

Introduction

The development of the phage display technology has provided an excellent means for construction of antibody libraries. Antibody genes can be inserted into a phagemid, a plasmid containing a phage-derived origin of replication. The phagemid is then introduced into competent *Escherichia coli* cells, which will express the antibody as a fusion protein to one of the bacteriophage coat proteins, thus providing a direct physical linkage between phenotype (antibody specificity) and genotype (sequence of the variable regions). Selection of specific antibodies can be achieved by a panning procedure, where phage particles carrying non-specific antibodies are culled, thereby enriching for those expressing specific antibodies. The aim of this experiment was to construct an antibody library against *Eimeria tenella* parasites, and to select and test the isolated antibodies.

Materials and methods

Except for minor modifications, methods described and recipes used for library construction, transformation, and selection follow protocols described in "Phage display: a laboratory manual" (Eds. Barbas, Burton, Scott, and Silverman, 2001, CSHL Press).

The carbenicillin (MP Biomedicals, Irvine, CA), tetracycline (Sigma, St Louis, MO), and kanamaycin (Invitrogen, Carlsbad, CA) stocks were 50 mg/ml, 10 mg/ml, and 50 mg/ml, respectively.

Immunization

Single comb white Leghorn (SCWL) chicks were obtained from a local commercial hatchery (Hyline International, Bryan, TX) on day of hatch. All chicks were randomized and placed in floor pens. Chicks were maintained at age-appropriate temperatures and given ad libitum access to water and a complete chick starter ration formulated to meet or exceed current NRC recommendations for poultry (National Research Council, 1994). Strain WLR-1 of *Eimeria tenella* was obtained from Dr. Harry Danforth (United States Department of Agriculture, Beltsville, MD). Birds were vaccinated on the day of hatch via spray cabinet with Coccivac-B (Schering-Plough Animal Health, Omaha, NE) according to the manufacturer's directions. At 21 days of age, the heaviest 30 birds received a clinical challenge with *E. tenella* oocysts ($2 \text{ X } 10^5$ oocysts per bird). On each of the following four, five, six, and seven days post-challenge, five birds were euthanized, and their spleens, ceca, and bone marrow were collected and stored in liquid nitrogen. Serum samples were also collected on days five and seven post-challenge. Two weeks after challenge, each of the remaining 10 birds was injected intravenously with $4 \text{ x}10^6$ *E. tenella* sporozoites to further activate the splenic B-lymphocytes. Three and five days later, the birds were sacrificed and their spleens were collected and stored in liquid nitrogen.

RNA extraction

Collected organs were ground in liquid nitrogen, and same-organ, same-day samples were pooled. Total RNA was extracted from one gram of tissue per sample using the Trizol method (Invitrogen, Carlsbad, CA). RNA samples were treated with DNase (Ambion, Austin, TX) to remove any genomic DNA contamination. Purity, integrity, and concentration of RNA samples were assessed by agarose gel electrophoresis and by absorbance readings at 260 and 280 nm.

$$\text{RNA Concentration } (\mu g/ml) = A_{260} \text{ x dilution factor x } 40$$

cDNA synthesis

First-strand cDNA was synthesized from each of the 14 total RNA samples using the RETROscript® kit from Ambion (Austin, TX). Briefly, 20 µg of RNA was mixed with 2 µl of oligo (dT) primer (50 µM) and nuclease-free water. The mix was incubated at 85ºC for 3 min, after which 2 µl of 10X RT buffer, 1 µl dNTP mix (at 10 mM each), 1 µl RNase inhibitor (10 U/µl), and 1 µl MMLV reverse transcriptase (100 U/µl) were added. The 20 µl reaction mixture was incubated at 44ºC for 1 h, followed by a 10-minute incubation at 92ºC. A cDNA pool was prepared using 10 µl of each cDNA sample.

Generation of scFv fragments

The cDNA pool was used to amplify V_H and V_L genes for the construction of a combinatorial scFv library. In a primary PCR step, individual V_H and V_L genes were

amplified using primer pairs that contain complementary sequences that encode a short linker (GGSSRSS), and are used in the secondary overlap PCR to create the final scFv product (Figure 3.1). A list of the primers used appears in Table 3.1. For both V_H and V_L amplification, 1 µl of cDNA was mixed with 60 pmole of each of the two appropriate primers, 10 µl of 10X PCR buffer, 8 µl dNTP mix (at 2.5 mM each), 0.5 µl Taq DNA Polymerase (5 units/µl, Qiagen, Valencia, CA), and water to yield a final volume of 100 µl. The V_L amplification reaction was carried out under the following conditions: denaturation at 94ºC for 5 min, followed by 30 cycles of 94ºC for 15 sec, 56ºC for 30 sec, and 72ºC for 1.5 min. The reaction was completed by a final extension step at 72ºC for 10 min. The settings for V_H amplification were the same, except that annealing conditions were adjusted to 52ºC for 40 sec. The V_L and V_H products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA), and quantified by absorbance readings at 260 nm.

$$\text{DNA concentration } (\mu g/ml) = A_{260} \times \text{dilution factor} \times 50$$

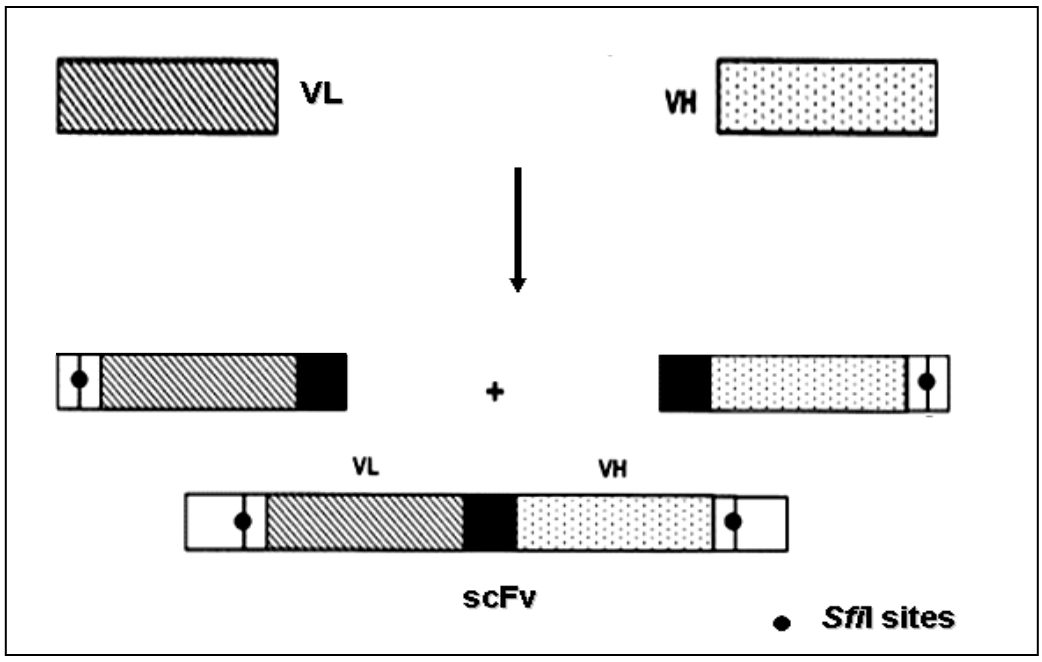

Figure 3.1. Amplification of scFv fragments. V_H and V_L fragments were amplified from cDNA using primer pairs that contain complementary linker sequences that are used to produce the full-length scFv fragment. The overlap primers incorporate *Sfi*I restriction sites for cloning into the phagemid vector (modified after Andris-Widhopf et al., 2000)

The secondary overlap PCR included 100 ng of each of the purified V_H and V_L products, and 60 pmole of each of the overlap primers (Table 3.1). These primers incorporate the *Sfi*I cloning sites into the final product, thus allowing for subsequent cloning into the phagemid vector (Figure 3.1). The remaining components were present in the same concentrations as in the primary PCR. The number of cycles was decreased to 25, the annealing conditions were 52ºC for 40 sec, and the extension time was increased to 2 min. The scFv product was purified and quantified as described for the V_H and V_L fragments. Identities of all PCR products were confirmed by agarose gel electrophoresis and sequencing.

Table 3.1. List of primers used in the primary and secondary PCRs (Andris-Widhopf et al., 2000).

Primers for V_H amplification
CSCVHo-F (forward)
5' GGTCAGTCCTCTAGATCTTCCGCCGTACGTTGGACGAG 3'
CSCG-B (reverse)
5' CTGGCCGGCCTGGCCACTAGTGGAGGAGACGATGACTTCGGTCC 3'
Primers for V_L amplification
CSCVK (forward)
5' GTGGCCCAGGCGGCCCTGACTCAGCCGTCCTCGGTGTC 3'
CKJo-B (reverse)
5' GGAAGATCTAGAGGACTGACCTAGGACGGTCAGG 3'
Primers for scFv amplification
CSC-F (forward)
5' GAGGAGGAGGAGGAGGAGGTGGCCCAGGCGGCCCTGACTCAG 3'
CSC-B (reverse)
5' GAGGAGGAGGAGGAGGAGGAGCTGGCCGGCCTGGCCACTAGTGGAGG 3'

Construction of the scFv test library

The phagemid pComb3X vector was obtained from Dr. Carlos F. Barbas III (The Scripps Research Institute, La Jolla, CA). The vector and the scFv insert were prepared for cloning by digestion with *Sfi*I. Fifteen µg of scFv product were mixed with 540 units of *Sfi*I (40U/µl, Roche Molecular Systems, Indianapolis, IN), 20 µl of 10X buffer M

(supplied with the enzyme), and water to yield a final volume of 200 µl. Twenty µg of vector were mixed with 120 units of *Sfi*I, 20 µl of 10X buffer M, and water to a final volume of 200 µl. Digestion reactions were incubated at 50°C for 6 h, followed by ethanol precipitation overnight at –80°C. The digested scFv product was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA). The digested vector and its stuffer fragment were purified on a 0.8% low melt agarose gel. The gel was run at 50V for 5 h. This long run allowed for clear separation between the single-cut and uncut vector and the desired double-cut vector. Small-scale ligations were performed to test for the cloning efficiency of the vector. Ligation of the vector and its stuffer fragment was used as a positive control. Background ligation was determined by self-ligation of the vector. Ligation reactions included 140 ng vector, either 140 ng stuffer or 70 ng scFv, 2 µl of 10X T4 DNA ligase buffer, 1µl ligase (Roche Molecular Systems, Indianapolis, IN), and water to yield a final volume of 20 µl. Ligation reactions were incubated overnight at room temperature. One µl from each ligation reaction was transformed into 50 µl of XL1-Blue electrocompetent cells (Stratagene, La Jolla, CA). Electroporation was performed using 1 mm gap BTX cuvettes, and a BTX square-wave generator (Electro Cell Manipulator ECM 2001, Harvard Apparatus, Holliston, MA), set at 1.7 KV to deliver 10 pulses of 99 µsec each. Immediately following electroporation, the cuvette was washed with 1 ml then twice with1 ml of prewarmed SOC medium. The 3-ml cultures were incubated for 1 h in an orbital incubator at 37°C and 250 rpm. The titer of the transformed bacteria was determined by plating 1:10 and 1:100 dilutions from

each transformation onto LB/carbenicillin plates, and incubating the plates overnight at 37°C. The number of transformants was calculated as follows:

$$\text{Total transformants} = \frac{\text{number of colonies} \times \text{culture volume} \times \text{ligation volume}}{\text{plating volume}}$$

where: culture volume = 3,000 µl, ligation volume = 20 µl, and plating volume = 1 µl (1:100 dilution) or 10 µl (1:10 dilution)

Transformants (cfu/µg) = Total transformants/0.14

Phagemids were purified from the cells transformed with vector and insert or stuffer using the Qiagen Miniprep plasmid purification kit (Qiagen, CA). Phagemids were then digested with *Sfi*I as described above, and the digestion products were run on a 1% agarose gel to check for presence of fragments of the appropriate size.

Preparation of helper phage

Two milliliters of SB medium containing 10 µg/ml tetracycline (hereafter referred to as SB/Tet medium) were inoculated with 2 µl of XL1-Blue *E. coli* cells, and the culture was shaken at 250 rpm at 37 °C for 1 h. Dilutions (10^{-6}, 10^{-7}, and 10^{-8}) of the kanamycin-resistant VCSM13 helper phage (Stratagene, La Jolla, CA) were prepared in SB medium. One microliter of each dilution was used to infect 50 µl of *E. coli* culture, followed by incubation at room temperature for 15 min. Three milliliters of liquefied LB top agar were then added and the culture was poured over pre-warmed LB agar plates. The plates were incubated overnight at 37°C. The following morning, an *E. coli* culture was prepared by inoculating 10 ml of pre-warmed SB/Tet medium with 10 µl of

XL1-Blue cells. The culture was incubated at 37°C and 250 rpm for 1 h. A single VCSM13 plaque was picked from the 10^{-8} plate and transferred to the culture which was shaken at 250 rpm for 2 h at 37°C. The culture was then divided over two 1-liter flasks each containing 250 ml of pre-warmed SB medium and 70µg/ml of kanamycin. The cultures were incubated overnight at 37°C and 250 rpm, and then transferred to 50-ml polypropylene tubes and centrifuged at 2,500*g* for 15 min. Supernatants were transferred to fresh 50-ml polypropylene tubes and incubated for 20 min in a water bath at 70°C. Following centrifugation at 2,500*g* for 15 min., the supernatants were collected, pooled, and stored at 4°C. The titer of the helper phage preparation was determined by inoculating XL1- Blue cultures with 10^{-6}, 10^{-7}, and 10^{-8} dilutions of the phage preparation as described above. The number of plaque-forming units/ml was calculated as follows:

$$\text{Titer (pfu/ml)} = \text{number of plaques} \times \text{dilution} \times 10^3$$

From this point on, extreme care was taken not to contaminate cultures with helper phage, the occurrence of which can be devastating.

Library construction and transformation

Four library ligations were prepared. Each ligation consisted of 1.4 µg of *Sfi*I-cut, purified pComb3X, 700 ng of *Sfi*I-cut, purified scFv product (a two-fold molar excess over the vector), 20 µl of 10X ligase buffer, 10 µl ligase (Roche Molecular Systems, Indianapolis, IN), and water to a final volume of 200 µl. Ligation reactions were incubated overnight at room temperature. Each library ligation was then precipitated by the addition of 0.1 volume (20 µl) sodium acetate (pH 5.2), 2.2 volumes (440 µl) of

absolute ethanol, and 1 µl of 20 mg/ml glycogen (Roche Molecular Systems, Indianapolis, IN), followed by incubation at -80°C overnight. After centrifugation at 14,000 rpm for 15 min at 4°C, the supernatant was discarded and the pellet was washed twice with 70% ethanol, dried, and dissolved in 16 µl of water. Each library was electroporated into 300 µl of XL1-Blue electrocompetent cells. Electroporation was performed as described for the test library. Because a 1-mm gap cuvette can only hold 90 µl, each ligation reaction was split into 4 aliquots. Each 4 µl aliquot was electroporated into 75 µl of electrocompetent cells. Immediately following electroporation, the cuvette was washed with 1 ml then with 250 µl of prewarmed SOC medium. The same procedure was carried out for the 3 remaining aliquots, using a fresh cuvette every time. Cells transformed with one ligation reaction were pooled. The 5 ml cultures were incubated for 1 h in an orbital incubator at 37°C and 250 rpm. To titer the transformed bacteria, 2 µl of each culture was diluted in 198 µl of SOC, and 10 and 100 µl of this dilution were plated on LB agar/carbenicillin plates. The plates were incubated overnight at 37°C. The size of each library was calculated by multiplying the number of colonies by the culture volume (5,000 µl) and dividing by the plating volume (0.1 µl for the 1 µl plate, or 1 µl for the 100 µl plate). Ten ml of prewarmed SB medium, 6 µl of 50 mg/ml carbenicillin, and 15 µl of 10 mg/ml tetracycline were added to each 5 ml culture. Cultures were incubated for 1 h at 37°C and 250 rpm. Carbenicillin was added to a final concentration of 50 µg/ml and the cultures were shaken for an additional hour at 37°C and 250 rpm. The cultures were combined in a 1-liter flask containing two ml of VCSM13 helper phage (prepared as above, 10^{12} pfu/ml),

185 µl carbenicillin, 185 µl tetracycline, and prewarmed SB medium to 200 ml. The culture was incubated at 37°C and 300 rpm for 2 h. Kanamycin was added to 70 µg/ml, and incubation was continued overnight. The culture was centrifuged for 15 min at 3,000*g* and 4°C. The supernatant was transferred to a 250-ml centrifuge bottle, and 50 ml of 5X PEG/NaCl were added, followed by gentle mixing and incubation on ice for 30 min. The phage was precipitated by spinning for 30 min at 15,000*g*, 4°C. The supernatant was discarded and the bottle was drained by inverting on a paper towel for 30 min. The phage pellet was resuspended in 1% BSA in PBS, followed by a 5-min spin at maximum speed and 4°C. The supernatant was passed through a 0.22 µm filter (Fisher Scientific, Pittsburgh, PA) and stored at 4ºC with 0.03% sodium azide.

Panning

Two *E. coli* cultures were prepared on the morning of each day of panning, one for the library, and one for input titering. Each culture was prepared by inoculating 2 ml prewarmed SB/Tet medium with 2 µl of XL1-Blue electrocompetent cells, and incubating for 2.5 h at 37°C and 250 rpm. Freshly prepared phage (50 µl) was blocked for 1 h with 150 µl of 3% BSA in PBS on ice. One vial of cryopreserved sporozoites (2x 10^6/ml) was thawed, and the cells were washed once in serum-free Dulbecco's Modified Eagle Medium (DMEM, Sigma, St Louis, MO) and once in PBS. Sporozoites were resupsended in 200 µl flow cytometry buffer (PBS containing 20 mM HEPES, 1% BSA, pH 7.4), and added to two wells of a V-bottom plate (100 µl of cell suspension/well). An equal volume of blocked phage was added to each well, mixed gently, and incubated for 1 h at room temperature with gentle rotation. The plate was centrifuged at room

temperature for 3 min. at 750g with the brake off. The supernatant was discarded and the cells in each well were resuspended in 180 µl of flow cytometry buffer. This washing step was repeated 4, 5, 6, 7, and 8 times in the first, second, third, fourth, and fifth round of panning, respectively. Cells were resuspended in 180 µl of PBS/well, and transferred to a 5-ml flow cytometry tube. Two ml of PBS were added, and the tube was centrifuged as above. The supernatant was discarded and the pelleted cells were gently resuspended in 100 µl of elution buffer (glycine-HCl, pH 2.2). After a 10-min incubation at room temperature, the cells were gently pipetted up and down, then pelleted as above. The eluate was transferred to a microfuge tube containing 6 µl of neutralization buffer (2 mM Tris in water). The neutralized eluate was added to a previously prepared 2 ml *E. coli* culture, and incubated for 15 min at room temperature. The culture was transferred into a 50-ml polypropylene tube containing 6 ml of prewarmed SB, 3.2 µl carbenicillin, and 6 µl tetracycline. The output of the panning round was determined by diluting 2 µl of the culture into 198 µl SB, and plating 10 and 100 µl of this dilution on LB/carbenicillin plates. The plates were incubated overnight at 37°C. The 8 ml culture was incubated for 1 h at 37°C, 250 rpm, at which point the carbenicillin concentration was increased to 40 µg/ml, and the culture was further incubated for 1 h at 37°C, 250 rpm. Meanwhile, the input was titered by infecting 50 µl of *E. coli* culture with 1 µl of each of 10^{-7}, 10^{-8}, and 10^{-9} dilutions of the phage preparation, followed by a 15-min incubation at room temperature, and plating on LB/carbenicillin plates. The plates were incubated overnight at 37°C. The 8 ml culture was transferred to a 1-liter flask containing 1 ml of VCSM13 helper phage (prepared as

described above, 10^{12} pfu/ml), 92 µl carbenicillin, 92 µl tetracycline, and 91 ml prewarmed SB medium. The culture was incubated at 37°C and 300 rpm for 2 h. Kanamycin was added to 70 µg/ml, and incubation was continued overnight. The next morning, phage was precipitated as described above, and stored at 4°C. Input and output titers were calculated for each panning round as follows:

Input (cfu/ml) = number of colonies x dilution x 10^3

Total input (cfu) = Input/ml x volume of phage used

(100 µl of 1:4 phage dilution from the blocking step = 25 µl phage).

Output (cfu) = number of colonies x culture volume / plating volume

where culture volume = 8,000 µl, and plating volume = 0.1 µl (10 µl plate) or 1 µl (100 µl plate).

Precipitation of antibody-displaying phage

Phage was precipitated from 10 clones randomly selected from the output plates of the last round of panning. Each colony was used to inoculate 5 ml of pre-warmed SB medium containing 50 µg/ml of carbenicillin. Cultures were incubated at 37°C and 300 rpm for 6 h. For backup, 5 µl from each culture were streaked on a pre-warmed LB/carbenicillin plate. Plates were incubated overnight at 37°C, sealed with Parafilm, and stored at 4°C. After addition of helper phage (50 µl/culture), cultures were incubated at 37°C and 300 rpm for 2 h. Kanamycin was then added to each culture to a final concentration of 70 µg/ml, and cultures were incubated overnight at 37°C and 300 rpm. Bacterial cells were pelleted by centrifugation at 3,000*g* for 15 min. at 4°C. Then, 1.2 ml of each supernatant was transferred to each of three microcentrifuge tubes

containing 300 µl of 5X PEG/NaCl, mixed, and incubated on ice for 30 min. PEG-precipitated phages were pelleted by centrifuging at 14,000 rpm and 4°C for 15 min. Supernatants were discarded and each pellet was resuspended in 50 µl of 1% BSA in PBS. After another 5-min spin at full speed and 4°C, supernatants (containing the specific phages) from the same clone were pooled, transferred to new tubes, and stored at 4 C with 0.03% sodium azide. Phagemids were purified form the bacterial pellets, digested with *Sfi*I, and visualized on a 1% agarose gel to check for presence of fragments of the appropriate size.

Sequence analysis of selected clones

Two clones were randomly selected from each of five backup plates. Clones from the same plate were designated by the plate number followed by "a" or "b". For example, clones 2a and 2b originated from plate 2, clones 3a and 3b form plate 3, and so on. Each colony was inoculated in 5 ml LB broth containing 50 µg/ml carbenicillin, and cultures were incubated overnight at 37°C and 250 rpm. Cells were pelleted at 14,000 rpm and 4°C for 5 min. Phagemids were extracted from the bacterial pellets using the Miniprep kit (Qiagen, Valencia, CA). Concentrations of the purified phagemids were measured by absorbance readings at 260 nm. To confirm the identity of the clones, 1 µg of each phagemid was digested with 6 units of *Sfi*I (Roche Molecular Systems, IN), and the digested phagemids were run on a 1% agarose gel. Phagemids were sequenced using vector-specific (ompseq and gback) primers (Table 3.2). Sequencing was performed using the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA)

at the Gene Technologies Lab, Institute of Developmental and Molecular Biology (Department of Biology, Texas A&M University, College Station, TX).

Table 3.2. Primers used for sequencing of individual clones (Andris-Widhopf et al., 2000).

ompseq	5' AAG ACA GCT ATC GCG ATT GCA GTG 3'
gback	5' GCC CCC TTA TTA GCG TTT GCC ATC 3'

Production of antibody fragments

The same clones (a total of 10, including the subclones) that were sequenced were used to produce antibody fragments. Each colony (picked from the clone backup plates) was inoculated into 2 ml pre-warmed SB medium containing 50 µg/ml carbenicillin. Cultures were incubated at 37°C and 300 rpm for 8 to 9 h (OD_{600} ~ 0.5), induced by adding Isopropyl-ß-D-thiogalactopyranoside (IPTG, Sigma, St Louis, MO) to 2 mM, and further incubated for 21 h at 37°C and 300 rpm. Cultures were centrifuged for 15 min at 2,800*g* and 4°C, and supernatants containing the antibody fragments were harvested and stored at -20°C. Cell pellets were resuspended in 300 µl of B-PER® Bacterial Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL), vortexed for 1 min, and centrifuged at 15,000*g* for 5 min. Supernatants (soluble fractions) were harvested, and pellets (insoluble fractions) were resuspended in 300µl of B-PER reagent. Samples from uninduced and induced cultures, induced culture supernatants, and soluble and insoluble fractions were checked for the presence of the antibody fragment by SDS-

PAGE. Ten µl of each sample was mixed with 3 µl reducing sample buffer, boiled for 10 min, cooled on ice, centrifuged, and loaded on a 10% polyacrylamide gel (Pierce Biotechnology, Rockford, IL). Samples were electrophoresed at 100 V for 45 mn, after which the gel was either stained with BioSafe Coomassie (Bio-Rad Laboratories, Hercules, CA), or transferred to a polyvinylidene fluoride (PVDF) membrane using the MiniTrans-Blot Cell (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked with 5% skimmed milk for 1 hour, then probed with mouse anti-HA (Roche Molecular Systems, Indianapolis, IN) at 1 µg/ml, overnight at 4ºC. The membrane was then washed with TBST for 4 times, 5 min each. The secondary antibody, goat anti-mouse IgG (Jackson Immunoresearch, Laboratories, West Grove, PA) was then added at a 1:6000 dilution, and the membrane was incubated for 1 h with gentle mixing. All dilutions were made in TBST. After washing as before, the membrane was further washed once with alkaline phosphatase buffer (0.1 M Tris-HCl, 0.1 M NaCl, and 50 mM $MgCl_2$, pH 9.5). One tablet of NBT/BCIP (Nitro-Blue Tetrazolium Chloride and 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt, Sigma, St Louis, MO) was dissolved in 20 ml of water. The membrane was incubated in this substrate solution until a suitable signal-to-noise ratio was achieved, at which point the reaction was stopped with 20 mM EDTA and the membrane was washed extensively in distilled water, then dried between paper towels, and imaged on an HP flatbed scanner.

Production of antibody fragments without the gene III fragment

Antibody fragments without pIII were produced by introducing phagemid DNA from 4 of the 10 selected clones into TOP10 cells (Invitrogen, Carlsbad, CA). Phagemid

DNA (20 ng) was transformed into 40 µl of competent TOP10 cells by electroporation as described for the library construction. Three milliliters of pre-warmed SOC medium were added to the cells, followed by incubation at 37°C and 300 rpm for 1 h. Cells were diluted 10- and 100-fold, and 100 µl of each dilution was plated on prewarmed LB/carbenicillin plates. Plates were incubated overnight at 37°C. Clones from the plates were used to inoculate and induce cultures; supernatants and cell lysates were prepared and tested by Western blot as described above.

Results

Construction of the test library

Reverse transcription of RNA extracted from spleens and bone marrow of chickens immunized with *E. tenella* parasites generated a cDNA pool which was subsequently used to amplify variable regions of the heavy and light antibody genes. V_H and V_L products were purified to eliminate primers, dNTPs and polymerase. A secondary PCR reaction was used to generate scFv fragments. This reaction included primers that incorporate the *Sfi*I cloning sites into the final product, thus allowing for subsequent cloning into the phagemid vector. The scFv product was successfully purified using the Qiagen PCR purification kit, which is also adequate for removal of restriction enzymes. Identities of V_H, V_L, and scFv products (400, 350, and 750 bp, respectively) were confirmed by agarose gel electrophoresis (Figure 3.2) and sequencing (data not shown).

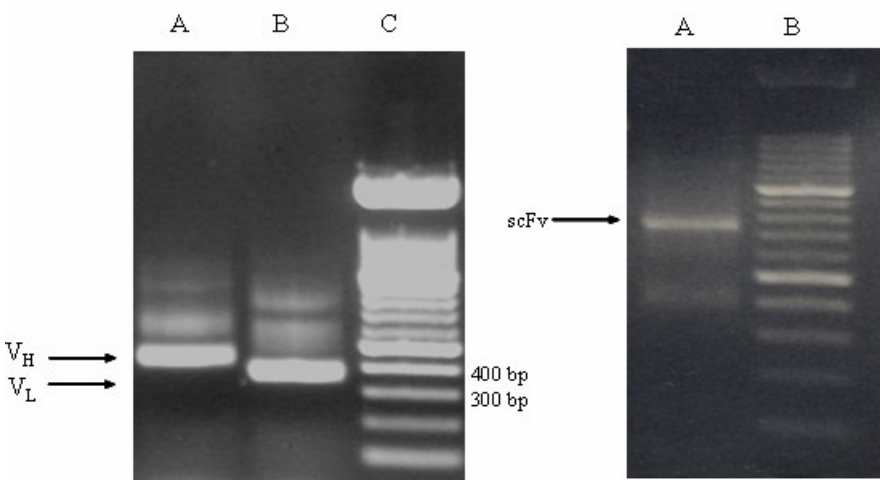

Figure 3.2. Amplification of V_H, V_L, and scFv fragments. (L) Lanes A and B: V_H (400 bp) and V_L (350 bp) fragments, respectively; Lane C: 100 bp ladder. (R) Lane A: scFv (750 bp); Lane B: 100 bp ladder.

The pComb3X vector and the scFv insert were prepared for cloning by digestion with *Sfi*I. *Sfi*I is a rare-cutting enzyme with the recognition sequence GGCCNNNN^NCCGG, a site that is almost never found in immunoglobulin genes. The use of unique 5' and 3' *Sfi*I restriction sites allows for directional cloning into the vector (Scott and Barbas, 2001). The source of the enzyme greatly influenced the efficiency of vector digestion. Enzyme obtained from Roche Molecular Systems, (Indianapolis, IL) was much more efficient than one from New England Biolabs, (Ipswich, MA). The former is sold in a more concentrated form (40U/µ) than the latter (20U/µl). Both were, however, present at the same final concentration in the digestion reactions (6U/µg vector DNA). Almost no uncut vector remained after digestion with the concentrated *Sfi*I. Even when the less concentrated *Sfi*I was used at double the amount recommended, more than half of the amount of vector was left uncut (Figure 3.3). The digested vector and its stuffer fragment were gel-purified, taking care to allow the gel to run slowly to

achieve good separation. The Qiagen kit used for purification produced clean preparations in less than 30 min, and was superior to other purification methods. Gel digestion with agarase (New England Biolabs, Ipswich, MA) gave clean preparations as well, but was time-consuming. Electroelution using the Quik-Pik capsules (Stratagene, La Jolla, CA) was time-consuming, cumbersome, and yielded less-than-average results.

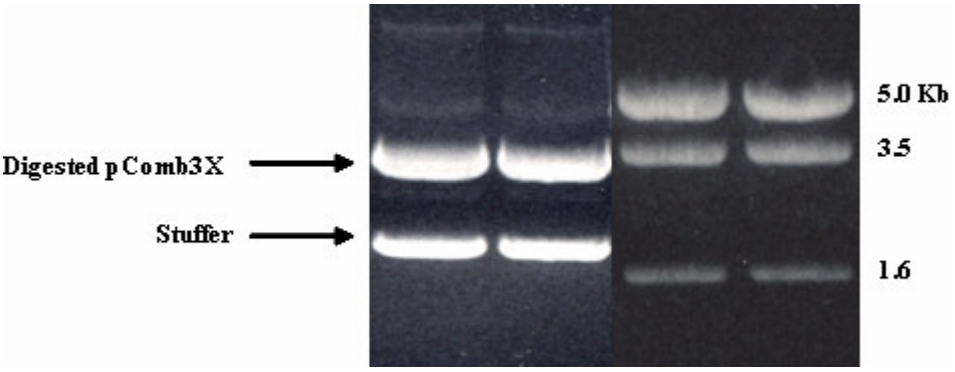

Figure 3.3. Comparison between digestion efficiencies of *Sfi*I obtained from two different sources. Lanes A and B: digestion with *Sfi*I (40U/µl) from Roche Molecular Systems, Indianapolis, IL. Lanes C and D: Digestion with *Sfi*I (20U/µl) from New England Biolabs Ipswich, MA.

The ligase buffer used in all ligation reactions was free of polyethylene glycol, which presence previously inhibited transformation into *E. coli* cells. Heat-inactivation of the ligation mixture prior to electroporation did not improve, and in some cases even decreased transformation efficiency, and was altogether skipped.

Electroporation resulted in higher transformation efficiency (number of transformants/µg DNA) than chemical transformation, and was hence used throughout. The number and duration of pulses needed for electroporation was empirically

determined, and 1-mm gap cuvettes were used as recommended by the manufacturer of the competent cells.

Ligation of the vector and its stuffer resulted in 1.5 x 10^7 cfu/µg. The vector-insert ligation produced 1.8 x 10^7 cfu/µg. Background ligation, as indicated by self-ligation of the vector, was less than 5%. Phagemids were purified from the cells transformed with vector and insert or vector and stuffer. Following *Sfi*I digestion, fragments of the expected size were visualized on a 1% agarose gel (Figure 3.4).

Library construction and transformation

Four library ligations were prepared, precipitated, and introduced into electrocompetent XL1-Blue cells by electroporation. The library size, i.e. the total number of independent transformants obtained from the four ligations combined was 7.4 x 10^7, or 5.3 **x** 10^7 cfu/µg vector DNA.

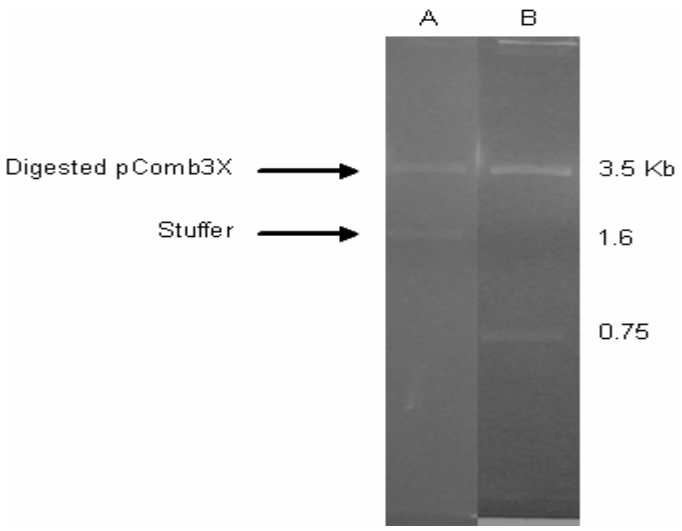

Figure 3.4. Digested plasmids purified from cells transformed with ligation reactions.
Cells were transformed with (A) vector (~3.5 Kbp) and stuffer (~1.6 Kbp), or (B) vector and scFv insert (750 bp), and digested with *Sfi*I.

Panning

The antibody library was taken through five rounds of panning on *E. tenella* cryopreserved sporozoites. Parasites had been previously cryopreserved and were thus available for each day of panning. After incubation of the library with the sporozoites and washing away unbound and weakly- bound phage, phage particles were eluted and used to infect an *E. coli* culture. Phage was then rescued by the addition of helper phage which provided the proteins and enzymes needed for replication. At this point, the addition of carbenicillin and kanamycin ensured the survival of only those cells containing both the helper and phage genomes. At the end of each panning round, phage was precipitated by the addition of a polyethylene glycol/sodium chloride solution. Before resuspending the phage pellet, care was taken to remove all traces of supernatant, because any remaining PEG can interfere with resuspension of the phage. The phage was then reamplified by repeating the panning cycle. In this manner, specific binding clones were selected and amplified. Input and output titers from each round appear in Table 3.3.

Table 3.3. Results of panning rounds of the antibody library against *E. tenella* sporozoites. % Bound = (output/input) x 100. Enrichment = fold increase of % bound compared to the previous round.

Round	Input	Output	% Bound (x 10^{-4})	Enrichment
1	1.65 x 10^{12}	2.4 x 10^{4}	0.015	
2	1.8 x 10^{11}	1 x 10^{5}	0.556	38.2
3	5 x 10^{11}	2.2 x 10^{7}	44	79.2
4	2x 10^{11}	7.6 x 10^{7}	380	8.6
5	7.5 x 10^{11}	8.5 x 10^{7}	113	0.6

A 1000-fold increase in output was obtained after 3 rounds of panning. There was clear enrichment of the positive clones over the panning rounds, where the increased washing stringency selected for specific-binding clones. The 3rd panning round resulted in a 3,000-fold enrichment over the first round, as the binding clones became the dominant population in the library.

Precipitation of antibody-displaying phage

Phage was precipitated from individual clones picked from the output plates of the last round of panning. Phagemids purified from these clones and digested with *Sfi*I yielded fragments of the expected size (Figure 3.5).

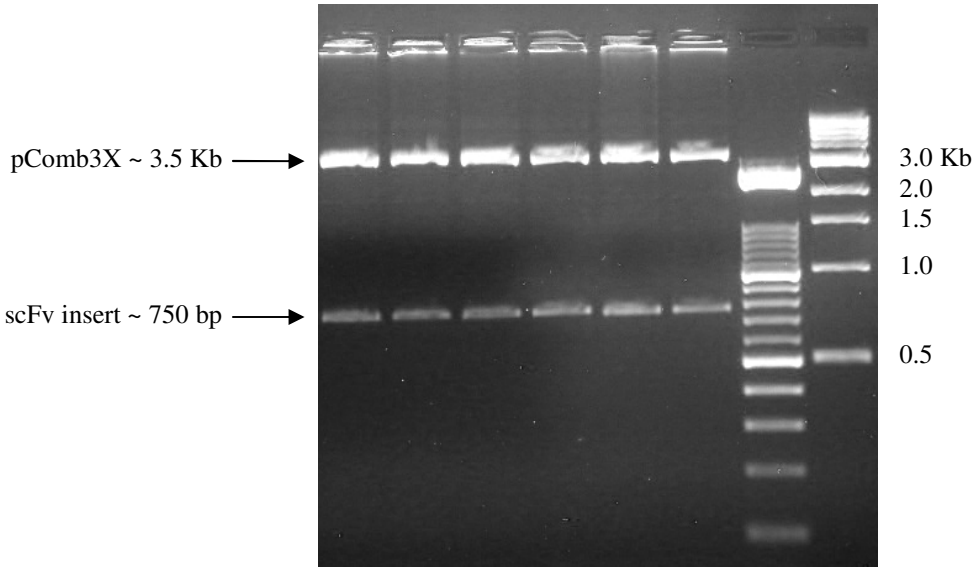

Figure 3.5. *Sfi*I-digested phagemids from individual clones of the fifth round of panning. Each digested phagemid yielded the vector fragment (~3.5 Kbp) and the scFv insert (750 bp).

Sequence analysis of selected clones

Sequence identification

Phagemids purified from all 10 clones were sequenced using vector-specific primers (Table 3.2). Ompseq is specific for the omp leader sequence, and gback is specific for the gIII fragment. A representative clone (2a) was chosen to delimit the location of primers, restriction sites, HA and 6-His tags, and the amber stop codon (Figure 3.6). Another clone (3a) was used to identify framework and complementarity-determining regions in the V_L (Figure 3.7) and V_H (Figure 3.8) chains, and compare each chain to the respective germline sequence.

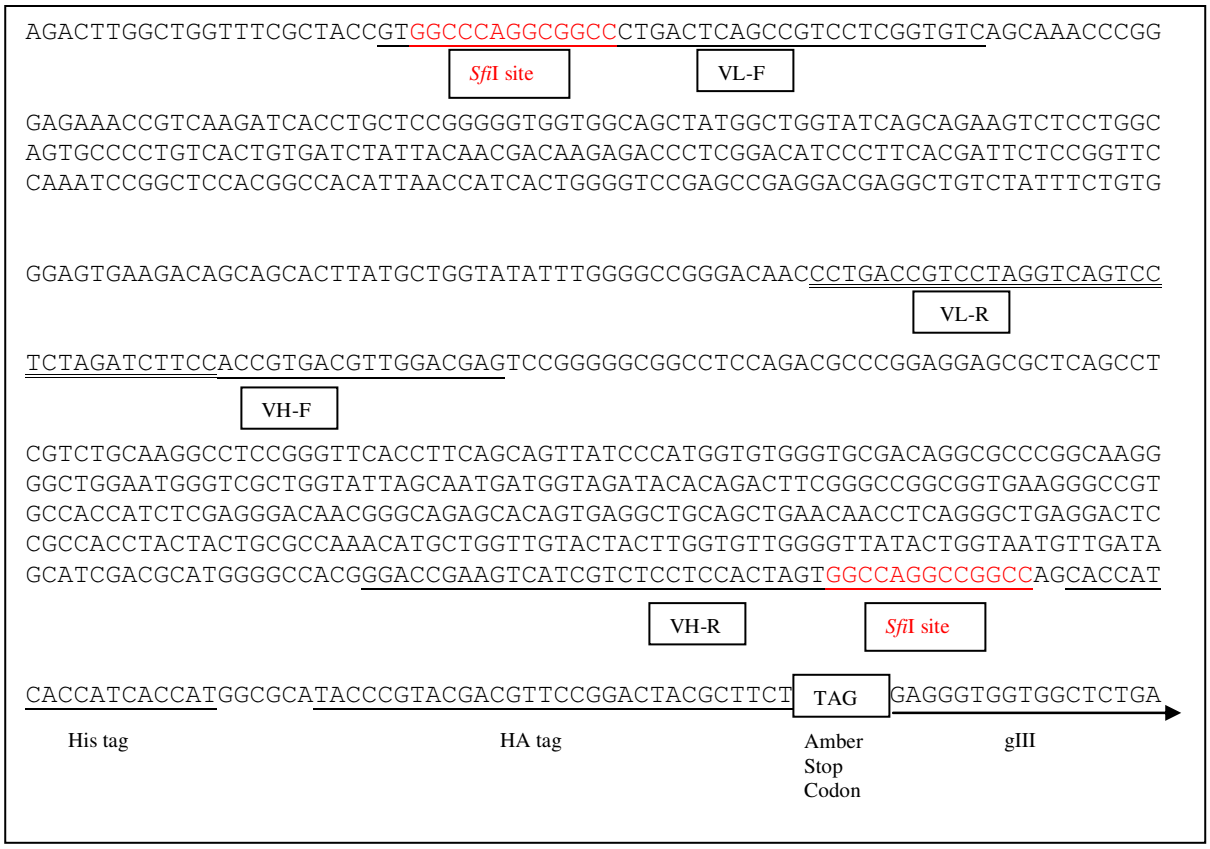

Figure 3.6. Delineation of key segments in a representative clone.

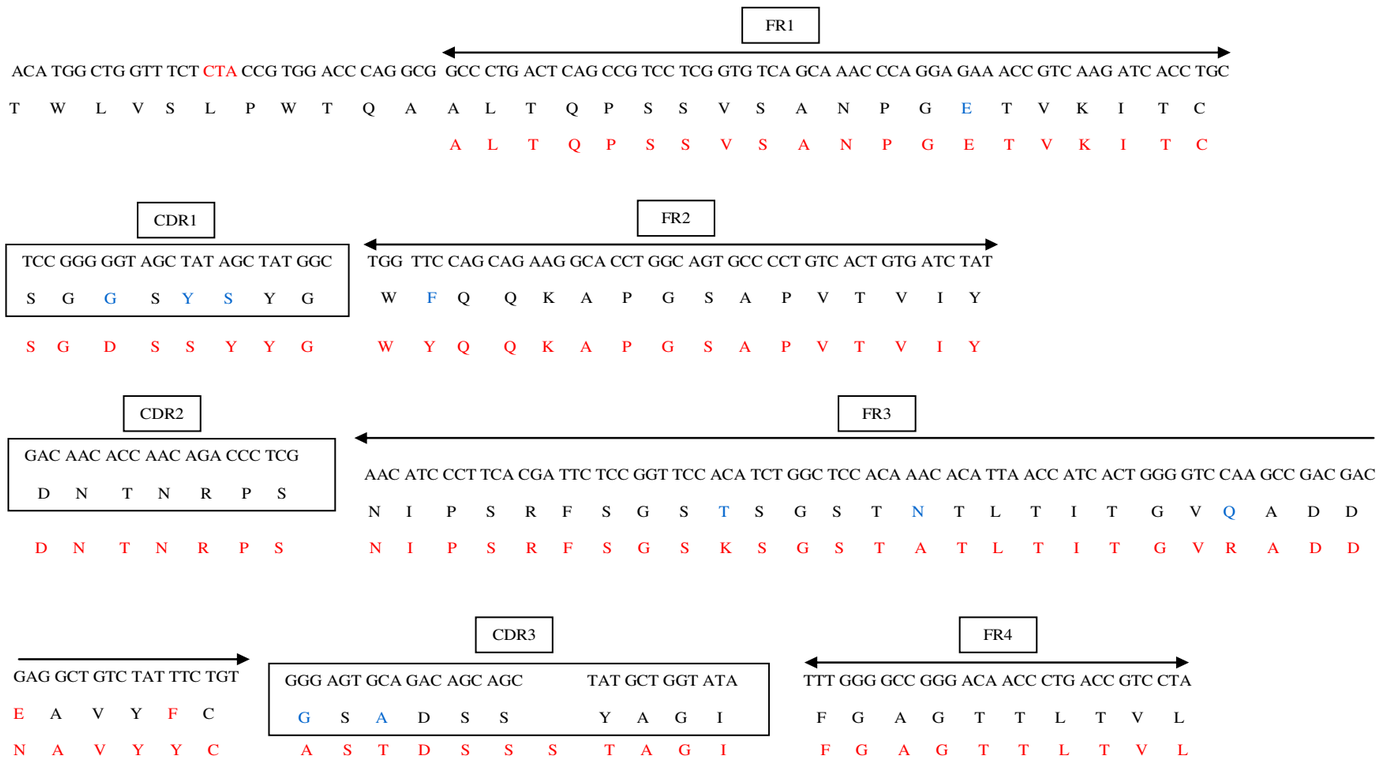

Figure 3.7. Alignment of the nucleotide and amino acid sequences of the V_L chain of clone 3a to the germline sequence (shown in red). Framework regions (FR) and Complementarity-determining regions (CDR) are also shown. Residues differing from the germline sequence are colored in blue.

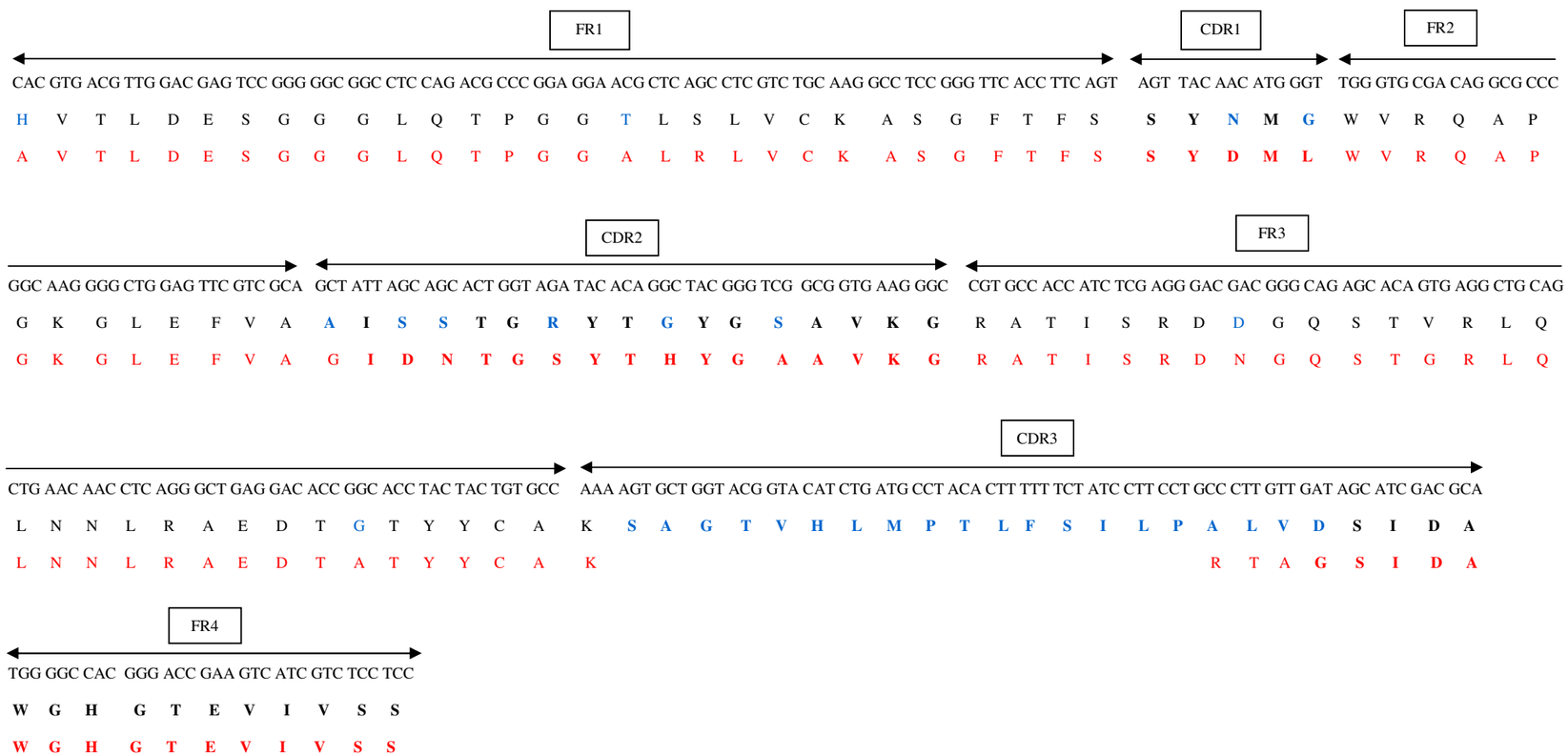

Figure 3.8. Alignment of the nucleotide and amino acid sequences of the V_H chain of clone 3a to the germline sequence (shown in red). Framework regions (FR) and Complementarity-determining regions (CDR) are also shown. Residues differing from the germline sequence are colored in blue.

Sequence alignment

Nucleotide sequence alignment and homology searches were performed with BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Subclones that originated from the same parent clone (e.g. 2a and 2b, 3a and 3b…) were 99% similar. An alignment example between clones 2a and 3a is shown in Figure 3.9. Although from different parent clones, these 2 clones were 91% identical.

```
3a Query  4    TGGCTGGTTTCTCTACCGTGGCCCAGGCGGCCCTGACTCAGCCGTCCTCGGTGTCAGCAA  63
2a Sbjct  4    ...........G.................................................  63

   Query  64   ACCCAGGAGAAACCGTCAAGATCACCTGCTCCGGGGGTAGCTAT-AGCTATGGCTGGT-T  121
   Sbjct  64   ....G.............................G.-.GGC.............A.  122

   Query  122  CCAGCAGAAGGCACCTGGCAGTGCCCCTGTCACTGTGATCTATGACAAC-ACCAACAGAC  180
   Sbjct  123  .-.........T.T..............................T.....G..-..G....  180

   Query  181  CCTCGAACATCCCTTCACGATTCTCCGGTTCCACATCTGGCTCCACAAACACATTAACCA  240
   Sbjct  181  .....G............................A...C.........GGC............  240

   Query  241  TCACTGGGGTCCAAGCCGACGACGAGGCTGTCTATTTCTGTGGGAGTGCAGACAGCAGC-  299
   Sbjct  241  ............G......G.............................A...........A  300

   Query  300  -T-ATGCTGGTATATTTGGGGCCGGGACAACCCTGACCGTCCTAGGTCAGTCCTCTAGAT  357
   Sbjct  301  C.T.........................................................  360

   Query  358  CTTCCACCGTGACGTTGGACGAGTCCGGGGGCGGCCTCCAGACGCCCGGAGGAACGCTCA  417
   Sbjct  361  ...........................................................G......  420

   Query  418  GCCTCGTCTGCAAGGCCTCCGGGTTCACCTTCAGTAGTTA-CAACATGG-GTTGGGTGCG  475
   Sbjct  421  ..................................C.....T.C-.....T..-.......  478

   Query  476  ACAGGCGCCCGGCAAGGGGCTGGAGTTCGTCGCAGCTATTAGCA--GCACTGGTAGATAC  533
   Sbjct  479  ........................A.GG.....T.G........AT.-.-..........  536

   Query  534  ACAGGCTACGGGTCGGCGGTGAAGGGCCGTGCCACCATCTCGAGGGACGACGGGCAGAGC  593
   Sbjct  537  ....A..T....C.....................................A...........  596

   Query  594  ACAGTGAGGCTGCAGCTGAACAACCTCAGGGCTGAGGACACCGGCACCTACTACTGTGCC  653
   Sbjct  597  ....................................T...C............C...  656

   Query  654  AAA-AGTGCTGGT  665
   Sbjct  657  ...C.-.......  668


   Query  710  TGTTGATAGCATCGACGCATGGGGCCACGGGACCGAAGTCATCGTCTCCTCC  761
   Sbjct  701  ..................................................  752
```

Figure 3.9. Nucleotide alignment of clones 2a and 3a. Identities are indicated with a dot and differences with insertion of the appropriate nucleotide. Sequence gaps were introduced to maximize alignment.

Amino acid sequences of V_H and V_L chains from 5 representative clones (2a, 3b, 5a, 8a, and 9b) were aligned and compared to the germline sequences using Clustal W program (European Bioinformatics Institute, Cambridge, UK). The sequences were grouped according to differences in their heavy chain CDR, and 3 groups were thus obtained (Figure 3.10). All 5 clones showed extensive gene conversion events, represented by amino acid insertions, in the CDR3 of the heavy chain. Gene conversion by amino acid deletions were also noted in the CDR3 of the light chains of clones 9b and 8a (one and two deletions, respectively). Somatic mutation events in the light and heavy chains were also apparent through amino acid substitutions, an indication of affinity maturation.

Production of antibody fragments

Ten clones obtained from the last selection round were used to produce antibody fragments. Cultures were induced with IPTG, and samples from uninduced and induced cultures were run on an SDS-PAGE gel. The antibody fragment banded at ~ 35 KDa, with much greater intensity in the induced versus the uninduced cultures (Figure 3.11).

(A)

```
VL          FR1                    CDR1        FR2            CDR2      FR3                                   CDR3        FR4
GL ALTQPSSVSANPGETVKITC SGDSSYYG WYQQKAPGSAPVTVIY DNTNRPS NIPSRFSGSKSGSTATLTITGVRADDNAVYYC ASTDSSSTAGI FGAGTTLTVL
2a  --------------------- --GGGS-- ------ S------------ Y--K--- D----------------------- E- E- - -F- G-E---TY--- ----------
5a  --------------------- --GGGS-- ------ S------------ Y-DK--- D------------------------ E- E- - -F- G-E---TY--- ----------
3b  --------------------- --G-YS-- -F--- ------------- -------  ---------------------Q--- E- --F- G-A--- Y--- ----------
9b  --------------------- --G--YS-- -F--- ------------- -------  ---------------------Q---E- -- F- G-A---Y--- ----------
8a  -----------------E-- --S-GNN- ------ S------------ ES-K---  ---------------------Q--- E- - -F- G-A--- GA- ----------
```

(B)

```
VH            FR1                          CDR1     FR2              CDR2                FR3
GL AVTLDESGGGLQTPGGALRLVCKASGFTFS SYDML WVRQAPGKGLEFVA GIDNTGSYTHGAAVKG RATISRDNGQSTGRLQLNNLRAEDTATYYCAK

2a  T-------------------S------------ --P-V ------------W-- --S-D-R--DF-P---- --------------------------------
5a  T-------------------S------------ -- P-V ------------W-- --S-D-R--DF-P---- --------------V-----------S-------
3b  T-------------------S------------ -- N-G ------------ F-- A-SS- R--GY-S---- -------D------------------------
9b  T-------------------S------------ -- N-G --------------- A-SS- R--GY-S---- -------D------------------------
8a  A-------------------S------------ G-N-G ------------ I- S-SN----DY------ --------------------------------
```

```
           CDR3                 FR4
GL                 RTAGSIDA WGHGTEVIVSS
2a  HAGCTTWCWGYTGN     VDSIDA -----------
5a  SAGCTTWCWGYTGN     VDSIDA --T-PKSSSLH
3b  SAGTVHLMPTLFSILPALVDSIDA -----------
9b  SAGTVHLMPTLFSILPALVDSIDA -----------
8a  SAGTVHLMPTLFSIFPALADSIDA -----------
```

Figure 10. Comparison of amino acid sequences of (A) V_L, and (B) V_H chains. Clones are aligned relative to the germline sequence (GL), and are grouped according to the heavy-chain CDR alignment. Identities are indicated by hyphens. Framework regions (FR) and Complementarity-determining regions (CDR) are shown.

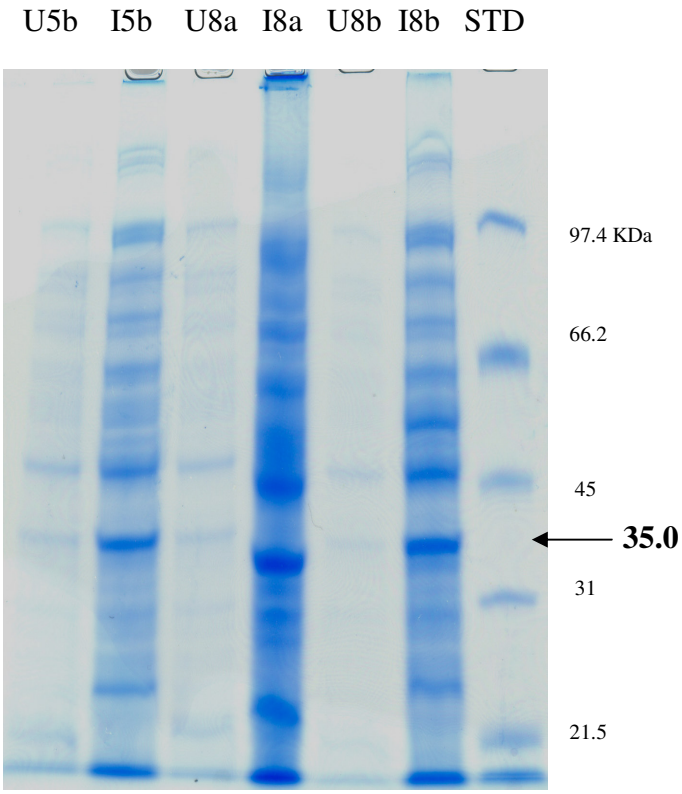

Figure 3.11. SDS-PAGE of uninduced (U) and induced (I) culture samples of clones 5b, 8a, and 8b. The gel was stained with Coomassie blue. STD = Molecular weight standard, low range (Bio-Rad Laboratories, Hercules, CA).

Induced culture supernatants containing antibody fragments were harvested. Supernatant proteins were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was probed with mouse anti-HA. This antibody recognizes the hemagglutinin peptide sequence (YPYDVDDYAS) that is inserted at the 3' carboxyl terminal of the fusion protein. Antibody fragments (~ 30 KDa) were detected in all tested samples, albeit at different banding intensities. For example, antibody concentration in supernatants of cultures 3, 8, and 9 was apparently higher than that of cultures 2 and 5 (Figure 3.12). This correlates well with the observation that cultures 2 and 5 did not grow as well as the others, and thus had not reached an OD_{600} of 0.5 at the time of induction.

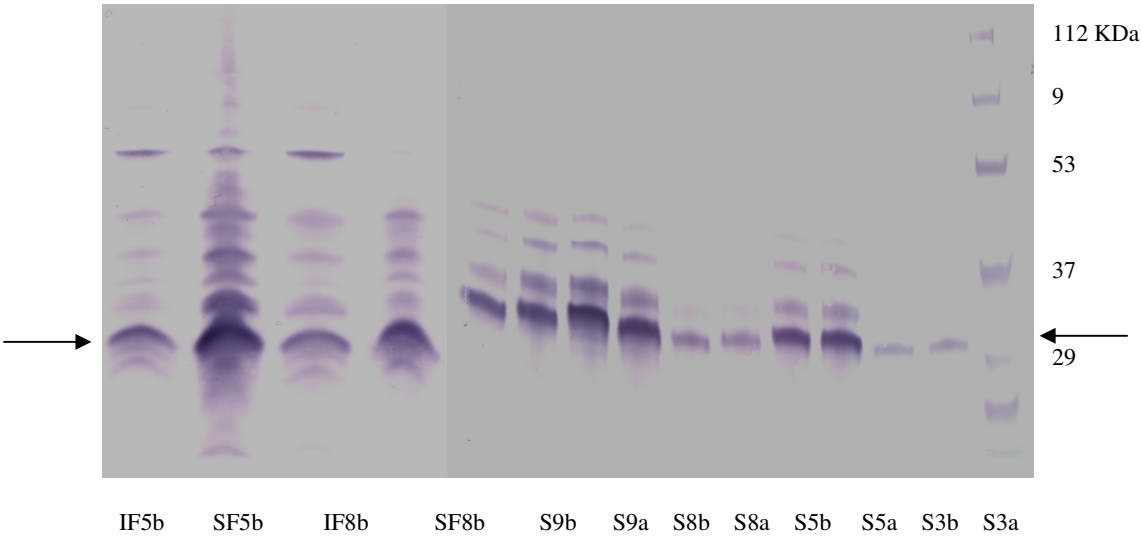

Figure 3.12. Detection of fusion proteins in induced culture supernatants (S), soluble (SF), and insoluble fractions (IF). Protein samples were separated by SDS-PAGE, transferred to a PVDF membrane, and detected by an antibody that recognizes the HA tag. STD = Prestained molecular weight standard (Bio-Rad Laboratories, Hercules, CA).

Antibody fragments were also recovered from soluble and insoluble fractions obtained after protein extraction from the induced cell pellets. Following SDS-PAGE and immunoblotting, fusion proteins were detected in the soluble fractions, and to a lesser extent in the insoluble fractions (Figure 3.12). In general, less of the fusion protein was present in the cell pellets than in the supernatants.

Production of antibody fragments without the gene III fragment

Antibody fragments without pIII were produced by introducing phagemid DNA form the 4 selected clones into TOP10 cells. When this non-suppressor *E. coli* strain is infected by phagemid DNA, the amber stop codon located just upstream of gIII is read, and soluble proteins (in this case scFv) are produced (Figure 3.13). Expression in TOP10 cells was higher than in XL1-Blue cells, as evidenced by presence of heavier bands in western blot of supernatants harvested from the former.

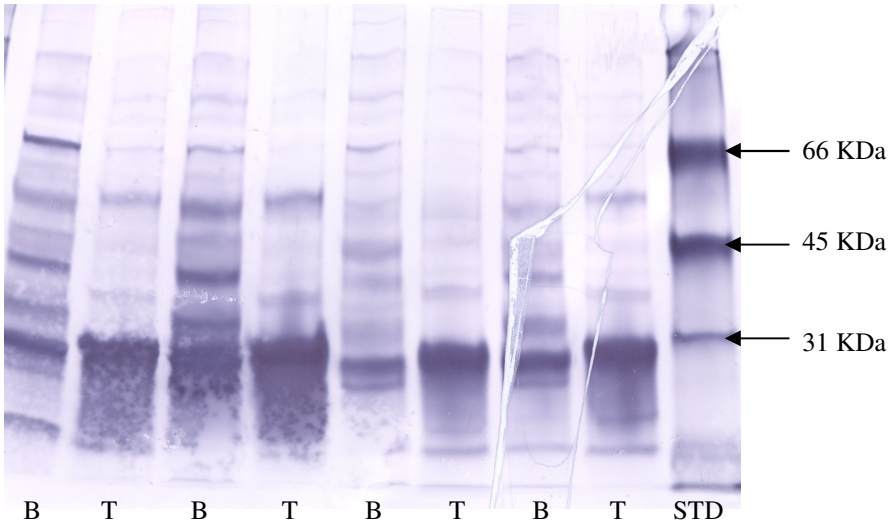

Figure 3.13. Western blot detection of antibody fragments from culture supernatants of XL1-Blue (B) or TOP10 (T) - transformed cells. STD = biotinylated molecular weight standard (Bio-Rad Laboratories, Hercules, CA).

Discussion

A chicken antibody library against *Eimeria tenella* sporozoites was constructed. The process was fraught with technical difficulties that were eventually surmounted, as "mistakes are the portals of discovery", according to James Joyce. These obstacles, and ways to dodge them, represent the kind of "details" that are generally left out of published Materials and Methods sections, but that are nevertheless essential to success. Each step was carried to completion by testing a host of approaches and choosing the one that best suited our needs and met our expectations. Familiarity with many of the techniques described in this chapter was acquired after attending the "Phage Display of Proteins and Peptides" course, offered yearly at the Cold Spring Harbor Laboratory,

Cold Spring Harbor, New York. However, in many cases, we digressed from the course recommendations, as we found approaches that worked better in our hands.

One major consideration for successfully constructing an antibody library is the quality of the vector. In this experiment, incorporation of *Sfi*I sites into the scFv insert allowed for directional cloning into the vector. Vector and insert were prepared for ligation by *Sfi*I digestion. The outcome of the vector digestion reaction was greatly influenced by the original concentration of the restriction enzyme, keeping in mind that it was always used at 6U/µg DNA. Concentrated *Sfi*I (40U/µl) constantly performed better than the less concentrated one (20U/µl), leaving almost no undigested vector behind. Digestion efficiency of the less concentrated enzyme was improved by using it at double the recommended amount, but was still not comparable to that of the concentrated enzyme. There is no explanation for this phenomenon that we are aware of. Digested vector and insert were then purified to eliminate contaminants such as salt and the restriction enzyme, the presence of which can inhibit ligation and transformation. The digested scFv was easily purified using Qiagen's PCR purification kit, which works equally well to eliminate restriction enzymes. Vector purification was a more crucial and a little bit more complicated step, which needed to be optimized to ensure high transformation efficiency and low background (Scott and Barbas, 2001). The double-cut vector had to be separated from its stuffer fragment and from any uncut or single-cut vector. Gel purification was thus the only possible purification method, and the gel was to be allowed to run long enough (4-5 hours at 50V) to achieve clear separation between all fragments. Purification of the DNA fragments from the gel was

best done using the Qiagen gel extraction kit. Other methods that were tried included digestion with β-agarase and electroelution with Strategene's Quik-Pik kit (recommended at the "Phage Display" course). The latter didn't perform well in our hands, in addition to being complicated and time-consuming. The β-agarase method was comparable to the Qiagen kit in terms of product yield and purity, but had the disadvantage of being time-consuming, requiring at least 2.5 hr to complete, compared to 30 min with the Qiagen kit.

Digested vector and insert were ligated overnight at room temperature, although incubation for a lesser amount of time would probably work equally well, as this was a cohesive-end ligation. The ligation mixture was not heat-inactivated prior to electroporation, as this practice did not seem to improve, and, in some cases, even decreased transformation efficiency. As expected, electroporation produced more transformants/μg DNA than chemical transformation. This is fundamental for antibody library construction, as the complexity of the library, i.e. its diversity, is considered to be equal to the number of independent transformants, i.e. the library size. The probability of selecting specific antibodies increases with the library size (Rader et al., 2001). Electroporation is straightforward and produces consistent results, as long as one works quickly, while still handling the competent cells as gently as possible. Arcing during electroporation can be prevented by tapping the cuvette, in order to make sure that its contents are dispersed evenly on the bottom, and that no air bubbles are present. The cuvette and the electric contacts of the electroporator also need to be dry. Some researchers also recommend cleaning up the DNA ligation mixture before

electroporation, to get rid of ligase and any salt present in the sample. In our experience, this is not necessary, as the DNA sample is too dilute to cause any arcing.

The library was panned against the cell surface of intact sporozoites, and specific antibodies were isolated. This option was the only one that could theoretically yield antibodies capable of blocking invasion of enterocytes by sporozoites. Panning offered an additional challenge, as it required freshly prepared phage and readily available sporozoites on each of the five days of the panning process. It is practically impossible to prepare sporozoites and use them for a panning round on the same day. This problem was circumvented by freeze-preservation of sporozoites in liquid nitrogen; the needed number could be thawed out on the day of panning and used directly (see Chapter II). Panning is a tedious process, and cell surface panning is even more so. Repetitive washing steps are critical in the panning process, as appropriate washing with increasing stringency is an absolute necessity to ensure the selection of highly specific-binding clones (Smith and Petrenko, 1997). Unless the cells are immobilized, the only way to perform the washing steps is by centrifugation. This only makes the process more onerous, and less user-friendly than panning on immobilized antigens. The latter is simply a variation on common ELISA procedures; the former resembles more the immunostaining for flow cytometry, with the exception that the washing steps are much more critical to success in a panning procedure.

The production of antibody fragments was achieved by inducing cell cultures with IPTG for 20 h. Induction for this long time should increase the secretion of antibody fragments into the culture supernatant (Steinberger et al., 2001). On an SDS-

PAGE gel, the antibody fragment banded at ~ 35 KDa, with greater intensity in the induced versus the uninduced cultures. However, the former were grown for a longer period of time, and induced samples subsequenlty contained more protein. As a future improvement to the procedure, and to properly evaluate induction, one clone will be grown to an OD of ~0.5, the culture will then be divided in two, and only one half will be induced. Both cultures will then be grown for 20h, after which samples from both will be tested by SDS-PAGE. In this manner, "housekeeping" proteins can be identified and will be expected to have similar intensities in both samples. The band corresponding to the antibody fragment, however, will be expected to be heavier in the induced sample, an observation that can be then interpreted with confidence as a result of IPTG induction. Characterization of antibody fragments in culture supernatants and cell lysates had to be done arbitrarily, in the sense that the antibody concentration in any given sample was not known. The optimal sample volume to be loaded on the gel for electrophoresis and subsequent immunodetection had to be determined empirically. Minor accessory bands were observed by immunblotting of antibody fragments (Figure 3.12), a result of gel overloading, and/or false detection by the anti-HA antibody. Unexpectedly, immunoblotted soluble antibodies appeared to have a higher molecular weight than fusion proteins (Figure 3.13). We are not aware of a definite explanation for this phenomenon, and can only hypothesize that it might have something to do with the amber stop codon being suppressed by TOP10 cells, or being read by XL1-Blue cells. Production of soluble antibody fragments has also been obtained by proteolysis of the antibody-gene III fragment in the periplasmic space of non-suppressor *E. coli* (Andris-

Widhopf et al., 2000). Another worthy observation is the fact that expression in TOP10 cells is higher than in XL1-Blue cells (as estimated by the broadness of the bands), possibly due to the fact that soluble antibodies fold better than fusion proteins (Arndt et al., 1998).

A potential improvement of this approach would be to exploit the presence of the 6-His tag to purify the antibodies to homogeneity by affinity chromatography. The pure end product could then be quantified, and the characterization tests could be standardized and optimized.

CHAPTER IV

IMMUNOCHEMICAL CHARACTERIZATION OF SELECTED ANTIBODIES

Introduction

The ultimate goal of antibody library construction is the isolation of specific, high-affinity antibodies against the antigen of interest. The success of the technology is hence gauged by analyzing selected antibodies at the polyclonal (phage pools), and at the clonal (phage clones and antibody fragments) levels (Steinberger et al., 2001). An antibody library against *Eimeria tenella* sporozoites was constructed and subjected to five selection rounds. The objective of this experiment was to characterize the selected antibodies. Phage pools, antibody-displaying phage clones, and antibody fragments were tested for their specificity by enzyme-linked immunosorbent assay and immunoblotting.

Materials and methods

Immunochemical analysis of phage pools and individual clones

Enzyme-linked immunosorbent assay

Antibody-displaying phage pools from the unpanned library and from all five rounds of panning, as well as 10 antibody-displaying clones (produced as described in Chapter III) were analyzed for binding to *Eimeria tenella* sporozoites. Phage pools were run in duplicate, while individual clones were not, due to the limited availability of sporozoites. Each of the 16 phage samples (30 µl) was blocked with 20 µl of 3%

BSA/PBS for one hour on ice. Cryopreserved sporozoites were thawed, washed once in serum-free DMEM and once in PBS, and resuspended in flow cytometry buffer (PBS containing 20 mM HEPES, 1% BSA, pH 7.4) at 10^7/ml. Fifty microliters of cell suspension ($5x10^5$ cells) were added to each of 17 wells of a V-bottom microtiter plate (Fisher Scientific, Pittsburgh, PA). Phage samples (50 µl) were added to the cells, mixed gently, and incubated at room temperature and 160 rpm for one hour. Omission of the primary antibody, in this case the phage, served as a negative control. Cells were pelleted at 750*g*, 3 min, with the centrifuge brake off. The supernatant was removed and the cells were resuspended in 200 µl flow cytometry buffer and centrifuged as above. This washing step was repeated two more times. All washes and dilutions were made in flow cytometry buffer. Cells were resuspended in 50 µl of mouse anti-M13 (GE Healthcare, Piscataway, NJ) at 10 µg/ml, and incubated as above. After washing, cells were incubated with 1 µg/ml peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoreserach Laboratories, West Grove, PA). Detection of bound antibody was completed by addition of BD OptEIA™ TMB substrate (BD Biosciences, San Diego, CA). The reaction was stopped by the addition of 10% sulfuric acid. Cells were pelleted, the supernatants were transferred to a flat-bottom ELISA plate, and absorbances were read at 450 nm Wallac plate reader (PerkinElmer, Boston, MA).

Immunofluorescence assay

This assay was carried out similarly to the ELISA, with the exception that the detection antibody was FITC-conjugated anti-mouse IPTG (Jackson Immunoreserach Laboratories, West Grove, PA) at 1:600. Following the last wash, cells were

resuspended in 50 µl flow cytometry buffer, transferred to a clean glass slide, and left to dry. Slides were then mounted with HardSet Vectashield mounting medium (Vector Labs, Burlingame, CA), and observed with an Olympus fluorescence microscope.

Analysis of antibody fragments

The specificity of antibody fragments produced after IPTG induction was evaluated. Two subclones from each of 5 clones were analyzed. Subclones were designated by an Arabic numeral followed by "a" or "b". For example, clones 2a and 2b were subclones of parent clone 2, clones 3a and 3b of parent clone 3, and so on. Tested samples included antibodies secreted in the culture supernatants (S), as well as those recovered from the cell pellets (P). Soluble and insoluble fractions were prepared from the cell pellets as described in Chapter III. For the purpose of this experiment, these fractions were pooled for each sample, as both originated from the same cell pellet.

Enzyme-linked immunosorbent assay

All samples were analyzed by ELISA, essentially as described for the phage ELISA. Sporozoites ($1x10^5$/well) were incubated with the different primary antibodies (culture supernatants or pellets) or with flow cytometry buffer (negative control) for 1 hour with gentle rotation. After washing with flow cytometry buffer, sporozoites were incubated with mouse anti-HA (Roche Molecular Systems, Indianapolis, IL) at 1 µg/ml. Cells were then washed and resuspended in a 1:10,000 dilution of peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoreserach Laboratories, West Grove, PA). Detection of bound antibody was achieved by the TMB substrate reaction, as described

above. The reaction was stopped by the addition of sulfuric acid, and absorbance readings of the supernatants were measured at 450 nm.

Immunoblotting

Supernatants obtained from induced cultures of clones 8a and 9b transformed in TOP10 cells (as described in Chapter III) were tested by Western blotting. Cryopreserved *Eimeria tenella* sporozoites ($2x10^6$) were thawed, washed and resuspended in 300 µl flow cytometry buffer. Sporozoites were flash frozen in liquid nitrogen, and then thawed in a water bath at 37ºC. This freeze-thaw cycle was repeated 3 times, and was followed by sonication, twice at 50% output and 20 sec each. Complete disruption of the sporozoites was ascertained by microscopy. After addition of 3 volumes of 4x SDS sample buffer, the sporozoite preparation was boiled for 10 min, cooled on ice, and pelleted at 400*g* for 3 min. The supernatants (10 µl per lane) were run on a Precise™ 10% acrylamide gel (Pierce Biotechnology, Rockford, IL) at 100 V for 45 min. These gels are different from the traditional ones (Laemmli, 1970) in that they are cast in neutral pH and run in Tris-SDS-HEPES running buffer. Proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane using the MiniTrans-Blot Cell (Bio-Rad Laboratories, CA) at 50 mAmp for 1h. Unless stated otherwise, all dilutions and washes were made in TBST, and all incubations were at room temperature with gentle mixing. The membrane was blocked for one hour with 5% skimmed milk, and cut in strips. Each strip was then incubated overnight at 4ºC with culture supernatants (diluted 1:10 in TBST) of TOP10 cells transformed with either of clones 5b, 8a, or 9b. Culture supernatant of an empty pComb3X-transformed clone was used as

a negative control. After washing, membranes were incubated for 1 h with a l:1000 dilution of mouse anti-$(His)_6$ (Covance, Berkeley, California), which recognizes the histidine tag on the antibody fragments. Membranes were again washed, then incubated with biotinylated goat anti-mouse IgG (Jackson Immunoresearch, Laboratories, West Grove, PA) at a 1:80,000 dilution for 1 h with gentle mixing. After washing, membranes were incubated with alkaline phosphatase-conjugated streptavidin (1:1000) for 20 min. Membranes were washed in TBST and then once more with alkaline phosphatase buffer (0.1 M Tris-HCl, 0.1 M NaCl, and 50 mM $MgCl_2$, pH 9.5). One tablet of NBT/BCIP (Nitro-Blue Tetrazolium Chloride and 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt, Sigma, St Louis, MO) was dissolved in 20 ml of water. Membranes were incubated in this substrate solution until a suitable signal-to-noise ratio was achieved, at which point the reaction was stopped with 20 mM EDTA and the membrane was washed extensively in distilled water, dried between paper towels, and imaged using an HP flatbed scanner.

Results

Analysis of phage pools and individual clones

Enzyme-linked immunosorbent assay

Phage pools from all panning rounds, as well as antibody-displaying phage clones tested positive (Figure 4.1). Specific binding increased along the panning rounds, reached a maximum at the third round, and then decreased slightly, reaching a plateau after 5 selection rounds. Moderate specific binding was observed with the unpanned

library, but it was still less significant than obtained with any of the panned phage pools or the individual clones. No signal was detected in the negative control.

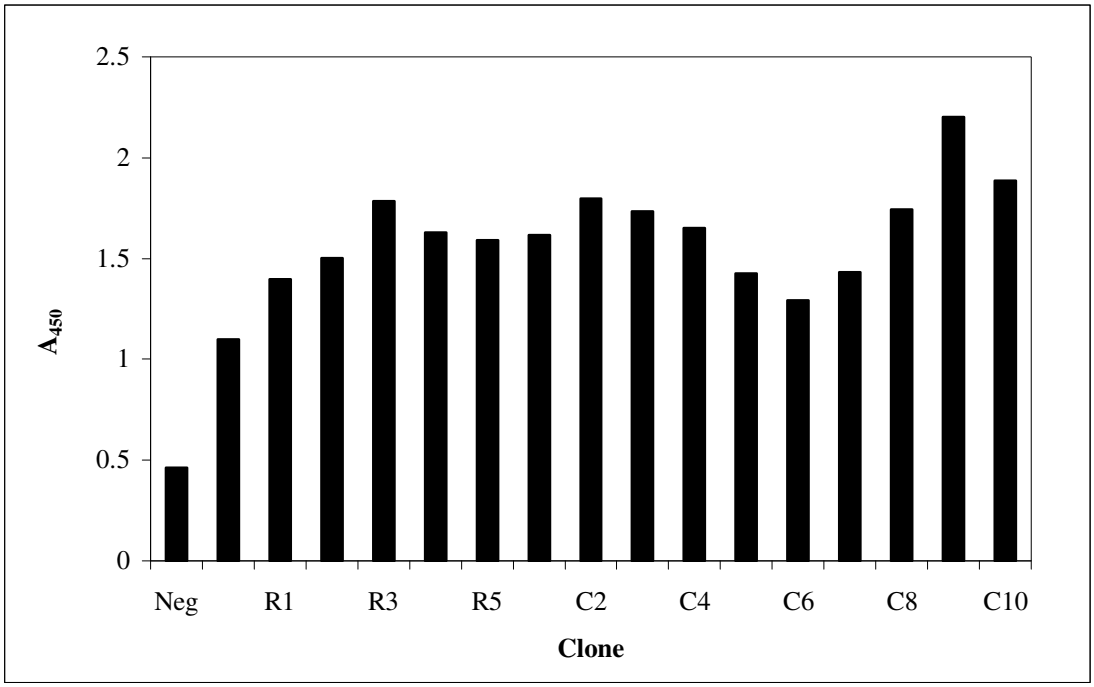

Figure 4.1. Specificity of antibody-displaying phage pools and individual phage clones to *E. tenella* sporozoites. Neg = Negative control; U = Unpanned library; R = Panning round; C = Clone. Binding was detected using anti-M13 antibody. Vertical bars represent absorbance readings at 450 nm. The reading of each phage pool (U, R1, R2, R3, R4, and R5) is an average of two replicates.

Immunofluorescence assay

Stained sporozoites were observed to agglutinate together, making it very difficult to observe fluorescence and obtain a clear picture (Figure 4.2).

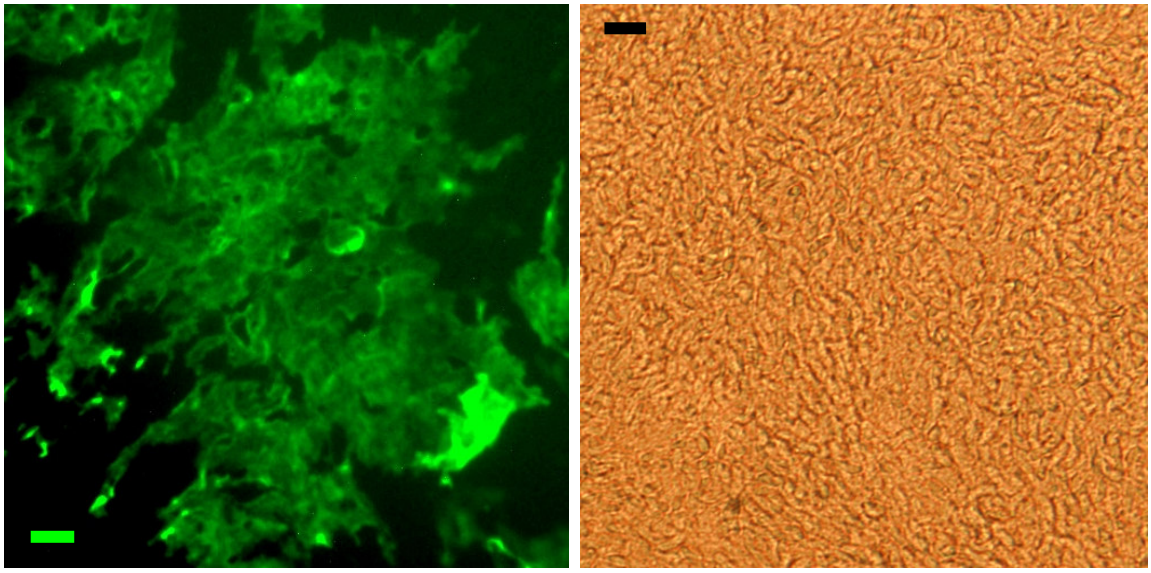

Figure 4.2. Agglutination of *E. tenella* sporozoites by antibody-displaying phage from clone 9. Staining was obtained with anti-M13 antibody and FITC-conjugated anti-IgG. (L) Sporozoites viewed with the FITC green filter; (R) Same view with bright field. Scale bars: 25 µm.

Analysis of antibody fragments

Enzyme-linked immunosorbent assay

Except for subclone 2a, all antibody fragments harvested from *E. coli* culture supernatants tested positive, while those from bacterial cell pellets did not, and had absorbance readings comparable to the negative control (Figure 4.3). Supernatants of clones 3a, 3b, 8a, 8b, 9a, and 9b were the best binders, with absorbance readings ranging from 0.833 to 1.27. Similar binding efficiency was observed between cell supernatant and pellet preparation from subclone 2b.

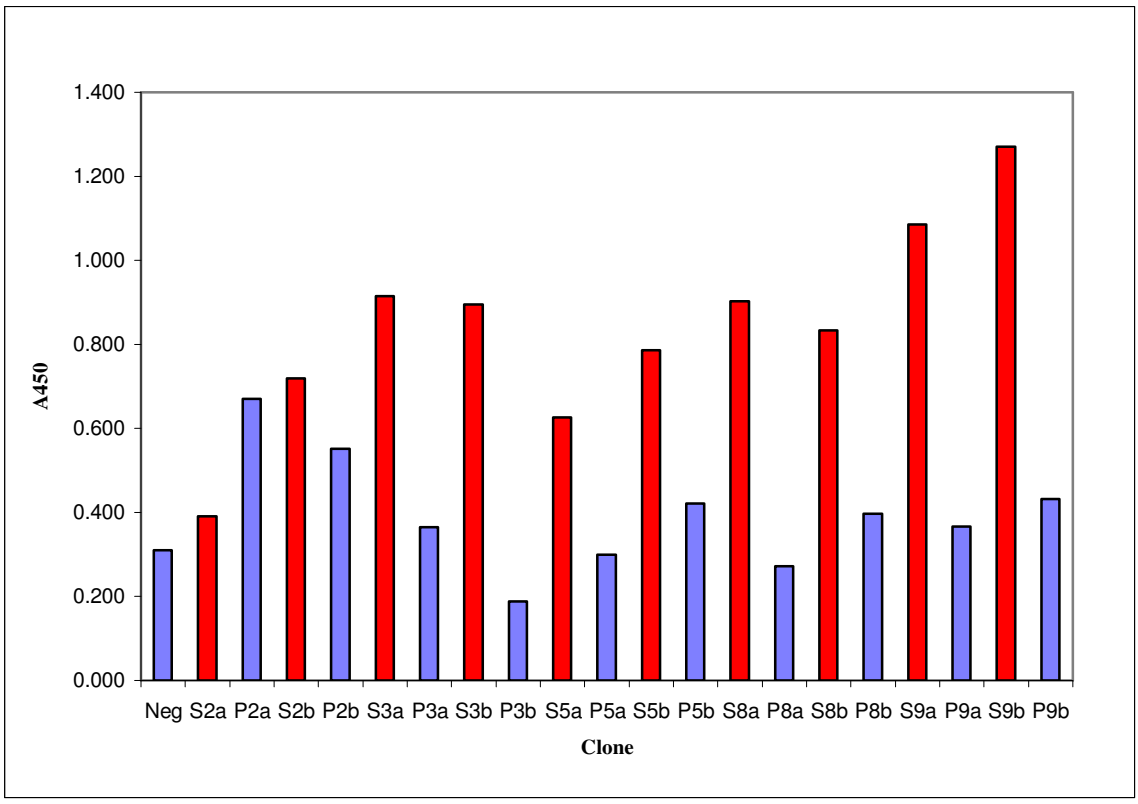

Figure 4.3. Specificity of antibody fragments to *E. tenella* sporozoites. Neg = Negative control; S = cell culture supernantant; P = cell pellet. Binding was detected using an anti-HA antibody and peroxidase-conjugated anti-IgG. Vertical bars (red for supernatants, blue for pellets) represent absorbance readings at 450 nm.

Immmunoblotting

Antibody fragments produced in TOP10 cells from clones 8a and 9b were tested for reactivity with *E. tenella* sporozoite crude protein extracts by Western blotting. These 2 clones were chosen because they constantly performed well, giving high readings in ELISA against sporozoites (Figure 4.3), and heavy bands when detected by Western blotting (Figure 3.13). Both clones strongly reacted with a 66-KDa sporozoite protein (Figure 4.4).

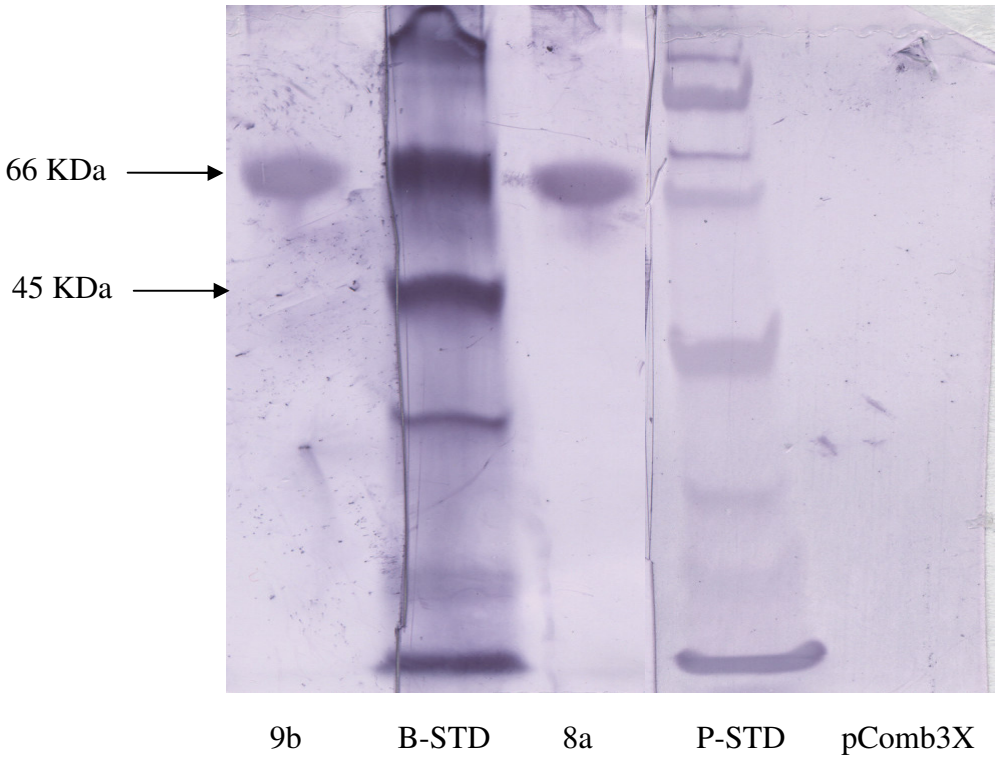

Figure 4.4. Western blotting of antibody fragments with *E. tenella* crude protein extracts. Detection was achieved with mouse anti-$(His)_6$, biotinylated anti-mouse IgG, and alkaline phosphatase-conjugated streptavidin. B-STD = Biotinylated molecular weight standard; P-STD = Prestained molecular weight standard; 9b, 8a, pComb3X = antibody fragments from cells transformed with 9b and 8a clones, and with the empty vector, respectively.

Discussion

An antibody library against *Eimeria tenella* sporozoites was constructed. Specific antibodies were selected from the library by repeated, increasingly stringent panning rounds against cryopreserved sporozoites. Phage pools from each selection round were tested for specific binding by ELISA. The highest signal was obtained from the third round's phage pool. This finding is consistent with the observation that the

highest enrichment of positive clones was obtained after the third panning round (Chapter III, Table 3.3). Phage was precipitated from individual clones randomly selected from the output plates of round 5. All antibody-displaying phage bound specifically to sporozoites, albeit to different degrees, with clone 9 giving the highest reading. A moderately high signal was also detected in the unpanned library. This is not surprising, as the unpanned library does contain specific antibody-displaying phages, although these only represent a small number of the total phage repertoire. They do not become the dominant population until the increased selection stringency eliminates non-specific, weakly or moderately bound clones.

In a similar experiment, sporozoites were incubated with either phage pools or single clones, and were stained with an FITC-conjugated secondary antibody. Microscopically, stained sporozoites appeared agglutinated. A phage particle displaying multiple copies of the fusion protein facilitates cross-linking of the displayed antibodies to the cell surfaces. In fact, during the assay, and upon incubation with the primary antibody, cells were observed to clump together, and were difficult to resuspend. A similar phenomenon was observed after the second panning round. Neither the negative control nor the unpanned library samples ever produced this agglutination effect.

Antibody fragments from single clones were produced by IPTG induction of TOP10 cells transformed with phagemid DNA. The long induction time (20h) ensured the secretion of most of the antibody fragments in the culture supernatants, with relatively few remaining in the periplasmic space or inside the bacterial cells. Almost all tested supernatants reacted strongly and bound specifically to sporozoite cell surfaces,

while cell pellet preparations did not. Clone 2a was the only clone which exhibited the opposite effect, with the cell supernatant and pellet testing negative and positive, respectively. Antibody fragments isolated from clone 2b were almost equally distributed between supernatant and pellet. In fact, cultures 2a and 2b were "slow growers", and at the time of induction had not reached an OD_{600} of 0.5, as recommended by the protocol. These results are in agreement with the immunoblot detection of culture supernatants, where supernatants from subclones 2a and 2b yielded lighter bands than the other clones (Figure 3.13).

Antibody fragments produced in clones 8a and 9b strongly reacted with a 66-KDa sporozoite protein, a possibly immunodominant epitope. The broadness of the bands could also be due to overloading of the gel or to the recognized antigen being heavily and heterogeneously glycosylated. Amino acid sequences of both 8a and 9b clones showed marked differences with the germline sequence, especially at the CDR3 of the V_H chain (Figure 3.10). Differences between both clones were mostly pronounced in the CDRs of the V_L chain and in CDR2 of the heavy chain. At the very least, both clones appear to be recognizing antigens with the same apparent molecular weight, if not the same antigen. It might thus be possible that the antigen specificity in this case is determined mainly by the heavy chain.

Attempts at staining sporozoites by immunofluoresence were met with limited success, as only single, few sporozoites were stained (results not shown). We can only hypothesize about the factors that might be responsible for this outcome, factors that are inherent to staining with monoclonal antibodies, especially those raised against native

epitopes. Immobilization of sporozoites on slides could only be achieved by using glutaraldehyde, a rather harsh fixative that could destroy, alter, or mask the epitope, making it inaccessible to the antibody. Staining of cell surface antigens is an additional challenge, as the epitope might be hidden within a cell structure, and/or the local antigen concentration might be too low to detect, as is the case with many cell surface molecules. In this case, staining in cell suspension is recommended (Harlow and Lane, 1999). This alternative, however, caused agglutination of the sporozoites and made imaging very difficult. Diffuse cell surface antigens, even when present at high concentrations, are difficult to detect (Harlow and Lane, 1999). In a few cases, sporocyst and oocyst shells were also stained, an observation in line with with previous reports of similar antigenic profiles between sporulated oocyts and sporozoites (Tomley, 1994a).

Further characterization of the selected antibodies can be achieved by producing the antibody fragments in large culture volumes, and exploiting the histidine tag to purify these antibodies by metal chelate chromatography. Panning on whole cells offers the advantage of identifying antibodies that recognize native structures. These antibody preparations (of known concentrations) could then for instance be tested for reactivity with individual candidate *Eimeria* proteins produced biosynthetically based on published sequence databases.

CHAPTER V

OPTIMIZATION OF AN IN VITRO CULTURE SYSTEM FOR CHICKEN ENTEROCYTES FOR POTENTIAL USE IN AN INVASION ASSAY FOR *EIMERIA TENELLA* SPOROZOITES

Introduction

Eimeria tenella is one of the most pathogenic *Eimeria* species that cause coccidiosis. *Eimeria* parasites invade and multiply in the avian intestinal tract. Invasion occurs when motile sporozoites make contact with and propel themselves into intestinal epithelial cells. During invasion, cellular internalization of sporozoites occurs within an hour of inoculation (Russell and Sinden, 1981). This short invasion time, as well as the intracellular nature of the sporozoites, leaves a small window of opportunity to visualize the dynamic host-parasite interaction. In vitro culture to study invasion has commonly relied on surrogate host cell lines such as Mardin-Darby Bovine Kidney cells (MDBK) and Baby Hamster Kidney cells (BHK), which can readily be cultured as monolayers and infected by sporozoites (Tierney and Mulcahy, 2003). The objective of this experiment was to propose a more realistic, homologous cell culture source for potential use in an in vitro invasion assay, using primary chicken intestinal cells, the natural host of *Eimeria* parasites. The rationale for this attempt is the assumption that adhesion to a chicken gut cell and adhesion to a heterologous kidney cell may not involve the same molecular interactions, and might therefore require a different blocking mechanism.

Materials and methods

Primary culture of intestinal cells

Small intestine and cecal segments were obtained from a 4-wk old laying hen, intestinal contents were immediately flushed out with Calcium and Magnesium-free Hank's Balanced Salt Solution (CMF-HBSS, Sigma, MO), and fat and mesenteric tissue were removed. Intestinal segments were then cleaned in several washes of CMF-HBSS with vigorous shaking at 37ºC. In a first method (Caldwell et al., 1993), tissue mucosal surface was exposed and incubated in isolation medium (Table 5.1) containing 1.5 mM EDTA for 30 min at 37ºC and 200 rpm. The cell suspension was filtered through a 60-µm nylon mesh (Spectrum Labs, Rancho Dominguez, CA), and the cells in the run-through were recovered by centrifugation, resuspended in growth medium (Table 5.1), and incubated at 37ºC for 2 hours. Non-adherent (epithelial) cells were transferred to another flask. In a variation of the above culture method (Dimier-Poisson et al., 2004), washed intestinal tissue was diced and incubated in growth medium at 37ºC and 200 rpm for 30 min. The remaining steps were the same as in the first method. For the third method (Grossmann et al., 1998a), washed intestinal tissue was incubated in growth medium at 37ºC for 5 hours. Mucosa was then removed and washed in 10 mM DTT in CMF-HBSS at 4ºC, and then incubated for 1 hour in 1 mM EDTA in CMF-HBSS at 4ºC. Intact epithelial crypts were detached by vigorously shaking the vessel. Cells were immediately filtered through a 60-µm nylon mesh (Spectrum Labs, Rancho Dominguez, CA), and the purified crypts (retained on the filter) were gently washed off the mesh with growth medium to avoid disruption of the crypt architecture. In all three methods,

isolated cells were seeded into Anopore cell culture inserts (NalgeNunc International, NY) placed in a 24-well plate, at $2x10^5$ cells/ml, and incubated at 37ºC and 5% CO_2.

Table 5.1 Composition of isolation and growth media*

	Isolation medium	**Growth medium**
DMEM	To 500 ml	To 500 ml
HEPES	25 mM	25 mM
BSA	1%	0.1%
Heat-inactivated Fetal bovine serum (FBS)	-	2% (increase to 5% after 1 week of incubation
Heat-inactivated chicken serum	2%	-
Sodium pyruvate	1 mM	1 mM
Glutamax®	2 mM	2 mM
Penicillin/Streptomycin	10,000 U/ml, 10 mg/ml	10,000 U/ml, 10 mg/ml
Amphotericin B	2.5 µg/ml	2.5 µg/ml
Hydrocortisone	5 µg/ml	5 µg/ml
Insulin/Transferrin/Selenium	5 µg, 5 µg, 5 ng/ml	5 µg, 5 µg, 5 ng/ml
Epidermal growth factor (EGF)	20 ng/ml	20 ng/ml
Gentamicin	50 µg/ml	50 µg/ml
pH	5.0	7.4

*All reagents were obtained from Sigma (St Louis, MO), except Glutamax (Invitrogen, Carlsbad, CA), FBS (Atlanta Biologicals, Lawrenceville, GA), and EGF (Peprotech, Rocky Hill, NJ).

Preparation and labeling of parasites

Eimeria tenella sporozoites were prepared as described in Chapter II. Excysted sporozoites were washed and labeled with the PKH67 Green Fluorescent Cell Linker Kit (Sigma, MO) according to the manufacturer's instructions (Fuller and McDougald, 2001). Briefly, $2x10^7$ sporozoites were washed with serum-free DMEM, and were resupsended in 1 ml of Diluent C (provided in the kit). Cells were then added to 1 ml of the dye (prepared at $4x10^{-6}$ M in Diluent C), immediately mixed by gentle pipetting, and incubated at room temperature for 5 min with periodic mixing. The staining reaction was stopped by incubating the cells for 1 min with an equal volume of fetal bovine

serum (Atlanta Biologicals, Lawrenceville, GA). The staining solution was removed by centrifugation, and the cells were transferred to a new tube and washed 4 times with complete DMEM.

Interaction with **Eimeria tenella** *sporozoites*

Medium was removed from the cell culture inserts and replaced with the PKH 67-labeled sporozoites (10^6/well). Cells were incubated at 41°C and 5% CO_2. Fifteen min, 30 min, 1, and 2 hours post-inoculation, free sporozoites were removed by gently washing the inserts with PBS. Cells were fixed with 4% paraformaldehyde for 15 min, washed with PBS, permeabilized with 0.3% Tween-20 for 15 min, and then rinsed gently with PBS. In a similar experiment, after the addition of sporozoites, cells were stained with 5µg/ml of FM® 4-64, a fluorescent lipophilic dye that is used for membrane labeling (Invitrogen, Carlsbad, CA). Cells were then fixed and washed as above. Membranes were separated from the inserts, mounted on glass slides with ProLong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA). Slides were observed with a Zeiss Axioplan fluorescence microscope at the Texas A&M University Image Analysis Laboratory.

Results

Primary culture of intestinal cells

The first two methods for isolation of enterocytes produced mostly individual cells, while method 3 resulted in recovery of whole intestinal crypts that remained viable and proliferated in cell culture. These were hence maintained for extended periods, and

destined for try-out in an invasion assay. Crypts were observed to attach to bare plastic, whereas cells isolated through methods 1 and 2 failed to attach**.** Figure 5.1 shows a crypt monolayer with cell membranes stained red with FM® 4-64.

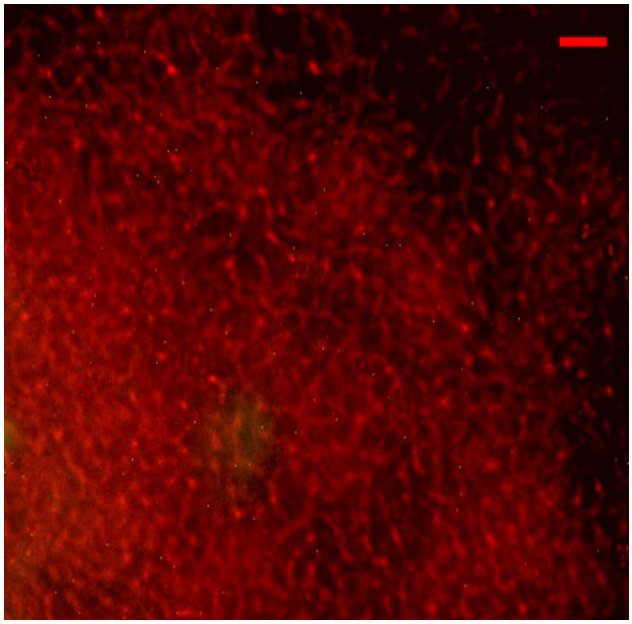

Figure 5.1. Monolayer of intestinal crypts. Cell membranes were stained with FM 4-64. Scale bar: 50 µm.

Interaction with **Eimeria tenella** *sporozoites*

Two hours following post-inoculation, *E. tenella* sporozoites were observed in very close proximity to host cell nuclei (Figure 5.2). Figure 5.3 shows white overlaps between stained nuclei and sporozoites.

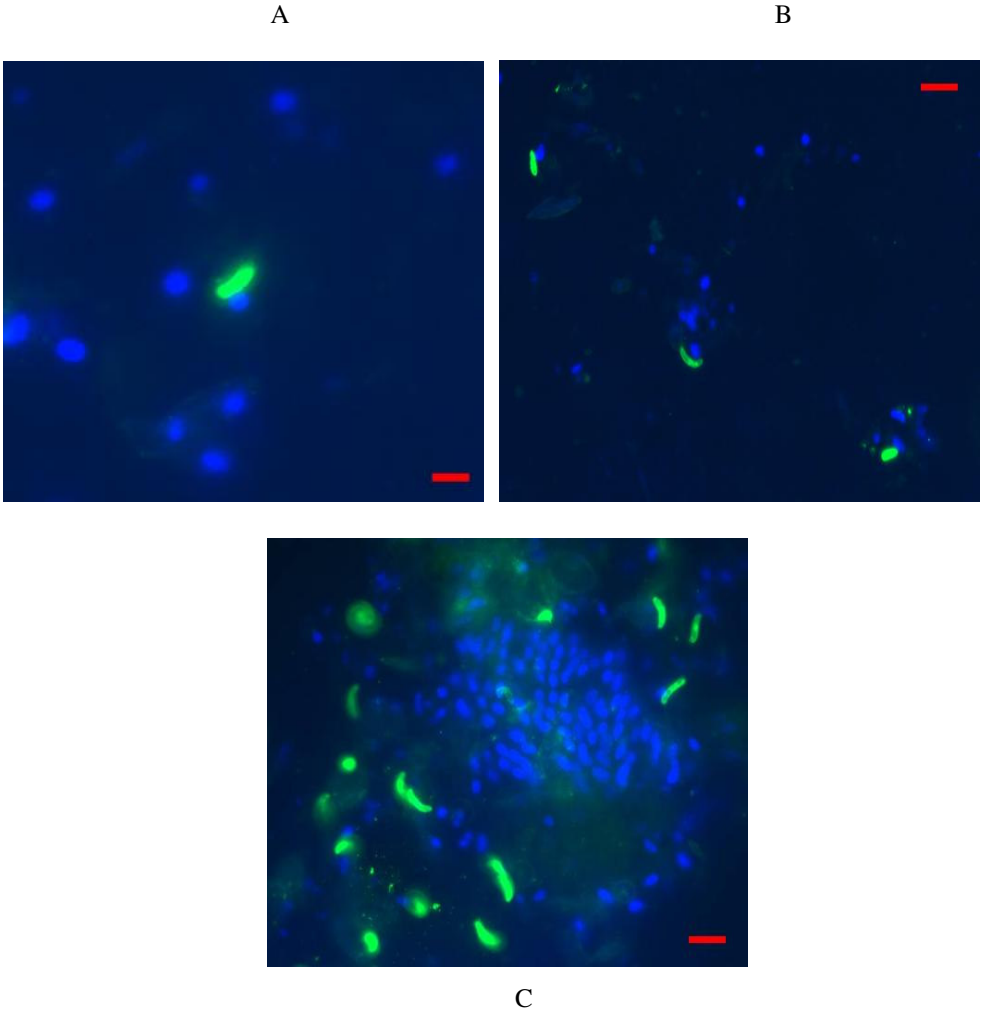

Figure 5.2. *E. tenella* sporozoites (green) seen in close proximity to enterocyte nuclei (blue), 2 hours post-inoculation. Scale bars: A and C: 25 µm, and (B) 62.5 µm

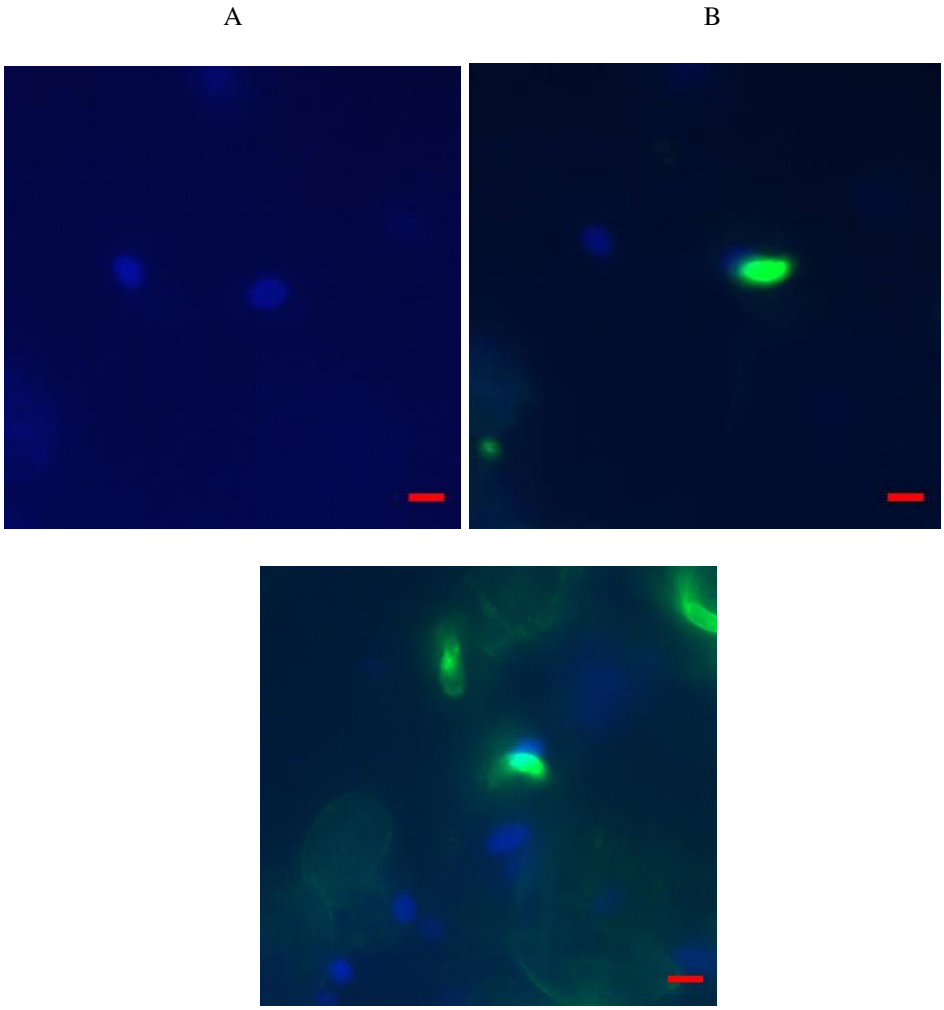

Figure 5.3. White overlap between *E. tenella* sporozoites and nuclei of enterocytes, 2 hrs post-inoculation. (A) Overview with only a blue filter shows the host cell nuclei; (B) The same overview with both blue and green filters shows a white overlap between a sporozoite and an enterocyte. Scale bar: 25 µm.

Discussion

A homologous (chicken) enterocyte system for potential use in an invasion assay for *Eimeria tenella* sporozoites was developed. Isolation of primary chicken enterocytes proved to be time consuming and cumbersome, which would make an invasion assay based on primary chicken cells considerably less user friendly than is the case with established cell lines. Anchorage to a substrate was found to be very critical for

epithelial cell survival. In our hands, isolated individual enterocytes quickly died in culture, while isolation of intestinal crypts, the source of epithelial stem cells, resulted in a viable proliferative cell population, which would eventually spread out and cover a substantial surface of the substrate with a monolayer of contingent epithelial cells. This method has been shown to allow for the isolation of human intestinal cells before activation of caspase 3 family members. Caspases, a family of cysteine proteases, play a central role in initiating, amplifying, and executing apoptosis. Activated caspases ultimately lead to activation of endonucleases, DNA fragmentation, and cell death (Grossmann, 2002). Within minutes of detachment, caspases initiated programmed cell death in human intestinal cells, resulting in DNA fragmentation in less than 2 h (Grossmann et al., 1998b). Detachment from neighboring cells was found to be the pivotal trigger of apoptosis in isolated human intestinal cells. Despite the lack of blood supply and incubation of the tissue at 37°C, prolonged anchorage to the mucosa (5 h) as well as reducing the isolation temperature to 4°C (presumably to reduce the metabolic needs of the cells), effectively prevented apoptosis (Grossmann et al., 1998a). A similar phenomenon that highlights the importance of cell anchorage has been shown to occur in bursal B-lymphocytes. Simple physical disruption of cell-to-cell contacts in bursal follicles noticeably stimulated apoptosis in normal bursal lymphoblasts within 2 hours of dispersion (Neiman et al., 1991).

Centrifugation steps were kept to a minimum in methods 1 and 2, and skipped altogether in method 3, since centrifugation had been observed to have a negative effect on cell survival (Grossmann et al., 1998a). DTT is a mucolytic agent that helps

eliminate mucus from the tissue, making cell filtration easier. EDTA is a chelating agent and is used to dissociate cells from their matrix. The incubation with EDTA is carried out at 4°C to inhibit caspase activation. A later study proposed that improvement on the isolation method could be attained by reducing the EDTA incubation from 1 hour to 10 min at 37°C, thus minimizing the detachment time and ensuring rapid reattachment of the isolated intestinal crypts (Grossmann et al., 2003).

Visualization of cell invasion by *E. tenella* sporozoites has traditionally been achieved by the use of monoclonal antibodies directed against parasite antigens (Fuller and McDougald, 2001). In this study an alternative strategy was tested; *E. tenella* sporozoites were labeled with the PKH67 cell linker kit, thus facilitating the visualization of their interaction with the host cells. The incorporation of highly aliphatic reporter molecules containing fluorochrome head groups into the cell cytoplasmic membranes of the sporozoites ensured stable and reproducible staining (Horan and Slezak, 1989).

The mechanism of invasion and of host specificity of *Eimeria* species is not very well understood, and is thought to be determined by both parasite and host. Most of the invasion assays described so far in the literature rely on surrogate cell lines, with varied results depending on the cell line and on the temperature at which invasion occurred (Tierney and Mulcahy, 2003). Given that invasion is a dynamic process that occurs in the complex environment of the intestine, it would seem logical to use the natural host cells for invasion assays, a scenario that would be closer to the real-life situation. This

seems to be all the more important when later assessing the potential of antibodies to inhibit cell invasion by sporozoites.

At the very least, our results demonstrate that after several rinses, sporozoites can, and do adhere to the target epithelial cells. In this aspect, the invasion assay proposed herein resembles the in vivo situation as closely as technically achievable. As such, it would provide a means to test the ability of specific anti-*Eimeria* antibodies to block invasion of chicken enterocytes by *Eimeria* sporozoites in vitro. It is, however, cumbersome and time-consuming, like we already mentioned above. Another problematic aspect that needs to be solved is the difficulty to discriminate with absolute certainty between attachment (adhesion) of sporozoites to the enterocyte layer and genuine invasion of the target cells. The technology used in our study strongly suggest adherence and potentially also invasion, but the latter would have to be verified by confocal microscopy: if sporozoites and enterocyte nuclei are located in the same plane, invasion has occurred; if sporozoites are superimposed on the monolayer, then adhesion is the correct interpretation.

The permeabilization step was necessary for visualization of the enterocytes' nuclei, and was achieved by treating cells with 0.3% Tween-20. Nuclei of the sporozoites were not stained, possibly because treatment with Tween-20, a mild detergent, is not sufficient to permeabilize the rather sturdy cell membrane of sporozoites. Further optimization of this cell culture system should forgo the permeabilization step, and include visualization of the boundaries of individual enterocytes to allow for unequivocal discrimination between adhesion and invasion.

CHAPTER VI

SUMMARY

The objective of this study was to produce recombinant antibodies against *Eimeria tenella* sporozoites by phage display. An immune library against *Eimeria tenella* sporozoites (7.4×10^7 total transformants) was constructed and panned against cryopreserved sporozoites. Specific, high-affinity antibodies were obtained, an advantage bestowed by immunization (Burton, 1995). Initially, a naïve library was constructed (results not shown) and could have been used instead, with the added advantage that such a library can be used for any antigen. However, antibodies isolated from naïve libraries are usually of low to moderate affinity (Azzazy and Highsmith, 2002), and strategies to improve affinity (such as mutagenesis and selection) are difficult and time-consuming (Burton, 2001).

To accommodate for the tedious panning process, we resorted to cryopreservation of *E. tenella* sporozoites. This method worked extremely well in our hands and provided us with a readily available daily supply of whole sporozoites. The sporozoite production process itself was a challenging task, given that the efficiency of sporozoite excystation was found to be heavily dependent on the age of the oocyst stock. Oocysts older than 3 months yielded dramatically lower numbers of usable sporozoites. Even after only 1 month of storage, decreased excystation efficiency set in.

Cell surface panning offered the advantage of isolating antibodies against corresponding antigens in a state as close as possible to that found in vivo. It should be

noted, however, that excystation in vitro does lead to the loss of some antigens (Wisher and Rose, 1987), possibly due to proteolysis by trypsin present in the excystation medium (Tomley, 1994b). Cell surface antigens were difficult to detect by immunofluorescence, which could be attributed to the low local concentration of many cell surface antigens, or the masking or altering of the epitope by the fixation method, which involved glutaraldehyde, a strong cross-linker that is deleterious to antigenicity.

Phage display, which remains to date the only reliable way to produce monoclonal antibodies in chickens, was employed for this purpose. It is not, however, without complications. Success in isolating a desirable antibody from a library depends on the size of the library, which in turn is determined by the quality of cDNA used to construct the insert, the quality of the vector, the efficiency of transformation and the selection strategy.

Future investigations will focus on purification and characterization of additional antibodies selected from the constructed library. Such antibodies can be tested, alone or in combination, for their ability to block in vitro the invasion mechanism of *E. tenella.* For this purpose, an attempt was made to establish a homologous in vitro model based on primary chicken enterocytes. While viable monolayers could be obtained, problems remained with unequivocal distinction between adhesion and invasion. Ultimately, surrogate kidney cell lines will likely prove more practical for use in in vitro invasion assays.

REFERENCES

Aikawa, M., Miller, L. H., Johnson, J., and Rabbege, J. (1978). Erythrocyte entry by malarial parasites. A moving junction between erythrocyte and parasite. J Cell Biol *77*, 72-82.

Allen, P. C., and Fetterer, R. H. (2002). Recent advances in biology and immunobiology of *Eimeria* species and in diagnosis and control of infection with these coccidian parasites of poultry. Clin Microbiol Rev *15*, 58-65.

Allen, P. C. (1997). Nitric oxide production during *Eimeria tenella* infections in chickens. Poult Sci *76*, 810-813.

Andris-Widhopf, J., Rader, C., Steinberger, P., Fuller, R., and Barbas, C. F., 3rd (2000). Methods for the generation of chicken monoclonal antibody fragments by phage display. J Immunol Methods *242*, 159-181.

Arakawa, H., and Buerstedde, J. M. (2004). Immunoglobulin gene conversion: insights from bursal B cells and the DT40 cell line. Dev Dyn *229*, 458-464.

Arakawa, H., Kuma, K., Yasuda, M., Ekino, S., Shimizu, A., and Yamagishi, H. (2002). Effect of environmental antigens on the Ig diversification and the selection of productive V-J joints in the bursa. J Immunol *169*, 818-828.

Arakawa, H., Kuma, K., Yasuda, M., Furusawa, S., Ekino, S., and Yamagishi, H. (1998). Oligoclonal development of B cells bearing discrete Ig chains in chicken single germinal centers. J Immunol *160*, 4232-4241.

Armstrong, N., Adey, N. B. McConnell, S. J., and Kay, B. K. (1996). Vectors for phage display. In Phage Display of Peptides and Proteins, B. Kay, J. Winter, and J. McCafferty, eds. (San Diego, California: Academic Press), pp. 35-53.

Arndt, K. M., Muller, K. M., and Pluckthun, A. (1998). Factors influencing the dimer to monomer transition of an antibody single-chain Fv fragment. Biochemistry *37*, 12918-12926.

Augustine, P. C. (2001). Cell: sporozoite interactions and invasion by apicomplexan parasites of the genus *Eimeria*. Int J Parasitol *31*, 1-8.

Augustine, P. C. (2000). Cellular invasion by avian *Eimeria* species. Avian and Poultry Biological Reviews *11*, 113-122.

Augustine, P. C., and Jenkins, M. C. (1998). Effect of conditioned media from chicken and turkey intestinal cell cultures on invasion by sporozoites of three species of avian coccidia. J Eukaryot Microbiol *45*, 344-346.

Augustine, P. C., and Danforth, H. D. (1990). Avian *Eimeria*: invasion in foreign host birds and generation of partial immunity against coccidiosis. Avian Dis *34*, 196-202.

Azzazy, H. M., and Highsmith, W. E., Jr. (2002). Phage display technology: clinical applications and recent innovations. Clin Biochem *35*, 425-445.

Barbas, C. F., 3rd, Burton, D. R., Scott, J. K., and Silverman, G. J. (2001). Phage display: A laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Barbas, C. F., and Wagner, J. (1995). Synthetic Human Antibodies: Selecting and Evolving Functional Proteins. Methods: A Companion to Methods in Enzymology *8*, 94-103.

Barbas, C. F., 3rd, Kang, A. S., Lerner, R. A., and Benkovic, S. J. (1991). Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. Proc Natl Acad Sci U S A *88*, 7978-7982.

Belli, S. I., Mai, K., Skene, C. D., Gleeson, M. T., Witcombe, D. M., Katrib, M., Finger, A., Wallach, M. G., and Smith, N. C. (2004). Characterisation of the antigenic and immunogenic properties of bacterially expressed, sexual stage antigens of the coccidian parasite, *Eimeria maxima*. Vaccine *22*, 4316-4325.

Breitling, F., and Dübel, S. (1999). Recombinant Antibodies. (New York, NY: John Wiley & Sons, Inc).

Budihardjo, I., Oliver, H., Lutter, M., Luo, X., and Wang, X. (1999). Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol *15*, 269-290.

Bumstead, J., and Tomley, F. (2000). Induction of secretion and surface capping of microneme proteins in *Eimeria tenella*. Mol Biochem Parasitol *110*, 311-321.

Burton, D. R. (2001). Antibody libraries. In Phage Display: A Laboratory Manual, C. F. Barbas III, D. R. Burton, J. K. Scott, and G. J. Silverman, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), pp. 3.1-3.18.

Burton, D. R. (1995). Phage display. Immunotechnology *1*, 87-94.

Butler, J. E. (1998). Immunoglobulin diversity, B-cell and antibody repertoire development in large farm animals. Rev Sci Tech *17*, 43-70.

Cai, X., Fuller, A. L., McDougald, L. R., and Zhu, G. (2003). Apicoplast genome of the coccidian *Eimeria tenella*. Gene *321*, 39-46.

Caldwell, D. J., Droleskey, R. E., Elissalde, M. H., Kogut, M. H., DeLoach, J. R., and Hargis, B. M. (1993). Isolation and primary culture of chicken intestinal epithelial cells retaining normal in vivo-like morphology. J Tissue Cult Meth *15*, 15-18.

Carruthers, V. B., Giddings, O. K., and Sibley, L. D. (1999). Secretion of micronemal proteins is associated with toxoplasma invasion of host cells. Cell Microbiol *1*, 225-235.

Chai, J. Y., and Lillehoj, H. S. (1988). Isolation and functional characterization of chicken intestinal intra-epithelial lymphocytes showing natural killer cell activity against tumour target cells. Immunology *63*, 111-117.

Clackson, T., Hoogenboom, H. R., Griffiths, A. D., and Winter, G. (1991). Making antibody fragments using phage display libraries. Nature *352*, 624-628.

Cox, F. E. (1994). The evolutionary expansion of the Sporozoa. Int J Parasitol *24*, 1301-1316.

Crane, M. S., Murray, P. K., Gnozzio, M. J., and MacDonald, T. T. (1988). Passive protection of chickens against *Eimeria tenella* infection by monoclonal antibody. Infect Immun *56*, 972-976.

Crane, M. S., and Dvorak, J. A. (1982). Influence of monosaccharides on the infection of vertebrate cells by *Trypanosoma cruzi* and *Toxoplasma gondii*. Mol Biochem Parasitol *5*, 333-341.

Daszak, P., Ball, S. J., Pittilo, R. M., and Norton, C. C. (1993). Ultrastructural observations on caecal epithelial cells invaded by first-generation merozoites of *Eimeria tenella* in vivo. Ann Trop Med Parasitol *87*, 359-364.

Dimier-Poisson, I. H., Bout, D. T., and Quere, P. (2004). Chicken primary enterocytes: inhibition of *Eimeria tenella* replication after activation with crude interferon-gamma supernatants. Avian Dis *48*, 617-624.

Doran, D. J. (1982). Behavior of coccidia in vitro. In The Biology of the Coccidia, P. L. Long, ed. (Baltimore, Maryland: University Park Press). pp. 229–285.

Doran, D. J., and Vetterling, J. M. (1968). Preservation of coccidial sporozoites by freezing. Nature *217*, 1262.

Dubremetz, J. F., Garcia-Reguet, N., Conseil, V., and Fourmaux, M. N. (1998). Apical organelles and host-cell invasion by Apicomplexa. Int J Parasitol *28*, 1007-1013.

Dulski, P. M. (1990). *Eimeria tenella*: incomplete excystation in the presence of EDTA in a taurodeoxycholate-based medium. J Protozool *37*, 524-528.

Dulski, P., and Turner, M. (1988). The purification of sporocysts and sporozoites from *Eimeria tenella* oocysts using Percoll density gradients. Avian Dis *32*, 235-239.

Ellis, J., and Revets, H. (1990). *Eimeria* species which infect the chicken contain virus-like RNA molecules. Parasitology *101 Pt 2*, 163-169.

Ermak, T. H., Steger, H. J., and Pappo, J. (1990). Phenotypically distinct subpopulations of T cells in domes and M-cell pockets of rabbit gut-associated lymphoid tissues. Immunology *71*, 530-537.

Farr, M. M., and Doran, D. J. (1962). Comparative excystation of four species of poultry coccidia. J Protozool *9*, 403-407.

FitzGerald, K., Holliger, P., and Winter, G. (1997). Improved tumour targeting by disulphide stabilized diabodies expressed in *Pichia pastoris*. Protein Eng *10*, 1221-1225.

Frisch, S. M., and Francis, H. (1994). Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol *124*, 619-626.

Feshney, R. I., and Freshney, M. G. (2002). Culture of epithelial cells. (New York, NY: Wiley-Liss, Inc).

Fuller, A. L., and McDougald, L. R. (2001). Cell membrane labeling of *Eimeria tenella* sporozoites with the fluorescent dye PKH-67 GL for tracking parasite-host interactions. Parasitol Res *87*, 521-525.

Girard, F., Fort, G., Yvore, P., and Quere, P. (1997). Kinetics of specific immunoglobulin A, M and G production in the duodenal and caecal mucosa of chickens infected with *Eimeria acervulina* or *Eimeria tenella*. Int J Parasitol *27*, 803-809.

Graat, E. A., van der Kooij, E., Frankena, K., Henken, A. M., Smeets, J. F., and Hekerman, M. T. (1998). Quantifying risk factors of coccidiosis in broilers using on-farm data based on a veterinary practice. Prev Vet Med *33*, 297-308.

Grossmann, J., Walther, K., Artinger, M., Kiessling, S., Steinkamp, M., Schmautz, W. K., Stadler, F., Bataille, F., Schultz, M., Scholmerich, J., and Rogler, G. (2003). Progress on isolation and short-term ex-vivo culture of highly purified non-apoptotic human intestinal epithelial cells (IEC). Eur J Cell Biol *82*, 262-270.

Grossmann, J. (2002). Molecular mechanisms of "detachment-induced apoptosis--Anoikis". Apoptosis *7*, 247-260.

Grossmann, J., Maxson, J. M., Whitacre, C. M., Orosz, D. E., Berger, N. A., Fiocchi, C., and Levine, A. D. (1998a). New isolation technique to study apoptosis in human intestinal epithelial cells. Am J Pathol *153*, 53-62.

Grossmann, J., Mohr, S., Lapentina, E. G., Fiocchi, C., and Levine, A. D. (1998b). Sequential and rapid activation of select caspases during apoptosis of normal intestinal epithelial cells. Am J Physiol *274*, G1117-1124.

Harlow, E., and Lane, D. (1999). Using Antibodies: A Laboratory Manual. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Hawkins, R. E., Russell, S. J., and Winter, G. (1992). Selection of phage antibodies by binding affinity. Mimicking affinity maturation. J Mol Biol *226*, 889-896.

Heussler, V. T., Kuenzi, P., and Rottenberg, S. (2001). Inhibition of apoptosis by intracellular protozoan parasites. Int J Parasitol *31*, 1166-1176.

Hoogenboom, H. R., and Chames, P. (2000). Natural and designer binding sites made by phage display technology. Immunol Today *21*, 371-378.

Horan, P. K., and Slezak, S. E. (1989). Stable cell membrane labelling. Nature *340*, 167-168.

Jeurissen, S. H., Janse, E. M., Vermeulen, A. N., and Vervelde, L. (1996). *Eimeria tenella* infections in chickens: aspects of host-parasite: interaction. Vet Immunol Immunopathol *54*, 231-238.

Joyner, L., 1982. Host and site specificity. In The Biology of the Coccidia, P. L. Long, ed. (Baltimore, Maryland: University Park Press). pp. 35-62.

Kaeffer, B. (2002). Mammalian intestinal epithelial cells in primary culture: a mini-review. In Vitro Cell Dev Biol Anim *38*, 123-134.

Kontermann, R. E., Wing, M. G., and Winter, G. (1997). Complement recruitment using bispecific diabodies. Nat Biotechnol *15*, 629-631.

Kruzel, M. L., Harari, Y., Chen, C. Y., and Castro, G. A. (1998). The gut. A key metabolic organ protected by lactoferrin during experimental systemic inflammation in mice. Adv Exp Med Biol *443*, 167-173.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Larsen, N. C., Rasmussen, K. R., and Healey, M. C. (1991). Production and partial characterization of monoclonal antibodies specific for the gamonts of *Eimeria tenella*. J Parasitol *77*, 1012-1015.

Lawn, A. M., and Rose, M. E. (1982). Mucosal transport of *Eimeria tenella* in the cecum of the chicken. J Parasitol *68*, 1117-1123.

Lee, S., Fernando, M. A., and Nagy, E. (1996). dsRNA associated with virus-like particles in *Eimeria* spp. of the domestic fowl. Parasitol Res *82*, 518-523.

Levine, N. D. (1988). Progress in taxonomy of the Apicomplexan protozoa. J Protozool *35*, 518-520.

Levine, N. D. (1982). The genus *Atoxoplasma* (Protozoa, Apicomplexa). J Parasitol *68*, 719-723.

Levine, N. D. (1970). Protozoan parasites of nonhuman primates as zoonotic agents. Lab Anim Care *20*, 377-382.

Lillehoj, H. S. (1987). Effects of immunosuppression on avian coccidiosis: cyclosporin A but not hormonal bursectomy abrogates host protective immunity. Infect Immun *55*, 1616-1621.

Lillehoj, H. S., and Lillehoj, E. P. (2000). Avian coccidiosis. A review of acquired intestinal immunity and vaccination strategies. Avian Dis *44*, 408-425.

Lillehoj, H. S., and Choi, K. D. (1998). Recombinant chicken interferon-gamma-mediated inhibition of *Eimeria tenella* development in vitro and reduction of oocyst production and body weight loss following *Eimeria acervulina* challenge infection. Avian Dis *42*, 307-314.

Lillehoj, H. S., and Trout, J. M. (1994). CD8+ T cell-coccidia interactions. Parasitol Today *10*, 10-14.

Lillehoj, H. S., and Ruff, M. D. (1987). Comparison of disease susceptibility and subclass-specific antibody response in SC and FP chickens experimentally inoculated with *Eimeria tenella*, *E. acervulina*, or *E. maxima*. Avian Dis *31*, 112-119.

Little, M., Kipriyanov, S. M., Le Gall, F., and Moldenhauer, G. (2000). Of mice and men: hybridoma and recombinant antibodies. Immunol Today *21*, 364-370.

Long, P. L. (1970). Anticoccidial drugs: factors affecting pathogenicity of avian coccidia. Exp Parasitol *28*, 4-10.

Mansikka, A., and Toivanen, P. (1991). D-D recombination diversifies the CDR 3 region of chicken immunoglobulin heavy chains. Scand J Immunol *33*, 543-548.

Martin, A. G., Danforth, H. D., Barta, J. R., and Fernando, M. A. (1997). Analysis of immunological cross-protection and sensitivities to anticoccidial drugs among five geographical and temporal strains of *Eimeria maxima*. Int J Parasitol *27*, 527-533.

Martin, A., Lillehoj, H. S., Kaspers, B., and Bacon, L. D. (1994). Mitogen-induced lymphocyte proliferation and interferon production following coccidia infection. Avian Dis *38*, 262-268.

Maynard, J., and Georgiou, G. (2000). Antibody engineering. Annu Rev Biomed Eng *2*, 339-376.

McCafferty, J., and Johnson, K. S. (1996). Vectors for phage display. In Phage Display of Peptides and Proteins, B. Kay, J. Winter, and J. McCafferty, eds. (San Diego, California: Academic Press), pp. 79-111.

McCormack, W. T., Tjoelker, L. W., and Thompson, C. B. (1991). Avian B-cell development: generation of an immunoglobulin repertoire by gene conversion. Annu Rev Immunol *9*, 219-241.

McDougald, L. R., Fuller, L., and Mattiello, R. (1997). A survey of Coccidia on 43 poultry farms in Argentina. Avian Dis *41*, 923-929.

McDougald, L. R., and Reid,W. M. (1997). Coccidiosis. In Diseases of Poultry, 10th ed., B. W. Calnek, H. J. Barnes, C. W. Beard, L. R. McDougald, and Y. M. Saif, eds. (Ames, Iowa: Iowa State University Press), pp. 865–883.

McDougald, L. R. and Reid, W. M. (1991). Coccidiosis. In Diseases of Poultry, B. W. Calnek., ed. (Ames, Iowa: Iowa State University Press), pp. 780-797.

Miller, L. H., Hudson, D., and Haynes, J. D. (1988). Identification of *Plasmodium knowlesi* erythrocyte binding proteins. Mol Biochem Parasitol *31*, 217-222.

Moore, M. A., and Owen, J. J. (1966). Experimental studies on the development of the bursa of Fabricius. Dev Biol *14*, 40-51.

Morrissette, N. S., and Sibley, L. D. (2002). Cytoskeleton of apicomplexan parasites. Microbiol Mol Biol Rev *66*, 21-38.

Naitza, S., Spano, F., Robson, K. J. H., and Crisanti, A. (1998). The Thrombospondin-related Protein Family of Apicomplexan Parasites: The Gears of the Cell Invasion Machinery. Parasitology *14*, 479-484.

Neiman, P. E., Thomas, S. J., and Loring, G. (1991). Induction of apoptosis during normal and neoplastic B-cell development in the bursa of Fabricius. Proc Natl Acad Sci U S A *88*, 5857-5861.

Nissim, A., Hoogenboom, H. R., Tomlinson, I. M., Flynn, G., Midgley, C., Lane, D., and Winter, G. (1994). Antibody fragments from a 'single pot' phage display library as immunochemical reagents. Embo J *13*, 692-698.

Norton, C. C., and Joyner, L. P. (1968). The freeze preservation of coccidia. Res Vet Sci *9*, 598-600.

Olah, I., and Glick, B. (1978). The number and size of the follicular epithelium (FE) and follicles in the bursa of Fabricius. Poult Sci *57*, 1445-1450.

Pasqualini, R., and Ruoslahti, E. (1996). Organ targeting in vivo using phage display peptide libraries. Nature *380*, 364-366.

Patton, W. H., and Brigman, G. P. (1979). The use of sodium taurodeoxycholate for excystation of *Eimeria tenella* sporozoites. J Parasitol *65*, 526-530.

Pini, A., and Bracci, L. (2000). Phage display of antibody fragments. Curr Protein Pept Sci *1*, 155-169.

Potten, C. S., and Allen, T. D. (1977). Ultrastructure of cell loss in intestinal mucosa. J Ultrastruct Res *60*, 272-277.

Rader, C., Steinberger, P., and Barbas, C. F., III (2001). Selction from antibody libraries In Phage Display: A Laboratory Manual, C. F. Barbas III, D. R. Burton, J. K. Scott, and G. J. Silverman, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), pp. 10.1-10.20.

Rader, C., and Barbas, C. F., III (1997). Phage display of combinatorial antibody libraries. Curr Opin Biotechnol *8*, 503-508.

Reynaud, C. A., Bertocci, B., Dahan, A., and Weill, J. C. (1994). Formation of the chicken B-cell repertoire: ontogenesis, regulation of Ig gene rearrangement, and diversification by gene conversion. Adv Immunol *57*, 353-378.

Rosenblum, J. S., and Barbas, C. F. (1995). Synthetic antibodies. In Antibody Engineering, 2nd edition, C. A. K. Borrebaeck, ed. (Oxford, UK: Oxford University Press), pp. 89-116.

Ruff, M. D. (1999). Important parasites in poultry production systems. Vet Parasitol *84*, 337-347.

Ruff, M. D. (1993). External and internal factors affecting the severity of avian coccidiosis. In Proceedings of the 6th International Coccidiosis Conference, J. R. Barta, and M. A. Fernando, eds., (Guelph, Ontario, Canada), pp. 73–79.

Ruff, M. D., Doran, D. J., and Wilkins, G. C. (1981). Effect of aging on survival and pathogenicity of *Eimeria acervulina* and *Eimeria tenella*. Avian Dis *25*, 595-599.

Russell, D. G., and Sinden, R. E. (1981). The role of the cytoskeleton in the motility of coccidian sporozoites. J Cell Sci *50*, 345-359.

Ryan, R., Shirley, M., and Tomley, F. (2000). Mapping and expression of microneme genes in *Eimeria tenella*. Int J Parasitol *30*, 1493-1499.

Sapats, S. I., Heine, H. G., Trinidad, L., Gould, G. J., Foord, A. J., Doolan, S. G., Prowse, S., and Ignjatovic, J. (2003). Generation of chicken single chain antibody variable fragments (scFv) that differentiate and neutralize infectious bursal disease virus (IBDV). Arch Virol *148*, 497-515.

Schier, R., McCall, A., Adams, G. P., Marshall, K. W., Merritt, H., Yim, M., Crawford, R. S., Weiner, L. M., Marks, C., and Marks, J. D. (1996). Isolation of picomolar affinity anti-c-erbB-2 single-chain Fv by molecular evolution of the complementarity determining regions in the center of the antibody binding site. J Mol Biol *263*, 551-567.

Scott, J. K., and Barbas, C. F. III. (2001). Phage-display vectors. In Phage Display: A Laboratory Manual, C. F. Barbas III, D. R. Burton, J. K. Scott, and G. J. Silverman, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), pp. 2.1-2.19.

Shaw, A. J. (1996). Epithelial Cell Culture. (New York, NY: Oxford University Press).

Shiotani, N., Baba, E., Fukata, T., Arakawa, A., and Nakanishi, T. (1992). Distribution of oocysts, sporocysts and sporozoites of *Eimeria tenella* and *Eimeria maxima* in the digestive tract of chicken. Vet Parasitol *41*, 17-22.

Shirley, M. W. (2000). The genome of *Eimeria* spp., with special reference to *Eimeria tenella*--a coccidium from the chicken. Int J Parasitol *30*, 485-493.

Shirley, M. W. (1994). The genome of *Eimeria tenella*: further studies on its molecular organisation. Parasitol Res *80*, 366-373.

Shirley, M. W., Smith, A. L., and Tomley, F. M. (2005). The biology of avian *Eimeria* with an emphasis on their control by vaccination. Adv Parasitol *60*, 285-330.

Shirley, M. W., and Harvey, D. A. (1996). *Eimeria tenella*: infection with a single sporocyst gives a clonal population. Parasitology *112 (Pt 6)*, 523-528.

Smith, A. L., and Hayday, A. C. (1998). Genetic analysis of the essential components of the immunoprotective response to infection with *Eimeria vermiformis*. Int J Parasitol *28*, 1061-1069.

Smith, G. P., and Petrenko, V. A. (1997). Phage Display. Chem Rev *97*, 391-410.

Smith, G. P. (1996). Foreword. In Phage Display of Peptides and Proteins, B. Kay, J. Winter, and J. McCafferty, eds. (San Diego, California: Academic Press), pp.xvii-xix.

Smith, G. P. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science *228*, 1315-1317.

Soldati, D., Dubremetz, J. F., and Lebrun, M. (2001). Microneme proteins: structural and functional requirements to promote adhesion and invasion by the apicomplexan parasite *Toxoplasma gondii*. Int J Parasitol *31*, 1293-1302.

Steinberger, P., Rader, C., and Barbas, C. F. (2001). Analysis of selected antibodies. In Phage Display: A Laboratory Manual, C. F. Barbas III, D. R. Burton, J. K. Scott, and G. J. Silverman, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), pp. 11.1-11.24.

Thornberry, N. A., and Lazebnik, Y. (1998). Caspases: enemies within. Science *281*, 1312-1316.

Tierney, J., and Mulcahy, G. (2003). Comparative development of *Eimeria tenella* (Apicomplexa) in host cells in vitro. Parasitol Res *90*, 301-304.

Tomley, F. M., and Soldati, D. S. (2001). Mix and match modules: structure and function of microneme proteins in apicomplexan parasites. Trends Parasitol *17*, 81-88.

Tomley, F. (1997). Techniques for isolation and characterization of apical organelles from *Eimeria tenella* sporozoites. Methods *13*, 171-176.

Tomley, F., Billington, K., and Bumstead, J. (1997). Progress on the characterization of micronemes of *Eimeria tenella*: novel targets for future control of coccidiosis. In Control of Coccidiosis into the next Millennium, M. Shirley, F. Tomley, and B. Freeman, eds. (Compton, Newbury Berks, UK: Institute for Animal Health), pp. 57-58.

Tomley, F. (1994a). Antigenic diversity of the asexual developmental stages of *Eimeria tenella* sporozoites. Methods *13*, 171-176.

Tomley, F. M. (1994b). Characterization of rhoptry proteins of *Eimeria tenella* sporozoites: antigenic diversity of rhoptry epitopes within species of the genus *Eimeria*

and among three asexual generations of a single species, *E. tenella*. Infect Immun 62, 4656-4658.

Trout, J. M., and Lillehoj, H. S. (1996). T lymphocyte roles during *Eimeria acervulina* and *Eimeria tenella* infections. Vet Immunol Immunopathol *53*, 163-172.

Vermeulen, A. N. (2004). Avian coccidiosis: a disturbed host-parasite relationship to be restored. Symp Soc Exp Biol, 211-241; discussion 243-215.

Vermeulen, A. N., Schaap, D. C., and Schetters, T. P. (2001). Control of coccidiosis in chickens by vaccination. Vet Parasitol *100*, 13-20.

Vervelde, L., Vermeulen, A. N., and Jeurissen, S. H. (1996). In situ characterization of leucocyte subpopulations after infection with *Eimeria tenella* in chickens. Parasite Immunol *18*, 247-256.

Vervelde, L., Vermeulen, A. N., and Jeurissen, S. H. (1993). Common epitopes on *Eimeria tenella* sporozoites and cecal epithelium of chickens. Infect Immun *61*, 4504-4506.

Voeten, A. C., Braunius, W. W., Orthel, F. W., and van Rijen, M. A. (1988). Influence of coccidiosis on growth rate and feed conversion in broilers after experimental infections with *Eimeria acervulina* and *Eimeria maxima*. Vet Q *10*, 256-264.

Wallach, M. (1997). The importance of transmission-blocking immunity in the control of infections by apicomplexan parasites. Int J Parasitol *27*, 1159-1167.

Wallach, M., Halabi, A., Pillemer, G., Sar-Shalom, O., Mencher, D., Gilad, M., Bendheim, U., Danforth, H. D., and Augustine, P. C. (1992). Maternal immunization with gametocyte antigens as a means of providing protective immunity against *Eimeria maxima* in chickens. Infect Immun *60*, 2036-2039.

Wallach, M., Pillemer, G., Yarus, S., Halabi, A., Pugatsch, T., and Mencher, D. (1990). Passive immunization of chickens against *Eimeria maxima* infection with a monoclonal antibody developed against a gametocyte antigen. Infect Immun *58*, 557-562.

Wang, C. C., and Stotish, R. L. (1975). Pancreatic chymotrypsin as the essential enzyme for excystation of *Eimeria tenella*. J Parasitol *61*, 923-927.

Webster, R. (2001). Filamentous phage biology. In Phage Display: A Laboratory Manual, C. F. Barbas III, D. R. Burton, J. K. Scott, and G. J. Silverman, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), pp. 1.1-1.37.

Williams, R. B. (2002). Anticoccidial vaccines for broiler chickens: pathways to success. Avian Pathol *31*, 317-353.

Williams, R. B. (1999). A compartmentalised model for the estimation of the cost of coccidiosis to the world's chicken production industry. Int J Parasitol *29*, 1209-1229.

Winter, G., Griffiths, A. D., Hawkins, R. E., and Hoogenboom, H. R. (1994). Making antibodies by phage display technology. Annu Rev Immunol *12*, 433-455.

Winter, G., and Milstein, C. (1991). Man-made antibodies. Nature *349*, 293-299.

Wisher, M. H., and Rose, M. E. (1987). *Eimeria tenella* sporozoites: the method of excystation affects the surface membrane proteins. Parasitology *95 (Pt 3)*, 479-489.

Yamanaka, H. I., Inoue, T., and Ikeda-Tanaka, O. (1996). Chicken monoclonal antibody isolated by a phage display system. J Immunol *157*, 1156-1162.

Yokota, T., Milenic, D. E., Whitlow, M., and Schlom, J. (1992). Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. Cancer Res *52*, 3402-3408.

Yun, C. H., Lillehoj, H. S., and Lillehoj, E. P. (2000). Intestinal immune responses to coccidiosis. Dev Comp Immunol *24*, 303-324.

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Daad Ali Abi Ghanem was born in Beirut, Lebanon. She was a frail child who sought refuge in books, a harbinger that she would someday end up jotting these lines. After high school, Daad joined the American University of Beirut (AUB) in 1991, and earned a B.S. in Nutrition and Dietetics (1994), and an M.S. in Poultry Science (1998). Thinking she has spent too much time in school, she decided to take a break, and worked for three years at AUB as a research assistant on a technology transfer and agricultural extension project, an experience that would shape her aspirations, and provide a decisive turning point in her life. Daad joined the Department of Poultry Science at Texas A&M University in September 2001, after a professor she had contacted forwarded her email to Dr. Luc R. Berghman. This fortunate happenstance was the beginning of a great collaboration, as she ended up working with Dr. Berghman, a most extraordinary advisor and mentor, who made her the scientist she is today. Daad is fascinated by the intricate fields of immunology, molecular biology and genetics. Her research interests include the application of antibody engineering in immunotherapy, and the development of strategies for control of infectious diseases.

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