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Genetic control of immune responsiveness to the avian coccidium Eimeria tenella

Robert Andrew Clare
University of New Hampshire, Durham

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GENETIC CONTROL OF IMMUNE RESPONSIVENESS
TO THE AVIAN COCCIDIUM
EIMERIA TENELLA

BY

Robert Andrew Clare
B.S., The Pennsylvania State University, 1983
M.S., University of New Hampshire, 1985

DISSERTATION

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This dissertation has been examined and approved.

_____
Dissertation director, Richard G. Strout,
Professor of Animal and Nutritional Sciences

_____
Walter M. Collins, Professor Emeritus of
Animal Sciences and Genetics

_____
Robert M. Zsigray, Professor of Microbiology

_____
Robert L. Taylor, Jr., Assistant Professor
of Animal and Nutritional Sciences

_____
Harry D. Danforth, Ph.D., Research Scientist
Protozoan Disease Laboratory,
Livestock and Poultry Health Institute,
Agricultural Research Service,
United States Department of Agriculture,
Beltsville, Maryland

_____
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ABSTRACT

GENETIC CONTROL OF IMMUNE RESPONSIVENESS TO THE AVIAN COCCIDIUM <u>EIMERIA</u> <u>TENELLA</u>

by

ROBERT A. CLARE

University of New Hampshire, May, 1988

Specific inbred lines of chickens expressing different major histocompatibility (<u>B</u>) complex haplotypes were studied for their immunogenetic response to the a‑ian coccidium <u>Eimeria</u> <u>tenella</u> and genetically-engineered antigens cloned from this parasite species. The relative influence of the <u>B-F</u> vs. <u>B-G</u> chromosomal regions on innate resistance and immunity to <u>E</u>. <u>tenella</u> was studied among six <u>B-F</u>/<u>B-G</u> recombinant hosts. F_1 chicks of similar genotypes, $\underline{B}^{R3}$ and $\underline{B}^{R4}$ ($\underline{B}^{F2-G23}\underline{B}^{17}$), gained significantly more weight than those with $\underline{B}^{R1}$ ($\underline{B}^{F24-G23}\underline{B}^{17}$) and $\underline{B}^{R5}$ ($\underline{B}^{F21-G19}\underline{B}^{17}$) following exposure to a single high dose of 25,000 oocysts, although no differences in cecal lesion scores were detected. Following exposure to a single low dose of 2500 oocysts, F_1 chicks $\underline{B}^{R3}$ and $\underline{B}^{R4}$ again gained significantly more weight and had significantly lower lesion scores compared to $\underline{B}^{R1}$ or $\underline{B}^{R5}$ F_1 chicks. Acquired immunity in F_2 homozygous recombinant chicks was studied using five consecutive daily exposures of 500 oocysts followed 21 days later by challenge with 10,000 oocysts. $\underline{B}^{R5}$ ($\underline{B}^{F21-G19}$) and $\underline{B}^{R6}$ ($\underline{B}^{F21-G23}$)

homozygous chicks, both carrying the $\underline{B}\text{-}\underline{F}^{21}$ allele, showed significant protection in terms of reduced lesion scores.

The immunogenicity of recombinant $\underline{E}$. tenella coccidial antigens 5401 and 3264 was studied in $6_1.\underline{B}$ congenic chickens derived from $\underline{B}^2\underline{B}^2$ and $\underline{B}^5\underline{B}^5$ parents segregating for haplotypes $\underline{B}^2$ and $\underline{B}^5$. In separate experiments, antigens 5401 and 3264, both inoculated subcutaneously in Freund's complete adjuvant, elicited a protective response only in $\underline{B}^5\underline{B}^5$ chickens. $\underline{B}^2\underline{B}^2$ and $\underline{B}^5\underline{B}^5$ chickens also responded differently in serum IgG titer and in the magnitude of lymphocyte proliferation. Western blot analysis showed differential labeling of low molecular weight proteins. Antibodies raised against antigen 3264 strongly labeled only the anterior portion of the sporozoite stage of $\underline{E}$. tenella. In contrast, these same antibodies strongly labeled only the posterior portion of second generation merozoites, while first generation merozoites were only slightly labeled, if at all. In addition, a single heavily labeled nodule, possibly an immune complex, was observed on the posterior surface of second generation merozoites.

Considered together, these findings demonstrate further that genetic responsiveness is linked to the major histocompatibility complex and has a profound influence on the host response to the parasite as well as the efficacy of recombinant protozoan vaccines.

INTRODUCTION

The avian coccidia are obligate intracellular protozoan parasites of intestinal epithelium. They belong to the Phylum Apicomplexa, including such genera as <u>Plasmodium</u>, <u>Sarcocystis</u>, <u>Toxoplasma</u>, and <u>Cryptosporidium</u>, all of which exhibit similar life cycles. Immunity to any one of these parasites appears to be directed at the sporozoite, the first parasitic stage to invade a host cell. Complete neutralization of the sporozoite would prevent the debilitating effects of these parasites. Because of the tremendous reproductive capacity of these organisms, even a few sporozoites evading the host response could still produce a fulminating infection.

Since sporozoite surface antigens are likely candidates to trigger the host immune response, it appears reasonable that these antigens could be used in the manufacture of a vaccine. Sporozoite antigens have been successfully cloned from <u>Plasmodium</u> and <u>Eimeria</u> species. The efficacy of these candidate vaccines depends upon priming the host's immune system such that an anamnestic response would be triggered by a natural infection with the parasite. As with other vaccines, responder and non-responder hosts exist. Therefore, a complete understanding of the molecular interactions between the host and a given vaccine antigen is

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necessary. For the host, a primary interaction is at the level of the major histocompatibility complex (MHC).

The chicken MHC, or B complex, is not well understood, and only recently has the molecular organization of this tightly-linked genetic region been investigated. The association of the B complex with several viral diseases in chickens is well established, as is its influence on immunity to avian coccidiosis. Since the coccidia are now the subject of intense vaccine research, it is appropriate to investigate the role the MHC may have on the efficacy of a candidate coccidial vaccine. Therefore, the purpose of this dissertation is to:

1. Investigate further the effect of the MHC on the fate of _Eimeria tenella_ parasitism by examining both a primary and subsequent secondary infection in six MHC B-F/B-G recombinant chicken hosts.

2. Investigate the immunogenic efficacy of two genetically-engineered coccidial proteins in chicken hosts congenic for the B locus.

3. Investigate the antigenic nature of sporozoites and merozoites of _E. tenella_ using antibodies raised against a recombinant coccidial antigen.

REVIEW OF THE LITERATURE

The Avian Major Histocompatibility Complex

The <u>B</u> complex, discovered by Briles and coworkers (1950), was originally identified as an avian blood group system of erythrocyte antigens. This highly polymorphic locus was later shown to be the marker for the chicken major histocompatibility complex (Schierman and Nordskog, 1961). At present, 27 <u>B</u> haplotypes have been identified in 32 different populations tested from laboratories in the United States and Europe. These studies are only a limited sampling, and the number of demonstrable haplotypes in chicken populations worldwide would probably exceed a hundred (Briles and Briles, 1987).

The <u>B</u> complex is understood to consist of three subregions, <u>B</u>-G, <u>B</u>-F, and <u>B</u>-L (Pink et al, 1977), each coding for serologically defined antigens of differing tissue distribution. <u>B</u>-F and <u>B</u>-L antigens code for avian homologs of mammalian class I and class II antigens, respectively (Nordskog et al., 1987). The <u>B</u>-F or class I molecule consists of a heavy chain having an apparent molecular weight of 40-43 kD, and is noncovalently associated with a beta-2-microglobulin chain having an apparent weight of 11-12 kD (Ziegler and Pink, 1976). <u>B</u>-F is expressed on all somatic cells, which include the nucleated avian erythrocyte.

The B-L or class II molecule is a dimer consisting of alpha and beta chains (Crone et al., 1981) having an apparent molecular weight of 27 and 32 kD, respectively (Ziegler and Pink, 1978). The B-L antigen, the equivalent of the Ia antigen in mice, is distributed on B cells, monocytes/macrophages, dendritic cells, and activated T cells (Hala et al., 1984).

The B-G subregion codes for a unique avian erythrocytic antigen, designated class IV, consisting of a 46-48 kD monomer in reduced form, and is expressed as an 85 kD dimer isolated from avian erythrocytes, or a 130 kD trimer isolated from erythrocyte membranes (Salomonsen et al., 1987). At present, no homologous mammalian counterpart to the B-G antigen has been identified (Briles and Briles, 1987).

The B complex has been shown to be linked to the nucleolus organizer region (NOR) on a medium-sized acrocentric chromosome of about 16th in size (Bloom and Bacon, 1985). Since the NOR occupies a majority of the long arm, Bloom and coworkers (1987) speculate that a likely subchromosomal location for the MHC resides on the long arm proximal to the centromere. Other possible locations include the distal long arm region, the short arm, or a pericentric location including both proximal long arm and short arm regions.

Infrequent recombinational events between the B-F and B-G regions have been described. Eight B-F/B-G recombinants

have been identified with the consistent transmission of B-F and B-L from the same parental chromosome (Briles et al., 1982). Moreover, gene duplication or recombination within the B-G subregion has been described (Miller et al., 1986). No recombination between B-F and B-L has yet been identified by serological methods suggesting that these regions may be tightly linked. It is possible that the limited frequency of recombination is directly related to the short chromosomal segment occupied by the avian MHC (Vainio et al., 1987). In addition, the B complex does not appear to contain hot spots of recombination as reported for the murine MHC (Steinmetz et al., 1986). Taken together, these findings indicate strong linkage disequilibrium with the transmission of associated B-F and B-L alleles (Simonsen et al., 1982).

The first functional description of the B complex as a marker for the avian MHC arose from studies examining the relationship of the B blood group system with skin graft rejection (Schierman and Nordskog, 1961). The B complex's association with other immune functions include graft-versus-host reaction (Lee and Nordskog, 1981), mixed lymphocyte reaction (Miggiano et al., 1974); total hemolytic complement (Chahn et al., 1976); immune responses to synthetic antigens (Benedict et al., 1975; Pevzner et al., 1978); serum IgG levels (Rees and Nordskog, 1981); interleukin-2 levels (Knudston and Lamont, 1987); and PHA-wattle response (Taylor et al., 1987).

The association between the B complex and disease resistance has been extensively reviewed (Bacon, 1987). Specific B haplotypes have been shown to control resistance and/or susceptibility to Marek's disease (Briles and Briles, 1982; Briles et al., 1977; Bacon et al., 1981); sarcoma tumors (reviewed by Collins and Zsigray, 1984; Schierman and Collins, 1987); avian lymphoid leukosis (Bacon et al., 1981); and fowl cholera (Lamont et al., 1987). Studies using B complex recombinant chickens have permitted the mapping of resistance to Marek's disease (Briles et al., 1983) and regression of Rous sarcoma virus-induced tumors (Auclair, 1982; Aeed, 1984) to the B-F subregion.

Genetic variation among hosts in the response to avian coccidia infection is well established (Jeffers and Shirley, 1982). Recent studies have investigated the specific role of blood group systems on resistance to Eimeria tenella infection (Johnson and Edgar, 1984; Johnson and Edgar, 1986; and Martin et al., 1986). The B complex has been shown to control, in part, innate resistance and acquired immunity to E. acervulina and/or E. tenella (Ruff and Bacon, 1984; Clare et al., 1985; and Lillehoj et al., 1985).

Genetically-Engineered Coccidia Vaccines

Coccidiosis is an enteric disease of domestic livestock causing acute morbidity resulting in reduced body weight gain, a decrease in feed efficiency, and some mortality. In the poultry industry this disease becomes economically

relevant as costs exceed $300 million annually in product losses, prophylactic medication (anticoccidial compounds) and research (Danforth and Augustine, 1985a). Indeed the success of today's poultry industry and the corresponding inexpensive cost of poultry meat is due to the anticoccidials. Although current drugs are highly effective, the coccidia are continuously evolving parasites, and resistant strains appear readily. Immunomodulation through the use of vaccines may permit a more complete method to effectively control these parasites. To be successful an economically feasible vaccine should cost no more than current means of prevention, require only a single treatment, and ultimately improve current production standards. To date, the most cost-effective means of vaccine production may involve recombinant DNA technology.

Most of the current understanding in protozoan vaccines evolved from research using species of <u>Plasmodium</u>, etiologic agents of malaria. It was originally discovered that immunity against sporozoites could be induced by the injection of irradiated parasites into humans, monkeys, or rodents (reviewed by Nussenzweig and Nussenzweig, 1984). Monoclonal antibodies to the major sporozoite surface antigen could also passively protect rodents from parasite challenge (Potocnjak et al., 1980), suggesting that high antibody titers to the sporozoite would block sporozoite invasion of hepatocytes and subsequent development. Since sporozoites are difficult to isolate or produce in large

quantities, characterization and cloning of the relevant antigens became a more feasible approach.

The gene coding for the major surface antigen or circumsporozoite (CS) protein has been cloned from P. falciparum (Dame et al., 1984) and P. vivax (Arnot et al., 1985) of human malaria, P. knowlesi (Ozaki et al., 1983) and P. cynomolgi (Enea et al., 1984) of simian malaria, and P. berghei of murine malaria (Weber et al., 1987). When sequenced, each of these genes consisted of a central region containing tandem repeats, shown to comprise an active epitope (Hollingdale et al., 1984). In addition, synthetic peptides based upon these repetitive epitopes (Ballou et al., 1985) or recombinant constructs in Escherichia coli containing these repeat sequences (Young et al., 1985) elicited antibodies which blocked hepatocyte invasion. The magnitude of protection was associated with dose, boosting, and the type of adjuvant administered with the constructs, aluminum hydroxide gel being the most effective (Mazier et al., 1986; Wirtz et al., 1987; Hollingdale et al., 1987).

These first candidate malarial vaccines were conceived without regard for T cell epitopes, assuming that antibody titers would be sufficient for protection. Studies in mice demonstrated that this omission created a problem because only mice bearing the $I-A^b$ haplotype responded to a given fusion protein (Del Giudice et al., 1986; Good et al., 1986). Moreover, of nine different congenic strains of mice, only $H-2^b$ and $H-2^k$ strains were high responders to the

recombinant CS protein of P. falciparum (Good et al., 1986).
Therefore, the immunizing ability of a candidate antigen was
directly related to the expression of the appropriate MHC
gene. Berzofsky and coworkers (1987) suggested a similar
situation could occur in humans, which would limit
considerably the usefulness of these vaccines.

Since $H-2^k$ mice do not respond to the repetitive
epitope, they must recognize a T epitope outside the
repetitive region. Studies were undertaken to identify the
specific T epitope recognized by $H-2^k$ mice and shown to map
to the $I-A^k$ region (Berzofsky et al., 1987). An amphipathic
model or algorithm has been proposed to functionally
describe a T reactive site (Margalit et al., 1987). Simply,
the immunodominant site recognized by T cells may be an
amphipathic structure consisting of hydrophilic and
hydrophobic residues which align, respectively, in an alpha-
helix (DeLisi and Berzofsky, 1985; Berzofsky et al., 1986;
DeLisi et al., 1987; Spouge et al., 1987). The hydrophilic
side would interact with the T cell receptor, while the
hydrophobic side would interact with either class I or II
MHC molecules (Berzofsky et al., 1987). An amphipathic
structure would result from antigen processing by the
appropriate antigen-presenting cell (APC), and would be
stabilized by the APC surface environment (Berzofsky et al.,
1987). Using this algorithm, a T site was predicted on the
carboxy terminal side of the CS epitope. A corresponding
peptide was manufactured which successfully primed $H-2^k$

helper T cells for a secondary antibody response. Having isolated the appropriate T epitope, a synthetic immunogen could be created consisting of both B and T epitopes.

The phenomena of MHC-restricted responsiveness must still be addressed. MHC restriction describes the interaction between lymphocytes and/or APC's, which requires identity at the level of expressed MHC haplotypes. The fact that only a few MHC haplotypes respond to the CS antigen suggests that associated vaccines would be limited in their immunizing capabilities. A greater understanding of the human MHC or HLA complex, which exceeds H-2 in polymorphism, is necessary as well as the specific interactions of HLA alleles and particular vaccine epitopes. Further immunomodulation may be required as indicated by Kawamura and coworkers (1985), who showed that MHC-linked unresponsiveness could be overcome by IL-2 treatment.

Immunity to the avian coccidia is understood to derive from a species-specific cell-mediated response augmented by locally produced antibody (reviewed by Rose, 1982; Powell, 1987). Rose (1985) speculated that the host immune response is evoked primarily by merozoite antigens and directed against subsequent sporozoite invasion. Murray and coworkers (1985) isolated and characterized sporozoite antigens of E. tenella. Twenty-three polypeptides were identified by polyacrylamide gel electrophoresis (PAGE), and of these bands, eight (235, 105, 94, 82, 71, 68, 45, and 26 kD) reacted strongly with polyclonal sporozoite antisera raised

in rabbits. An extract produced from ground, sporulated oocysts, injected intramuscularly without adjuvant into chickens, caused a reduction in lesion scores following homologous parasite challenge. In addition, a similar antigen preparation from E. acervulina elicited protection through reduced lesion scores, against E. tenella and E. maxima. These findings are in direct contrast to studies (reviewed by Rose, 1982) which concluded that non-viable coccidia antigens were not immunogenic. Moreover, the existence of species cross-protective antigens had not been described previously.

Unlike Plasmodia, little information exists concerning a major sporozoite surface antigen (Paul et al., 1986). A 21-23 kD polypeptide was isolated from E. tenella and used to produce serum antibodies in chickens which neutralized sporozoites in vitro. The effect of this antigen in vivo was not examined.

Hybridoma antibodies have been used extensively to characterize both species- and stage-specific coccidia antigens (Wisher, 1983; Danforth, 1985; Truitt et al. 1986). E. tenella has probably been characterized the most thoroughly, including monoclonal antibodies (MAb) raised against sporozoite surface antigens (McAndrew et al., 1986), both sporozoites and merozoites (Danforth and McAndrew, 1987), and microgametocytes (Laxer et al., 1987). Although several of these MAb have been shown to neutralize parasite

stages _in vitro_, none have been shown to elicit passive protection _in vivo_.

The promise of a genetically-engineered malarial vaccine has encouraged work toward the production of a similar vaccine for the coccidia (reviewed by Danforth and Augustine, 1988). Cloning technology has enabled the production of cDNA libraries for E. tenella sporozoites and/or merozoites (Danforth and Augustine, 1985b; Binger et al., 1986; Clarke et al., 1986). Stage-specific MAb have been used to screen these libraries, and a few recombinant antigens have been isolated and tested _in vivo_.

One of these proteins, designated 5401, was cloned from sporulated E. tenella oocysts and has an apparent molecular weight of 35 kD (Danforth and Augustine, 1988). 5401 contains a nine amino acid sequence consisting of Ala-Glu-Glu-Leu-Pro-Gly-Glu-Glu-Gly, which is repeated five times. Following subcutaneous injection along with adjuvant into birds, 5401 elicits serum antibodies which produce a surface-internal fluorescent pattern on air-dried sporo-zoites. Protein 5401, given at doses of 2.4-4.8 ug per bird, also induced partial protection against a 25,000 oocyst challenge, although this protection could be overwhelmed by a challenge of 300,000 or more oocysts. When the 5401 gene was expressed in another vector, designated 3264, it induced protection in terms of reduced lesion scores at this higher challenge dose (Danforth and Augustine, unpublished

results). Neither protein, however, elicited any cross-species protection with E. acervulina.

These immunity trials with 5401 and 3264 are encouraging with respect to producing a genetically-engineered coccidial vaccine. Several problems exist, however, which must be addressed before an effective vaccine will be marketed. Similar to the first cloned malarial antigens, these coccidia antigens have been characterized only by antibody or B cell recognition. Since B cell epitopes do not appear to be sufficient, reactive T cell epitopes must be explored and identified. The amphipathic algorithm previously mentioned could be useful in predicting reactive T sites in the cloned gene products.

The lack of species-cross reactive antigens identified to date presents a problem since nine species of chicken coccidia exist. Conceivably, a vaccine would have to be made for each one, a consequence which could severely limit the practicality of this cloning approach. The work reported by Murray and coworkers (1985) shows promise in this area since E. acervulina antigens were cross-protective against E. maxima and E. tenella. Little recombinant work has been done with species other than E. tenella, and possibly other species may provide a more complete antigen.

Finally, the host response cannot be ignored. Host immunity is a manifestation of several complex cellular events integrated and controlled by molecules encoded by the MHC. Malarial research has shown that only a few H-2

haplotypes respond to certain CS antigens. This same phenomenon may be occurring in chickens since certain B haplotypes have been shown to respond differently to the E. tenella parasite (Clare et al., 1985). Only recently have highly inbred congenic populations of chickens been available to study directly the interaction of a given B allele and the expression of immunity elicited by cloned antigens. These congenic chickens are produced by the sequential backcrossing of a donor line, which supplies the differential gene(s) or in this case the B complex, on a highly inbred recipient line. This mating scheme produces birds whose genomes differ only at the region of the MHC. Since congenic chickens which differ in B complex genotype possess identical background gene complements, differential immune responsiveness may be attributable to the expressed MHC alleles. Information gained from this type of research may be applied ultimately to all vaccine programs in the field.

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CHAPTER ONE

Characterization of Resistance and Immunity

to _Eimeria_ _tenella_ Among

Major Histocompatibility Complex

_B-F_/_B-G_ Recombinant Hosts

Abstract

The relative influence of the B-F vs. B-G chromosomal regions on innate resistance and immunity to *Eimeria tenella* was studied among six B-F/B-G recombinants, designated $\underline{B}^{R1}$, $\underline{B}^{R3}$, $\underline{B}^{R4}$, $\underline{B}^{R5}$, $\underline{B}^{R6}$, and $\underline{B}^{R8}$. In one experiment, resistance was studied among 340 F_1 chicks, each carrying the $\underline{B}^{17}$ allele derived from line UCD.003, and therefore also heterozygous for one of the $\underline{B}$ recombinant haplotypes. In a second experiment, acquired immunity was studied among 161 F_2 chicks each carrying one $\underline{B}^{17}$ allele, and segregating for one of the recombinant alleles.

In Experiment 1, F_1 chicks of similar haplotypes, $\underline{B}^{R3}$ and $\underline{B}^{R4}$ ($\underline{B}^{F2-G23}$), gained significantly more weight than those with $\underline{B}^{R1}$ ($\underline{B}^{F24-G23}$) and $\underline{B}^{R5}$ ($\underline{B}^{F21-G19}$) following exposure to a single high dose of 25,000 oocysts, although no differences in lesion scores were detected among the six recombinants. Following exposure to a single low dose of 2500 oocysts, F_1 chicks $\underline{B}^{R3}$ and $\underline{B}^{R4}$ also gained significantly more weight and had significantly lower lesion scores compared to $\underline{B}^{R1}$ or $\underline{B}^{R5}$ F_1 chicks. On the other hand, when all six heterozygous recombinants were immunized with a single exposure of 2500 oocysts followed by challenge with 10,000 oocysts 21 days later, $\underline{B}^{R5}$ chicks had the lowest mean lesion scores. To study acquired immunity in the F_2 homozygous chicks, five consecutive daily exposures of 500 oocysts were followed 21 days later by challenge with 10,000 oocysts. $\underline{B}^{R5}$ ($\underline{B}^{F21-G19}$) and $\underline{B}^{R6}$ ($\underline{B}^{F21-G23}$) homozygous

chicks, both carrying the $\underline{B-F}^{21}$ allele, showed significant protection in terms of reduced lesion scores. These results suggest that the $\underline{B-F}$ chromosomal subregion imparts some control over the mechanism of innate resistance and also acquired immunity to $\underline{E}$. $\underline{tenella}$.

KEY WORDS: Coccidiosis, $\underline{Eimeria}$, $\underline{B}$ complex recombinants

Introduction

Genetic variation among hosts in the response to avian coccidia infection is well established (Jeffers and Shirley, 1982). More recent studies have investigated the specific role of blood group system genes on resistance to *Eimeria tenella* infection (Johnson and Edgar, 1986; Martin *et al.*, 1986; and Johnson Edgar, 1984). The B blood group system, discovered by Briles and coworkers (1950) and established as the marker for the chicken major histocompatibility complex (Schierman and Nordskog, 1961), has been shown to control, in part, innate resistance and acquired immunity to E. acervulina or E. tenella (Ruff and Bacon, 1984; Lillehoj *et al.*, 1985; Clare *et al.*, 1985).

The B complex is understood to consist of three subregions, B-G, B-F, and B-L (Pink *et al.*, 1977), each coding for serologically defined antigens of differing tissue distribution. B-F and B-L antigens code for avian homologs of mammalian class I and class II antigens, respectively. The B-G subregion codes for a unique avian erythrocytic antigen, having no homologous mammalian counterpart. No recombination between B-F and B-L has yet been identified in progeny from experimental matings by serological methods, suggesting that these subregions are very tightly linked. Studies using B-F/B-G recombinant chickens have permitted the mapping of resistance to Marek's disease (Briles *et al.*, 1983) and regression of Rous sarcoma

virus-induced tumors (Auclair, 1982; Aeed, 1984) to the B-F subregion.

The present study examines the relative influence of the B-F and B-G chromosomal regions on innate resistance and immunity to E. tenella, among six B complex recombinants, designated B^{R1}, B^{R3}, B^{R4}, B^{R5}, B^{R6}, and B^{R8}.

Materials and Methods

Experiment 1. Two hatches totaling 340 F_1 generation chicks were produced from single sire matings designed to place each of the six B-F/B-G recombinant haplotypes on a line UCD.003 ($B^{17}B^{17}$) background. Therefore, all chicks produced from each of the six matings were either full-sibs or half-sibs. At 5 weeks of age, chicks heterozygous for each separate recombinant were divided among three treatment groups to receive the following primary inocula of E. tenella: 25,000 oocysts, 2500 oocysts, and uninfected controls. The Lilly 65 strain of E. tenella was used throughout this study. Twice as many chicks were included in groups exposed to 2500 oocysts and uninfected controls as in the group exposed to 25,000 oocysts. Half of the chicks in the former groups were challenged with 10,000 oocysts 21 days after the primary exposure. Cecal lesion scores (Johnson and Reid, 1970), prepatent body weight gain, and percent mortality were measured 6 days following both primary and secondary exposures.

Experiment 2. Adults of each of four heterozygous genotypes $\underline{B}^{17}\underline{B}^{R3}$, $\underline{B}^{17}\underline{B}^{R5}$, $\underline{B}^{17}\underline{B}^{R6}$, and $\underline{B}^{17}\underline{B}^{R8}$ resulting from matings in Experiment 1 were segregated into single sire breeding groups. The progeny of each of the four groups consisted of sib and half-sib individuals homozygous for recombinant $\underline{B}^{R3}$, $\underline{B}^{R5}$, $\underline{B}^{R6}$, or $\underline{B}^{R8}$ together with sibs and half-sibs heterozygous for the respective recombinants ($\underline{B}^{R3}\underline{B}^{17}$, etc.) and homozygous for the haplotype $\underline{B}^{17}$. A total of 161 F_2 chicks were placed on experiment. At five weeks of age, chicks of each of these $\underline{B}$ haplotype combinations were immunized with 500 $\underline{E}$. tenella oocysts for each of five consecutive days. Unimmunized chicks from each mating type were maintained as controls. Twenty-one days following the last 500 oocyst exposure, all chicks were challenged with 10,000 oocysts. Cecal lesion scores and prepatent body weight gain were determined 6 days following the challenge exposure.

All birds were typed at 2 weeks of age for $\underline{B}$ locus alloantigens by hemagglutination (Briles and Briles, 1982; Briles, unpublished). Data were analyzed by analysis of variance for genotype and treatment effects, as well as for any genotype x treatment interaction. Means were separated using Fisher's protected LSD test at $P\leq0.05$.

Results

Experiment 1. $\underline{B}^{17}$ heterozygotes possessing serologically similar haplotypes $\underline{B}^{R3}$ and $\underline{B}^{R4}$ (both $\underline{B}^{F2-G23}$) gained

significantly more weight than those with $\underline{B}^{R1}$ ($\underline{B}^{F24-G23}$) and $\underline{B}^{R5}$ ($\underline{B}^{F21-G19}$) following exposure to 25,000 oocysts, although no significant differences in lesion scores were detected among the six recombinants (Table 1). The 25,000 oocyst exposure produced a severe infection as indicated by cecal lesion scores averaging between 3.6 and 4.0 as well as high mortality. Such a heavy infection may have overwhelmed any genetic effect upon innate resistance.

A more distinct genotype effect on resistance was shown following the exposure to 2500 oocysts. $\underline{B}^{R3}$ and $\underline{B}^{R4}$ gained significantly more weight and had significantly lower lesion scores than $\underline{B}^{R1}$ and $\underline{B}^{R5}$ (Table 1). Although $\underline{B}^{R6}$ ($\underline{B}^{F21-G23}$) and $\underline{B}^{R8}$ ($\underline{B}^{F2-G2,23}$) exhibited greater weight gains at each exposure level than did $\underline{B}^{R1}$ and $\underline{B}^{R5}$, both manifested the highest lesion scores following 2500 oocysts. Only 5% of $\underline{B}^{R6}$ chicks died as a result of the lower dose (data not shown).

No significant differences among genotypes in either lesion score or weight gain following the secondary 10,000 oocyst challenge were detected (Table 2). Chicks previously exposed to 2500 oocysts had reduced lesion scores compared to susceptible controls.

Experiment 2. Homozygous $\underline{B}^{R5}\underline{B}^{R5}$ chicks had significantly lower lesion scores following challenge than either $\underline{B}^{R3}\underline{B}^{R3}$ or $\underline{B}^{R8}\underline{B}^{R8}$ homozygotes, while homozygous $\underline{B}^{R6}\underline{B}^{R6}$ chicks had significantly lower lesion scores than $\underline{B}^{R8}\underline{B}^{R8}$ chicks (Table 3). Both $\underline{B}^{R5}\underline{B}^{R5}$ and $\underline{B}^{R6}\underline{B}^{R6}$ chicks had

significantly lower lesion scores than their respective unimmunized controls while $\underline{B}^{R3}\underline{B}^{R3}$ and $\underline{B}^{R8}\underline{B}^{R8}$ chicks did not.

Heterozygotes $\underline{B}^{17}\underline{B}^{R5}$ and $\underline{B}^{17}\underline{B}^{R6}$ (Table 3) had significantly lower lesion scores than their respective controls, although neither differed significantly from either $\underline{B}^{17}\underline{B}^{R3}$ or $\underline{B}^{17}\underline{B}^{R8}$. Among the $\underline{B}^{17}\underline{B}^{17}$ homozygotes, the segregants from the $\underline{B}^{R3}$ mating showed for significant protection to challenge and significantly lower lesion scores than $\underline{B}^{17}\underline{B}^{17}$ segregants from either $\underline{B}^{R5}$ or $\underline{B}^{R6}$ matings.

Immunized chicks gained significantly more weight following challenge than respective unimmunized controls (Table 4). No significant effect of $\underline{B}$ genotype on weight gain was measured among the recombinant $\underline{B}^{R}\underline{B}^{R}$ homozygotes or the $\underline{B}^{17}\underline{B}^{R}$ heterozygotes. The $\underline{B}^{17}\underline{B}^{17}$ segregants from the $\underline{B}^{R3}$ mating did gain significantly more weight than the $\underline{B}^{17}\underline{B}^{17}$ segregants from the $\underline{B}^{R5}$ mating, an effect paralleling the magnitude of protection also seen with reduced cecal lesions.

Discussion

These data suggest that different genetic mechanisms may be involved with innate resistance to cecal coccidiosis as compared to acquired immunity. Recombinant chicks possessing the B-F2 segment from the haplotype $\underline{B}^{2}$ (Briles and Briles, 1977; Briles and Briles, 1980) exhibited greater innate resistance to E. tenella parasitism, but after priming showed little protection against a challenge

infection. Conversely, chicks carrying the $\underline{B-F21}$ segment of the haplotype $\underline{B}^{21}$ (Briles $\underline{et}$ $\underline{al}$., 1983) were susceptible to an initial exposure, but following immunization were protected against a challenge infection. Therefore, the $\underline{B-F2}$ segment was associated with resistance, while the $\underline{B-F21}$ segment was associated with acquired immunity. Taken together, these findings confirm previous studies (Aeed, 1984; Auclair, 1982; Briles $\underline{et}$ $\underline{al}$., 1983) which implicate the $\underline{B-F}$ subregion as having a definite role in disease susceptibility.

While the recombinant $\underline{B}^{R8}$ possesses the chromosome segments $\underline{B-F2}$ and $\underline{B-G23}$ in addition to $\underline{B-G2a}$ (Briles $\underline{et}$ $\underline{al}$., 1982; Miller $\underline{et}$ $\underline{al}$., 1987; Briles, unpublished), these chicks did not show the same protective response to the initial exposure as did serologically similar $\underline{B}^{R3}$ and $\underline{B}^{R4}$ chicks also possessing $\underline{B-F2}$ and $\underline{B-G23}$. These data suggest that $\underline{B}^{R8}$ chickens were not as resistant as $\underline{B}^{R3}$ and $\underline{B}^{R4}$ chickens and may indicate an influence of the $\underline{B-G}$ subregion.

Previous studies in this and other laboratories (Davis $\underline{et}$ $\underline{al}$., 1985) have shown that repeated low dose exposures of oocysts induce greater immunity to challenge than a single inoculation; a differential $\underline{B}$ genotype effect was shown following such an immunizing protocol (Clare $\underline{et}$ $\underline{al}$., 1985). In Experiment 1, a single primary inoculum of 2500 oocysts induced equal protection to challenge among the six recombinants tested. However, all recombinants used in this experiment were F_1 progeny, and therefore 50% of their

genetic composition came from inbred line UCD.003. This may explain the uniformity of response to the challenge exposure.

Experiment 2 used F_2 chicks segregating at the $\underline{B}$ locus for both the recombinant haplotype and $\underline{B}^{17}$. This protocol permitted an evaluation of recombinant haplotypes expressed in the heterozygote condition as in Experiment 1, and also a comparison of $\underline{B}^R\underline{B}^R$ and $\underline{B}^{17}\underline{B}^{17}$ homozygotes. Five consecutive daily exposures of 500 oocysts produced a protective response to challenge in $\underline{B}^{R5}$ and $\underline{B}^{R6}$ homozygotes, $\underline{B}^{17}\underline{B}^{R5}$ and $\underline{B}^{17}\underline{B}^{R6}$ heterozygotes, and all $\underline{B}^{17}\underline{B}^{17}$ homozygotes. These results support a role for the $\underline{B-F}^{21}$ allele and suggest that $\underline{B-F}^{17}$, from the $\underline{B}^{17}$ haplotype contributed by line UCD.003, also influences acquired immunity to $\underline{E}$. tenella. In addition, since all progeny were produced from single-sire matings, individual sire effects may be contributing to the differential response to parasitism.

The present findings continue to support previous work implicating a general role for $\underline{B}$ alloantigens in controlling resistance or immunity to coccidiosis (Ruff and Bacon, 1984; Lillehoj et al., 1985; Clare et al., 1985). All of these investigations found the response to parasite infection was associated with the expression of a specific $\underline{B}$ haplotype. In contrast, studies completed by Johnson and Edgar (1984) described the frequencies of $\underline{A}$ and $\underline{E}$ blood group system haplotypes expressed in the the R and S Auburn White Leghorn lines, which were selected for resistance and

susceptibility, respectively, to cecal coccidiosis (Johnson and Edgar, 1982). The R line possessed two AE allele combinations in high frequency as opposed to completely different AE alleles being expressed in the S line. These investigators concluded that the interaction of A and E was necessary for these loci to factor in genetic resistance to E. tenella. In addition, the frequencies of B and C blood group system alleles in these lines were examined (Johnson and Edgar, 1986), and collectively, A, E, C, and B alloantigen systems appear to influence mortality and body weight gain in response to cecal coccidiosis. Therefore, it seems that at least three alloantigen systems in addition to the chicken MHC may influence the complex host response to E. tenella parasitism.

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TABLE 1. <u>Mean cecal lesion scores, mean body weight gain and percent mortality 6 days following exposure B genotypes to either 25,000 or 2500 Eimeria tenella oocysts</u>

Genotype	No. of chicks	Lesion score	Weight gain (g) (25,000 Oocyst dose)	Mortality %	Lesion score	Weight gain (g) (2500 Oocyst dose)
B^{17}/B^{R3} (F2-G23)	22	$3.9 \pm .1^{ns}$	24 ± 3^{c}	38	$2.8 \pm .2^{a}$	39 ± 2^{c}
B^{17}/B^{R4} (F2-G23)	22	$3.6 \pm .2$	29 ± 3^{c}	23	$2.8 \pm .2^{a}$	42 ± 2^{c}
B^{17}/B^{R8} (F2-G2a,23)	14	4.0 ± 0	21 ± 4^{bc}	43	$3.5 \pm .1^{bc}$	32 ± 4^{bc}
$B17/B^{R1}$ (F24-G23)	14	$3.6 \pm .6$	-20 ± 9^{a}	7	$3.3 \pm .2^{bc}$	25 ± 4^{ab}
B^{17}/B^{R6} (F21-G23)	21	4.0 ± 0	16 ± 3^{bc}	48	$3.5 \pm .2^{c}$	40 ± 3^{c}
B^{17}/B^{R5} (F21-G19)	21	$3.6 \pm .2$	6 ± 6^{b}	38	$3.2 \pm .2^{b}$	15 ± 8^{a}

[abc]Means within columns not followed by a common letter are significantly different at $P \leq 0.05$.

[ns]not significant

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TABLE 2. Mean cecal lesion scores and mean body weight gain of B genotypes 6 days following a secondary 10,000 oocyst challenge exposure given 21 days after an immunizing dose of 2500 oocysts

Genotype	No. of chicks	Lesion score	Weight gain (g) (Primed)	Lesion score (Naive)	Weight gain (g)
B^{17}/B^{R3} (F2-G23)	21	$1.4 \pm .3^{ns}$	56 ± 5^{ns}	$3.7 \pm .1^{ns}$	62 ± 4^{ns}
B^{17}/B^{R4} (F2-G23)	22	$1.2 \pm .3$	53 ± 3	$3.2 \pm .2$	63 ± 5
B^{17}/B^{R8} (F2-G2a,23)	15	$1.9 \pm .3$	50 ± 4	$3.1 \pm .2$	48 ± 4
$B17/B^{R1}$ (F24-G23)	16	$1.3 \pm .3$	56 ± 3	$3.1 \pm .2$	55 ± 7
B^{17}/B^{R6} (F21-G23)	17	$1.5 \pm .2$	53 ± 4	$3.5 \pm .2$	58 ± 6
B^{17}/B^{R5} (F21-G19)	20	$1.2 \pm .2$	50 ± 3	$3.3 \pm .2$	64 ± 7

[ns]not significant

TABLE 3. <u>Mean cecal lesion scores of B genotypes 6 days following a 10,000 E. tenella oocyst challenge given 21 days after the fifth consecutive daily immunizing exposure of 500 oocysts</u>

Genotype	Recombinant mating			
	R3 (F2-G23)	R8 (F2-G2a,23)	R5 (F21-G23)	R6 (F21-G19)
$\underline{B}^R\underline{B}^R$	$2.8 \pm .6^{bc}$ (9)	$3.4 \pm .3^{c}$ (11)	$1.4 \pm .4^{a}$ (11)	$1.9 \pm .6^{ab}$ (9)
$\underline{B}^{17}\underline{B}^R$	$2.7 \pm .4^{bc}$ (11)	$2.7 \pm .5^{bc}$ (10)	$1.9 \pm .5^{ab}$ (10)	$2.7 \pm .4^{b}$ (9)
$\underline{B}^{17}\underline{B}^{17}$	$0.9 \pm .4^{a}$ (10)	$2.0 \pm .6^{b}$ (9)	$2.1 \pm .4^{b}$ (9)	$2.8 \pm .5^{b}$ (9)
Control	$3.5 \pm .2^{c}$ (11)	$3.5 \pm .2^{c}$ (15)	$3.8 \pm .1^{c}$ (10)	$3.9 \pm .1^{c}$ (8)

[abc]Means within columns or rows not followed by a common letter are significantly different at $P \leq 0.05$.

(n) = number of chicks

TABLE 4. <u>Mean body weight gain (g) of B genotypes 6 days</u> <u>following a 10,000 E. tenella oocyst challenge given 21 days</u> <u>after the fifth consecutive daily immunizing exposure of 500</u> <u>oocysts</u>

Genotype	Recombinant mating			
	R3 (F2-G23)	R8 (F2-G2a,23)	R5 (F21-G23)	R6 (F21-G19)
R/R	54 ± 4^{cd} (9)	59 ± 8^{c} (11)	56 ± 5^{c} (11)	51 ± 7^{c} (9)
17/R	53 ± 9^{cd} (11)	51 ± 7^{c} (10)	49 ± 8^{c} (10)	47 ± 10^{c} (9)
17/17	61 ± 5^{d} (10)	58 ± 6^{cd} (9)	43 ± 4^{bc} (9)	52 ± 8^{c} (9)
Control	5 ± 8^{b} (11)	8 ± 7^{b} (15)	-28 ± 10^{a} (10)	-9 ± 11^{ab} (8)

[abc]Means within columns or rows not followed by a common letter are significantly different at $P \leq 0.05$.

(n) = number of chicks

CHAPTER TWO

Immunogenicity of a Recombinant *Eimeria* *tenella* Protein in $6_1 \cdot \underline{B}$ Congenic Chickens

Abstract

The immunogenicity of a recombinant _Eimeria tenella_ coccidial antigen-5401 was studied in $6_1.B$ congenic chickens derived from $\underline{B}^2\underline{B}^2$ and $\underline{B}^5\underline{B}^5$ parents segregating for haplotypes $\underline{B}^2$ and $\underline{B}^5$. Five week old chickens were immunized with 2.4 ug of recombinant protein (designated 5401) and challenged with 75,000 oocysts 28 days post immunization (DPI) to determine the degree of elicited protective immunity. Serum samples were collected weekly for 5 weeks post immunization for ELISA, IFA and Western blot analysis.

Lesion scores following oocyst challenge were significantly reduced in $\underline{B}^5\underline{B}^5$ chickens compared to $\underline{B}^2\underline{B}^2$ chickens. Immunization induced a sporozoite-specific serum IgG titer detected by ELISA that peaked at 28 DPI, the day of challenge, in $\underline{B}^5\underline{B}^5$ chickens and at 42 DPI in $\underline{B}^2\underline{B}^2$ chickens. Following challenge, this titer declined for each genotype. Anti-sporozoite IgG detected by IFA attained peak titer at 21 DPI in $\underline{B}^2\underline{B}^2$ chickens and 28 DPI in $\underline{B}^5\underline{B}^5$ chickens. Serum from immunized $\underline{B}^5\underline{B}^5$ birds reacted strongly in Western blots with several high molecular weight soluble proteins (>100 kD), prepared from sporozoites. Serum from $\underline{B}^2\underline{B}^2$ birds reacted with similar proteins as well as a 51-53 kD protein not labeled by $\underline{B}^5\underline{B}^5$ serum. These results demonstrate further the role of host genetics on anti-coccidial immunity, and suggest that a peak anti-sporozoite IgG titer on the day of challenge is associated with a protective response.

Introduction

Coccidiosis is a parasitic intestinal disease affecting poultry and livestock worldwide. Infection with this obligate, intracellular protozoan causes a species-specific protective immune response at the expense of feed conversion, weight gain, and occasional mortality. Several studies have examined the role of host genetics in controlling both innate susceptibility to the disease (1-4) as well as acquired immunity (5,6). Specifically, the $\underline{B}^5$ haplotype (7) of the chicken major histocompatibility complex (8) or $\underline{B}$ locus (9) has been implicated in conferring immunocompetence against *Eimeria tenella* (5), the etiological agent of cecal coccidiosis in domestic fowl. In contrast, chicken hosts possessing the $\underline{B}^2$ haplotype were not protected against parasite challenge.

A recombinant *E. tenella* antigen cloned from the oocyst, the stage of this parasite ingested by its avian host, has been produced and characterized (10). This 35 kD protein designated 5401 contains several amino acid repeat sequences, a trait also observed of the circumsporozoite antigen of *Plasmodium falciparum*, another Apicomplexan protozoan (11). Immunization of chickens with this antigen induced partial clinical protection to oral parasite challenge in a commercial outbred line of chickens (10). The present study reports the differential humoral and associated clinical responses elicited by 5401 in congenic chickens segregating for haplotypes $\underline{B}^2$ and $\underline{B}^5$.

Materials and Methods

Chickens. Inbred Single Comb White Leghorn (SCWL) line 6_1 ($\underline{B}^2\underline{B}^2$), developed at the Regional Poultry Research Laboratory (RPRL), East Lansing, MI, was selected as the background strain for the developing $\underline{B}$ congenic line. This line is histocompatible, resistant to Marek's disease, susceptible to subgroup A, B and C leukosis viruses, and regresses Rous sarcoma virus (RSV)-induced. tumors (12). Females of inbred SCWL line 15_1 ($\underline{B}^5\underline{B}^5$), also developed at RPRL, were crossed with line 6_1 to produce the F_1 generation. Line 15_1 is susceptible to Marek's disease and subgroup A leukosis virus, and progresses RSV-induced tumors (13). For subsequent backcross generations, heterozygous $\underline{B}^2\underline{B}^5$ females were mated to line 6_1 males. Eighth backcross generation heterozygous males and females were mated inter se to produce $\underline{B}^2\underline{B}^2$ or $\underline{B}^5\underline{B}^5$ homozygous progeny with >99% background gene uniformity (unpublished data).

A total of 55 chickens, divided among three trials, were used in this study. All progeny were typed for $\underline{B}$ alloantigens by hemagglutination (7). Chicks were raised coccidia-free to 5 weeks of age on wire floor brooding batteries and fed an non-medicated all-mash starter ad libitum.

Recombinant antigen and parasite. The recombinant coccidial antigen 5401, kindly provided by Genex Corporation, Gaithersburg, MD, was cloned from non-sporulated and sporulated oocysts of L.S. #24 strain

E. tenella (Animal Parasitology Institute, Beltsville, MD),
and produced in Escherichia coli (10). The gene coding for
this antigen was expressed in a lambda gt11 bacteriophage
vector, yielding a fusion protein of approximately 150 kD
corresponding to about 35 kD coccidial antigen fused to
Beta-galactosidase. Sequencing indicated that the antigen
contained a primary structure of five repeating segments of
nine amino acids each.

Immunization of chickens. Five week old chickens were
immunized individually with 2.4 ug of antigen-5401 in
saline, emulsified 1:1 in Freund's complete adjuvant (FCA),
and injected in a 0.5 ml volume subcutaneously at the base
of the neck. Previous titration studies showed a single dose
of 2.4 ug was maximally protective (14). Unimmunized birds
of each genotype were maintained as controls. Chickens were
challenged at 28 days post immunization (DPI) by an oral
inoculation of 75,000 L.S. #24 E. tenella oocysts (API) to
determine the efficacy of antigen-5401 immunization.

Clinical criteria. Clinical disease was monitored 6
days post oral inoculation by cecal lesion scores [scored 0
to 4] (15) and body weight gain.

ELISA. Pooled serum samples within genotypes were
taken weekly, beginning the day of immunization (Day 0)
through day of termination (Day 42) to assess sporozoite-
specific IgG titers. Control serum was obtained from
chickens immunized with saline emulsified 1:1 in FCA. ELISA
analysis was carried out essentially as previously described

(16). Briefly, sporozoites were excysted by standard procedures, passed over a scrubbed nylon wool column to remove debris, centrifuged, and resuspended in phosphate-buffered saline (PBS). Sporozoites were then disrupted with glass beads (100 um in diameter) by vortexing for 2 min. The material was centrifuged at 11,600 x g for 2 min and the supernatant fluid collected. A protein concentration was determined using the method outlined by Biorad (Biorad, Richmond, CA). Soluble sporozoite antigen was then diluted in carbonate-bicarbonate buffer (pH 9.6) and adsorbed to 96 well EIA plates (Costar, Cambridge, MA) at a concentration of 1 ug/well overnight at 4 C. All subsequent incubations were carried out at 41 C for 1 h. Plates were blocked with 3% bovine serum albumen in PBS then rinsed three times with PBS-Tween 20. Serial dilutions of individual serum samples in PBS-Tween 20 were reacted with adsorbed antigen followed by three rinses in PBS-Tween 20. Plates were then exposed to a 1:1000 dilution of goat anti-chicken IgG horseradish peroxidase conjugate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) in PBS. Plates were washed three times with PBS-Tween 20 and developed with tetramethyl benzidine (TMB) reagent (ICN Biomedicals, Costa Mesa, CA). Absorbances were read on a microelisa reader (Dynatech, Alexandria, VA) at 450 nm and corrected for background. Mean absorbances from quadruplicate wells per trial are reported for 1/1600 serum dilutions.

IFA. Sporozoites were obtained as described and fixed by air-drying to Toxoplasma titer slides at a concentration of 1 x 10^6/ml. Sporozoites were reacted with serial dilutions of immune sera and incubated at room temperature in a humid chamber for 1 h. Slides were washed for 10 min in PBS, then reacted with rabbit anti-chicken IgG (Kirkegaard and Perry Laboratories) diluted 1:30 in PBS and incubated for 1 h in humidity. Again slides were washed for 10 min in PBS, then reacted with fluorescein (FITC) conjugated goat anti-rabbit IgG (Kirkegaard and Perry Laboratories) diluted 1:30 for 1 h in humidity. Slides were washed a final time in PBS, mounted with glycerol and observed under UV light microscopy.

PAGE and Western blot analysis. Approximately 2.0 X 10^7 E. tenella sporozoites were disrupted with glass beads (100 um in diameter) by vortexing for 2 min in sample buffer (17). The suspension was centrifuged at 11,600 x g for 2 min, and the supernatant fluid was applied to a 10% SDS-polyacrylamide gel. The gel was electrophoresed at 35 mA for 6 h. Separated sporozoite proteins were transferred from 10% SDS-gels onto nitrocellulose paper overnight at 30 V (18). The paper was blocked with 3% gelatin in Tris-buffered saline (TBS) for 2 h at 37 C, rinsed in water and exposed for 2 h at room temperature to 1:1000 dilutions of 5401-immunized chicken serum in antibody buffer. All subsequent incubations were for 2 h at room temperature. The paper was rinsed twice in PBS-Tween 20 and exposed to a 1:1000

dilution of rabbit anti-chicken IgG in antibody buffer. Following another two rinses in PBS-Tween, the paper was labeled with goat anti-rabbit horseradish peroxidase conjugate diluted 1:1000 in antibody buffer, then washed twice in PBS-Tween 20 and developed with 4-chloronaphthol reagent.

Statistics. All data were analyzed by analysis of variance, and means were tested using Student-Newman-Keul's (SNK) test at the 0.05 level of probability.

Results

Clinical response to parasite challenge. Oral challenge at 28 DPI induced moderately severe cecal lesions 6 days later at 34 DPI in 5401 immunized $\underline{B}^2\underline{B}^2$ chickens compared to essentially no infection in $\underline{B}^5\underline{B}^5$ chickens (Table I). All unimmunized controls showed lesion scores of +4. Although immunized birds of each genotype gained more weight than unimmunized controls, this difference was not significant ($P \leq 0.05$) (Table I).

Serum antibody production-ELISA. A sporozoite-specific IgG titer was first detected by ELISA in $\underline{B}^5\underline{B}^5$ chickens at 14 days post immunization (DPI) and continued to increase through 21 DPI with a peak at 28 DPI, the day of oral challenge (Fig. 1). In unchallenged $\underline{B}^5\underline{B}^5$ birds, the IgG titer fell sharply by 35 DPI and continued its decline to 42 DPI. The IgG titer of challenged $\underline{B}^5\underline{B}^5$ chickens at 34 DPI, the day these birds were sacrificed for cecal lesion

scoring, declined 50% (0.141) following challenge, but at a slower rate than unchallenged $\underline{B}^5\underline{B}^5$ birds. A sporozoite-specific IgG titer was barely detectable in $\underline{B}^2\underline{B}^2$ chickens at 14 DPI but continued to rise slowly to a peak concentration at 42 DPI in unchallenged birds. Following oral challenge on 28 DPI, the IgG titer of $\underline{B}^2\underline{B}^2$ chicks fell sharply at 34 DPI (day of lesion scoring), to a level (0.082) approximately one-half of that measured in $\underline{B}^5\underline{B}^5$ chickens. No anti-sporozoite titers were measured in chickens of either genotype following immunization with saline and FCA (data not shown).

Serum antibody production-IFA. Immune sera from both genotypes produced a surface-internal immunofluorescent antibody pattern on air dried sporozoites, but each showed a different pattern of titer reactivity by IFA than ELISA. No IFA titers were detected at 7 DPI from birds of each genotype, while equivalent IFA titers were measured for each genotype at 14 DPI (Table II). In the absence of challenge, $\underline{B}^2\underline{B}^2$ sera peaked in anti-sporozoite titer at 21 DPI then declined from 28 to 35 DPI. In contrast, the $\underline{B}^5\underline{B}^5$ response which was lower than that of $\underline{B}^2\underline{B}^2$ peaked at 28 DPI and then declined. Following challenge at 28 DPI, however, $\underline{B}^2\underline{B}^2$ titers were elevated to their highest levels on 35 DPI while $\underline{B}^5\underline{B}^5$ titers remained at their pre-challenge level.

Serum profiles to sporozoite antigen. Western blot analysis was used to characterize differential serum antibody reactivities following immunization with 5401.

Soluble sporozoite antigen was resolved on a 10% PAGE slab into several protein bands ranging in molecular weight between 15 and >200 kD (Fig. 2). Upon Western transfer, serum from 5401-immunized $\underline{B}^5\underline{B}^5$ chickens consistently labeled several high molecular weight bands (>100 kD) beginning at 14 DPI with strongest labeling or signal seen at 28 DPI (Fig. 2). A weaker signal was observed with serum from unchallenged $\underline{B}^5\underline{B}^5$ birds at 35 and 42 DPI. Serum from 5401-immunized $\underline{B}^2\underline{B}^2$ chickens labeled similar high molecular weight proteins although labeling was not detected until 21 DPI. Unchallenged $\underline{B}^2\underline{B}^2$ serum gave the strongest signal at 35 and 42 DPI. $\underline{B}^2\underline{B}^2$ serum also reacted with a lower molecular weight band (approximately 51-53 kD) not labeled with $\underline{B}^5\underline{B}^5$ serum. Serum from chickens of both genotypes immunized with saline and FCA did not react with Western blots (data not shown).

Discussion

The present study confirms the results of earlier clinical studies implicating the role of the chicken $\underline{B}$ (MHC) complex on immunity to coccidiosis (5,19). Chickens congenic for the $\underline{B}$ complex exhibited a differential response to a cloned coccidial protein following both immunization and parasite challenge. Immunization with recombinant antigen 5401 reduced cecal pathology following challenge in $\underline{B}^5\underline{B}^5$ birds while eliciting little protection in $\underline{B}^2\underline{B}^2$ hosts. Body weights were neither significantly influenced by parasite

challenge nor genotype, although immunized birds gained numerically more weight than unimmunized birds. This apparent uniformity in weight gain may reflect the highly inbred character of these lines.

Previous immunity studies using 5401 in a commercial outbred line demonstrated high IgG titers following immunization (9). In the present study anti-sporozoite IgG titers elicited by 5401, measured weekly by both ELISA and IFA, differed between both genotype and assays. ELISA and IFA exhibit inherently different antibody concentration sensitivities and specificities, and these patterns may explain the interassay differences in the titer profile. The ELISA technique used in this study would detect antibody specific for soluble sporozoite antigen only, while the IFA technique using air-dried sporozoites would measure antibody directed against antigens present in or on the intact parasites. There may be several antigens in common for each assay, and the different antigen preparations may select for or eliminate certain antigenic moieties. ELISA and IFA may measure an IgG titer to different antigens. In addition, the more sensitive ELISA may detect small quantities of antigens not detected by IFA. Both assays detected genotype differences in the magnitude and kinetics of the response. $\underline{B}^5\underline{B}^5$ chickens exhibited a peak IgG titer by ELISA at 28 DPI, the day of the 75,000 oocyst challenge, and showed protection against that challenge 6 days later (34 DPI). On the other hand, $\underline{B}^2\underline{B}^2$ chickens produced a significantly lower

IgG titer at 28 DPI, and were not protected against this same challenge dose. Six days following challenge (34 DPI), the $\underline{B}^2\underline{B}^2$ serum titer dropped to its lowest concentration. Therefore, a peak serum ELISA IgG titer at day of challenge was associated with a protective response to clinical disease in $\underline{B}^5\underline{B}^5$ chickens. Compared to the IgG titer in unchallenged birds, oral challenge in $\underline{B}^5\underline{B}^5$ chickens sustained an IgG titer, but challenge in $\underline{B}^2\underline{B}^2$ birds either suppressed the humoral response or bound circulating antibody. In contrast, a noninbred Light Sussex line showed a peak serum IgG titer 11-15 days following an oral inoculation of 10^4 E. tenella oocysts and protection against subsequent parasite challenge (20), suggesting that the parasite elicited both a protective and more rapid response than the cloned antigen. However, this protection was at the expense of a severe clinical disease produced by the primary oocyst infection.

A different IgG titer profile was measured by IFA. The $\underline{B}^2\underline{B}^2$ serum IgG titer peaked 21 DPI and then declined in unchallenged birds. Following challenge, $\underline{B}^2\underline{B}^2$ serum titer increased to its highest level while the $\underline{B}^5\underline{B}^5$ IgG titer remained unchanged. An elevated IgG titer measured by IFA was not associated with protection to challenge in $\underline{B}^2\underline{B}^2$ birds. These results may indicate the $\underline{B}^2$ haplotype is mediating a strong response to a non-protective antigen. A similar "decoy" scenario has been suggested in the response to the circumsporozoite antigen of malaria (21). Moreover,

B^2B^2 birds respond most strongly to sporozoite antigen after challenge, a phenomenon which appears to abrogate a protective response.

Western blot analysis indicated a response similar to that measured by the ELISA, because the magnitude of antibody labeling or signal corresponded directly with measured titers; both assays utilized similar solid support antigens. Immune serum from both genotypes labeled similar high molecular weight protein bands. B^2B^2 serum collected 21 and 28 DPI also labeled a 45 kd band not labeled by B^5B^5 serum. Again, the B^2 haplotype may be mediating a response against an antigen which either misdirects or inhibits protection.

Immunity to the avian eimeria has been shown to be a T cell-mediated response (22). Previously, there were little data supporting a direct humoral role in the protective response to coccidiosis, although immunity to E. tenella may be augmented by secretory IgA (23,24). The present data clearly demonstrate the association between a peak serum anti-sporozoite IgG titer on day of parasite challenge and reduced cecal pathology following immunization with a recombinant antigen.

In addition, the B^5 haplotype appears to impart a greater efficiency in mounting the IgG response than does B^2. Although parasite neutralization may ultimately involve T cells, the efficiency of the response may be inherent in antigen presentation as a result of the interaction between

parasite antigen and MHC gene products on antigen presenting cells (APC) (25). In this system, antigen-5401 may have a stronger affinity for the class II molecule encoded by the $\underline{B}^5$ haplotype than the corresponding molecule encoded by the $\underline{B}^2$ haplotype. Thus, $\underline{B}^5\underline{B}^5$ chickens may be considered high responders to antigen-5401 and $\underline{B}^2\underline{B}^2$ chickens low responders. Similar genetic responsiveness was described in mice immunized with a P. falciparum sporozoite vaccine and shown to map to the I-A region of the murine MHC (26).

The initial response to antigen-5401, a soluble antigen, may be B cell mediated, as the B cell may serve as the APC along with an obligatory production of parasite-specific IgG and subsequent secretory IgA (27). It is not known what direct role these antibodies may have, if any, in arresting parasite development. Antigen presentation during a natural infection may involve macrophages (28) or intraepithelial lymphocytes (29). Therefore, the use of a recombinant antigen such as antigen-5401 may invoke an additional cellular component, B cells, which may serve in some capacity to neutralize parasitism.

These results demonstrate the role of host genetics in ascertaining the efficacy of a recombinant protein as a vaccine. The use of these congenic lines of chickens indicate that a high anti-sporozoite IgG titer at the time of challenge is associated with protection elicited by the genetically-engineered antigen. The ability of a recombinant

coccidial protein to stimulate a IgG response may indicate an effective vaccine component.

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Table I. Mean cecal lesion scores and body weight gain (g) 6 days following oral $\underline{E}$. $\underline{tenella}$ oocyst challenge (28 DPI) of $6_1.\underline{B}$ congenic chickens previously immunized with recombinant $\underline{E}$. $\underline{tenella}$ antigen-5401.

B Genotype	No. Birds	Mean Lesion Score	Mean Weight Gain ($\pm$SEM)
B2/B2-immune	10	3.2^b	99.5 ± 17.3 g
B5/B5-immune	9	$<1^a$	86.7 ± 14.6 g
Control-infected	10	4.0^b	66.1 ± 3.7 g

[a,b]Means not followed by a common letter are significant at $P\leq0.05$.

Table II. Anti-sporozoite serum IgG titers, detected by IFA and reported as reciprocal dilutions, from $6_1.\underline{B}$ congenic chickens immunized with recombinant $\underline{E}$. tenella antigen-5401 on day 0 and challenged with 75,000 $\underline{E}$. tenella oocysts 28 DPI.

B Genotype	0	7	14	21	28	35	34C[*]
				Day			
B2/B2	0	0	80	320	160	40	500
B5/B5	0	0	80	160	160	80	160

[*]Serum IgG titers of birds challenged at 28 DPI and scored

for cecal lesions at 34 DPI.

Figure 1. Anti-sporozoite serum IgG titers, detected by ELISA and reported as Abs.$_{450}$, of $6_1.\underline{B}$ congenic chickens immunized with recombinant $\underline{E}$. $\underline{tenella}$ antigen-5401.
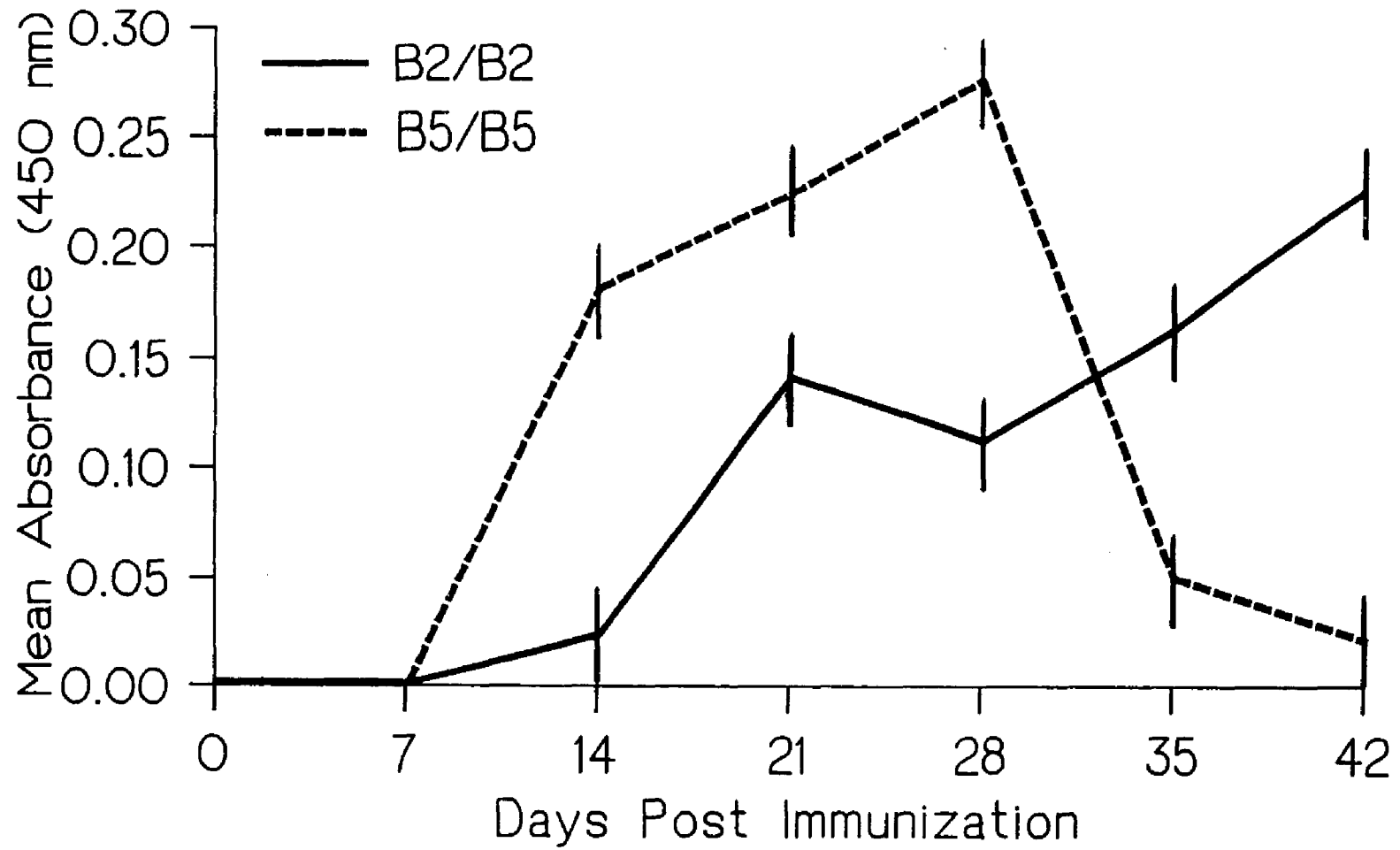
Figure 2. Western blot analysis of serum from $\underline{B}^5\underline{B}^5$ or $\underline{B}^2\underline{B}^2$ birds immunized with antigen-5401 on $\underline{E}$. tenella sporozoite preparation. Lanes 1 and 7 at 7 DPI, lanes 2 and 8 at 14 DPI, lane 3 and 9 at 21 DPI, lanes 4 and 10 at 28 DPI, lanes 5 and 11 at 35 DPI, and lanes 6 and 12 at 42 DPI. Asterisks in lanes 9-12 placed beside 51-53 kD antigen reacting with serum from $\underline{B}^2\underline{B}^2$ immunized birds. Positions of M_r markers are identified on left of figure, and from top to bottom correspond to myosin, 200 kD; phosphorylase B, 97 kD; serum albumen, 68 kD; ovalbumen, 43 kD; and alpha-chymotrypsinogen, 25 kD.
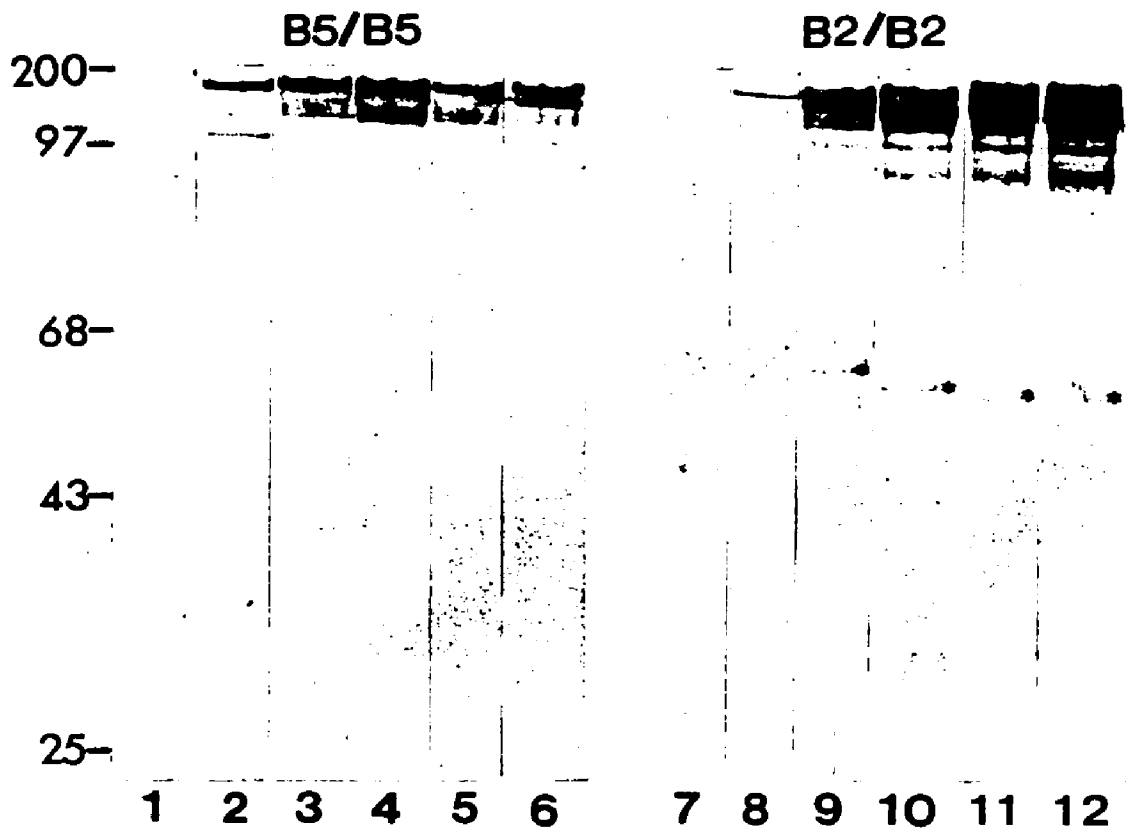
CHAPTER THREE

Major Histocompatibility Complex Control
of Immunity Elicited By Genetically Engineered
Eimeria _tenella_ (Apicomplexa) Antigen in Chickens

Abstract

Immunity elicited by a recombinant coccidial antigen #3264 was studied in $6_1.\underline{B}$ congenic chickens segregating for haplotypes $\underline{B}^2$ and $\underline{B}^5$. Antigen 3264, cloned from <u>Eimeria tenella</u> oocysts, was administered in Freund's complete adjuvant to 5 week old chickens in doses of either 2.4 ug, 10 ug, or 20 ug. Immunized chicks were then given a boost dose of 10 ug, 10 ug, or 20 ug, respectively. Seven days following the boost all chicks were exposed to 25,000 oocysts. Body weight gain and cecal lesion scores in response to parasite challenge were measured. Serum was collected weekly for ELISA and western blot analysis. Peripheral lymphocytes were also collected weekly for an <u>in vitro</u> proliferation assay.

In all three immunization protocols, 3264 elicited a protective response only in $\underline{B}^5\underline{B}^5$ chickens. $\underline{B}^2\underline{B}^2$ and $\underline{B}^5\underline{B}^5$ chickens responded differently in serum IgG titer and in the magnitude of lymphocyte proliferation. Western blot analysis showed differential labeling of certain low molecular weight proteins. Together, these findings demonstrate how genetic responsiveness linked to the major histocompatibility complex will have a profound effect on the efficacy of candidate protozoan vaccines.

Introduction

Eimeria tenella, an obligate intracellular protozoan parasite, is the etiologic agent of cecal coccidiosis in domestic chickens. Avian coccidia (Family Eimeriidae) belong to the Phylum Apicomplexa, as do the Plasmodia (Family Plasmodiidae), and both share similar development patterns. To date, both genera are the subject of intense vaccine research (7,16), with considerable emphasis being placed upon the ability to clone reactive sporozoite surface antigens (6,20). The sporozoite is the first parasitic stage to penetrate host cells and would be a likely candidate to trigger the host's immune system.

Recent studies have examined the host's response to candidate malarial antigens at the level of the major histocompatibilty complex in mice (8,9). These studies demonstrated that high and low responders occurred in a given mouse population, and that these responses were MHC restricted. Similarly, avian host immune responses to coccidiosis have been shown to be MHC-linked (3,11,13, Clare, unpublished).

A candidate coccidial antigen, designated 3264, has been successfully cloned from sporulated oocysts of E. tenella (Danforth and Augustine, unpublished). The response to 3264 in vivo elicits partial protection in a commercially available strain of chickens (Danforth and Augustine, unpublished). It is quite possible that the host variation which leads to this incomplete protection is derived, in

part, from MHC effects. The purpose of the present study is to evaluate the response to the 3264 antigen in chicken hosts congenic for the MHC using different antigen dose levels. These studies may provide an additional insight into the interaction between host and potential protozoan vaccines.

Materials and Methods

Chicken hosts. Five week old line 6_1.$\underline{B}$ partially congenic chicks segregating at the $\underline{B}$ locus for alleles $\underline{B}^2$ and $\underline{B}^5$ were used in this study. Inbred Single Comb White Leghorn (SCWL) line 6_1 ($\underline{B}^2\underline{B}^2$), developed at the Regional Poultry Research Laboratory (RPRL), East Lansing, MI, was selected as the background strain for the developing $\underline{B}$ congenic line. Females of inbred SCWL line 15_1 ($\underline{B}^5\underline{B}^5$), also developed at RPRL, were crossed with line 6_1 to produce the F_1 generation. Subsequent backcross generations were derived from heterozygous $\underline{B}^2\underline{B}^5$ females mated to line 6_1 males. Line 6_1 is histocompatible, resistant to Marek's disease, susceptible to subgroup A, B and C leukosis viruses, and regresses Rous sarcoma virus (RSV)-induced tumors (18), while line 15_1 is susceptible to Marek's disease and subgroup A leukosis virus, and progresses RSV-induced tumors (4). Eighth backcross generation heterozygous males and females were mated inter se to produce $\underline{B}^2\underline{B}^2$ or $\underline{B}^5\underline{B}^5$ homozygous progeny with >99% background gene uniformity (unpublished data).

Chicks were typed at 2 weeks of age for $\underline{B}$ alloantigens by hemagglutination (2). All chicks were housed in wire-floored cages and provided unmedicated feed and water <u>ad libitum</u>.

<u>Antigen and parasites</u>. The genetically engineered antigen used in this study, designated 3264, was cloned from sporulated oocysts of <u>E</u>. <u>tenella</u> (L.S. #24 strain, Animal Parasitology Institute, Beltsville, MD). The gene coding for this antigen was expressed in a lambda gt11 bacteriophage vector which yielded a fusion protein of approximately 150 kD corresponding to about 35 kD coccidial antigen fused to Beta-galactosidase (Danforth and Augustine, unpublished). An oral inoculum of 25,000 sporulated L.S. #24 <u>E</u>. <u>tenella</u> oocysts was used as a parasite challenge to test the immunogenicity of the cloned antigen.

<u>Immunization studies</u>. Three experiments using different immunization protocols were completed. In all studies, antigen-3264 emulsified in Freund's complete adjuvant was injected subcutaneously at the base of the neck into 5 week old 6_1.B congenic chicks. In Experiment 1, birds were immunized on Day 0 with 2.4 ug of antigen-3264 and received a booster injection of 10 ug antigen-3264 at Day 28. Seven days following the booster injection, chicks were challenged with 30,000 oocysts. In Experiment 2, birds were immunized with 10 ug 3264 on Day 0, boosted with 10 ug 3264 on Day 21, and exposed to oocysts on Day 28. In Experiment 3, birds were immunized with 20 ug 3264 on Day 0, boosted with 20 ug

3264 on Day 14, and exposed to oocysts on Day 21. In all experiments, age-matched birds not receiving antigen treatment were maintained as controls for the challenge exposure.

Clinical disease. Body weight gain was measured during the 6 day prepatent period immediately following parasite challenge, afterwhich immunized and non-immunized control birds were killed and cecal lesion scores determined (10).

ELISA. Serum samples pooled within genotypes were taken weekly, beginning the day of immunization (Day 0) through the sixth day following oocyst challenge to assess sporozoite-specific IgG titers. Control serum was obtained from chickens immunized with saline emulsified 1:1 in FCA.

Serum antibody titer analysis was carried out using a modification of the procedure reported by Rose and Mockett (14). Sporozoites were excysted aseptically by standard procedures, passed over a scrubbed nylon wool column to remove debris, centrifuged, and resuspended in phosphate-buffered saline (PBS). Sporozoites were then disrupted with glass beads (100 um in diameter) by vortexing for 2 min. The material was centrifuged at 11,600 x g for 2 min and the supernatant fluid collected. The protein concentration was determined using the method outlined by Biorad (Biorad, Richmond, CA). Soluble sporozoite antigen was then diluted in carbonate-bicarbonate buffer (pH 9.6) and adsorbed to 96 well EIA plates (Costar, Cambridge, MA) at a concentration of 1 ug/well overnight at 4 C. All subsequent incubations

were carried out at 41 C for 1 h. Plates were blocked with 3% bovine serum albumen in PBS, then rinsed three times with PBS-Tween 20. Serial dilutions of pooled serum samples in PBS-Tween 20 were reacted with adsorbed antigen followed by three rinses in PBS-Tween 20. Plates were then exposed to a 1:1000 dilution of goat anti-chicken IgG horseradish peroxidase conjugate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) in PBS. Plates were washed three times with PBS-Tween 20 and developed with tetramethyl benzidine (TMB) reagent (ICN Biomedicals, Costa Mesa, CA). Absorbances were read on a microelisa reader (Dynatech, Alexandria, VA) at 450 nm and corrected for background. Mean absorbances from quadruplicate wells per trial are reported for 1/1600 serum dilutions.

PAGE and Western blot analysis. Approximately 2.0×10^7 E. tenella sporozoites were disrupted with glass beads (100 um in diameter) by vortexing for 2 min in sample buffer (12). The suspension was centrifuged at 11,600 x g for 2 min, and the supernatant fluid was applied to a 10% SDS-polyacrylamide gel. The gel was electrophoresed at 35 mA for 6 h. Separated sporozoite proteins were transferred from 10% SDS-gels onto nitrocellulose paper overnight at 30 V (19). The paper was blocked with 3% gelatin in Tris-buffered saline (TBS) for 2 h at 37 C, rinsed in water and exposed for 2 h at room temperature to 1:500 dilutions of 3264-immunized chicken serum in antibody buffer. All subsequent incubations were for 2 h at room temperature. The paper was

rinsed twice in PBS-Tween 20 and exposed to a 1:1000 dilution of rabbit anti-chicken IgG in antibody buffer. Following another two rinses in PBS-Tween, the paper was labeled with goat anti-rabbit horseradish peroxidase conjugate diluted 1:1000 in antibody buffer, then washed twice in PBS-Tween 20 and developed with 4-chloronaphthol reagent.

Lymphocyte proliferation assay. Blood from sensitized birds was collected weekly and assayed for the degree of in vitro lymphocyte stimulation by soluble sporozoite antigen (1). A lymphocyte-enriched culture was prepared using a colloidal silica suspension (Sepratech, Oklahoma City, OK). Mononuclear cells were suspended in RPMI 1640 medium, containing ITS [insulin/transferrin/selinium] (Collaborative Research, Cambridge, MA) and 5% fetal bovine serum, at a concentration of 5 x 10^6 viable cells/ml. Microcultures were established in tissue culture microtitre plates (Costar, Cambridge, MA) by the addition of 100 ul (5 x 10^5 cells) of cell suspension. The three treatments, each repeated six times, were as follows: 1) 1 ug/well of soluble sporozoite antigen in 100 ul of medium; 2) 25 ug/well of phytohemagglutinin (PHA) in 100 ul of medium; 3) 100 ul/well of control media. Cultures were incubated for 72 h at 41 C in 5% CO_2. Mitogenic responses were assayed by the addition of 0.5 uCi ^{3}H-[methyl]-thymidine (^{3}H-TdR) (6.7 Ci/mmole; New England Nuclear, Boston, MA) in 25 ul medium 18 h prior to harvesting. Cells were harvested on to glass fiber filters

using an automated cell harvester. Dried filter discs were prepared in toluene-PPO and counted in a Beckman liquid scintillation counter. Results are expressed as a stimulation index (SI) as follows:

SI = avg. cpm antigen or PHA/avg. cpm control.

Statistics. All data were analyzed by analysis of variance to test genotype and treatment as main effects. Means were separated using Student-Newman-Keul's (SNK) test at the 0.05 level of probability.

Results

Effect of B genotype and immunization on clinical disease. Preliminary studies revealed that 3264 did not elicit a protective response to challenge when administered in a single primary injection in the $6_1.B$ congenic segregants (data not shown). Thus, three different immunization regimens, consisting of a primary injection and a secondary boost, were used to assess protection to parasite challenge elicited by 3264. In all three experiments, immunized homozygous B^5B^5 chicks showed protection in terms of significantly lower cecal lesion scores than similarly immunized homozygous B^2B^2 chicks (Table 1). In addition, B^5B^5 chicks gained significantly more weight following challenge than B^2B^2 chicks in Experiments 2 and 3.

Anti-sporozoite antibodies elicited by 3264. In Experiment 1 (Fig. 1), birds of both genotypes, primed with

2.4 ug and boosted with 10 ug on Day 28, exhibited a similar serum IgG titer response with titers peaking on Day 14, declining to Day 28, then increasing following the 10 ug boost to Day 35. At Day 35, $\underline{B}^5\underline{B}^5$ serum had a significantly higher titer than $\underline{B}^2\underline{B}^2$ serum.

In contrast, $\underline{B}^5\underline{B}^5$ serum showed a significantly higher titer at Days 14 and 21 than $\underline{B}^2\underline{B}^2$ serum following a primary injection of 10 ug 3264 in Experiment 2 (Fig. 2). Following the 10 Ug boost on Day 21, IgG titer in $\underline{B}^5\underline{B}^5$ chicks declined while IgG levels remained essentially the same in $\underline{B}^2\underline{B}^2$ chicks.

When the primary and secondary antigen doses were increased to 20 ug each on Days 0 and 14, respectively in Experiment 3, $\underline{B}^2\underline{B}^2$ serum IgG titer appeared on Day 14, was essentially unaffected by the booster injection, but increased markedly following challenge, to a titer significantly greater than that of $\underline{B}^5\underline{B}^5$ birds (Fig. 3). $\underline{B}^5\underline{B}^5$ titer was significantly higher than $\underline{B}^2\underline{B}^2$ titer on Day 21 following the Day 14 boost, but its titer declined following challenge.

<u>Western blot analysis of 3264 elicited antibody</u>. Western analysis of serum samples permitted a characterization of different Ag-Ab specificities induced by 3264 immunization. Soluble sporozoite antigen was resolved on a 10% PAGE slab into several protein bands ranging in molecular weight from 15 to > 200 kD. In Experiment 2 (Fig. 4), $\underline{B}^2\underline{B}^2$ serum collected on Days 14, 21, and 28 labeled several protein

bands in excess of 68 kD, with the most intense signal given at Day 28, seven days following the 10 ug boost. In addition, $\underline{B}^2\underline{B}^2$ serum collected on Day 14 labeled a low molecular weight band, approximately 15-18 kD, not labeled on Days 21 and 28, and never labeled by $\underline{B}^5\underline{B}^5$ serum. $\underline{B}^5\underline{B}^5$ serum labeled similar protein bands >68 kD; however, a very weak signal was detected on Day 14 that intensified on Days 21 and 28, but not to the magnitude of the $\underline{B}^2\underline{B}^2$ signal. In contrast, only $\underline{B}^5\underline{B}^5$ serum collected on Day 28 labeled two bands of approximately 51-53 kD and 58-60 kD, respectively.

Similar protein bands >68 kD were labeled with serum of both genotypes collected in Experiment 3 (Fig. 5). The $B^2\underline{B}^2$ signal was weak on Days 14 and 21, and was completely absent 7 days following the challenge at Day 28. The $\underline{B}^5\underline{B}^5$ signal was strongest on Day 14 and weakened progressively on Days 21 and 28. However, $\underline{B}^5\underline{B}^5$ serum collected on Day 28, 7 days following challenge, again labeled a low molecular weight band, approximately 51-53 kD, not labeled by $\underline{B}^2\underline{B}^2$ serum.

3264 induced lymphoproliferation. Peripheral lymphocytes were collected from birds of both genotypes in Experiment 2 on Days 0, 14 and 21 and were subjected to in vitro stimulation with soluble sporozoite antigen (Fig. 6). Lymphocytes collected prior to 3264 injection responded minimally for each genotype. Fourteen days following sensitization, $\underline{B}^5\underline{B}^5$ lymphocytes showed a significantly greater proliferative response to sporozoite antigen than $\underline{B}^2\underline{B}^2$ lymphocytes. By Day 21, however, this $\underline{B}^5\underline{B}^5$

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proliferative response had returned to the level measured in unsensitized (Day 0) lymphocytes. PHA stimulated lymphocytes from birds of both genotypes responded essentially to the same magnitude with proliferation indices of 35 for $\underline{B}^2\underline{B}^2$ cells and 39 for $\underline{B}^5\underline{B}^5$ cells (not shown).

Discussion

In this study, we investigated the immunogenicity of recombinant E. tenella protein 3264 and its ability to elicit a protective response in $6_1.\underline{B}$ congenic chickens segregating for alleles $\underline{B}^2$ and $\underline{B}^5$. Previous studies have shown injected antigen extracts prepared from lysed oocysts or sporozoites of E. tenella, to effectively elicit protective immunity against parasite challenge in chickens (15). Recombinant forms of similar antigens induced only partial protection to challenge (6). Although recombinant antigens may be produced efficiently in large quantities, this technique may inherently alter protein conformation and limit the number of epitopes expressed to the host. This consequence could affect the response pattern of certain associated MHC alleles.

In all cases, chickens homozygous for the $\underline{B}^5$ allele showed significantly greater protection to parasite challenge following immunization than $\underline{B}^2\underline{B}^2$ homozygotes. These results support previous studies (3,11,13) demonstrating that the $\underline{B}$ complex plays a profound role in the host response to cecal coccidiosis. Studies by Clare and

coworkers (3) first reported that the $\underline{B}^5$ allele was associated with a greater protective immune response to $\underline{E}$. $\underline{tenella}$ parasitism than the $\underline{B}^2$ allele. Ruff and Bacon (17) suggested that expression of the $\underline{B}^5$ allele may also be associated with innate resistance to parasitism. In addition, work in this laboratory, using similar congenic chickens, established the identical response pattern of these $\underline{B}$ alleles to another recombinant $\underline{E}$. $\underline{tenella}$ antigen, 5401 (Clare, unpublished).

Both 3264 and 5401 are derived from the same cloned gene, coding for a protein molecule consisting of several repeats of a nine amino acid sequence, but are expressed in different plasmids (Danforth and Augustine, unpublished). The use of different plasmids changes the number of total repeats which appear in the final fusion protein. Although both 3264 and 5401 have a molecular weight of approximately 35 KD, 3264 appears to be expressed missing at least one repeat sequence.

A single dose of 2.4 ug 5401 elicited a protective response in $\underline{B}^5\underline{B}^5$ chickens (Clare, unpublished) and partial protection in a commercial strain (6). This same dose of 3264 was not sufficient to establish immunity to challenge in the $\underline{B}$ congenics. In fact, a primary injection of 3264 had to be combined with a secondary booster to obtain protection in $\underline{B}^5\underline{B}^5$ chickens. The difference in plasmid expression between 3264 and 5401 appears to alter the immunogenicity of these proteins.

The serum IgG response to 3264 detected by ELISA was influenced by the dose of antigen administered. Following 2.4 ug of 3264 in Experiment 1, birds of both genotypes responded essentially to the same magnitude. Increasing the primary dose to 10 ug in Experiment 2 appeared to suppress the $\underline{B}^2\underline{B}^2$ response at Day 14, while stimulating a marked increased titer in $\underline{B}^5\underline{B}^5$ birds at Day 21. The 20 ug dose given in Experiment 3 appeared to suppress IgG titers in birds of both genotypes at Days 14 and 21. Following the 25,000 oocyst challenge on Day 21, the $\underline{B}^2\underline{B}^2$ titer increased approximately two-fold at Day 28. What these anti-sporozoite IgG titers mean in terms of the immune status of the chicken is not clear. In previous studies with antigen 5401 a peak titer in $\underline{B}^5\underline{B}^5$ birds on the day of challenge was associated with protection (Clare, unpublished). This observation may be the case with Experiments 1 and 3 of the present study, but not in Experiment 2 where titers of both genotypes were essentially the same on the day of challenge.

Western analysis showed that birds of both genotypes respond to similar sporozoite proteins >68 kD, although the magnitude of antibody labeling changes with time following immunization. The large number of protein bands labeled indicates that epitopes associated with 3264 are expressed on several different sporozoite proteins, or that these bands represent subunits of a larger protein resulting from soluble antigen preparation. In contrast, $\underline{B}^5\underline{B}^5$ serum consistently labeled one or two low molecular weight bands

on Days 21 and 28, never labeled by $\underline{B}^2\underline{B}^2$ serum. Recognition of these bands may be MHC-restricted and ultimately associated with protection.

Results from the lymphocyte stimulation study supported further an increased level of immunocompetence among $\underline{B}^5\underline{B}^5$ chickens immunized with 3264. A significant increase in stimulation was observed in $\underline{B}^5\underline{B}^5$ chickens over $\underline{B}^2\underline{B}^2$ chickens at Day 14 in Experiment 2, but returned to control levels at Day 21. The Day 14 response may be signaling a period of peak immune activity.

The exact nature of this MHC mediated response requires further analysis. Similar responsiveness has been described in mice immunized with a candidate malaria vaccine (8,9). Our results conclude that the efficacy of a recombinant coccidial vaccine may be limited by its degree of interaction with a given MHC genotype.

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TABLE 1. Effect of immunization with antigen-3264 on a
25,000 E. tenella oocyst challenge.

Expt.	B Geno.	N	Immunization Treatment[a]	Lesion Score±SEM	Weight Gain±SEM
1	2/2	7	2.4/10	3.2 ± .3	71 ± 11 g
1	5/5	10	2.4/10	2.4 ± .2	63 ± 5 g
2	2/2	7	10/10	3.4 ± .3	59 ± 8 g
2	5/5	11	10/10	1.6 ± .3	77 ± 3 g
3	2/2	11	20/20	3.0 ± .2	56 ± 8 g
3	5/5	7	20/20	1.7 ± .2	77 ± 5 g

[a]Primary/boost (ug) injections of Ag-3264 given on the following days, respectively: Expt. 1-Day 0/Day 28; Expt. 2-Day 0/Day 21; Expt. 3-Day 0/Day 14. All challenge exposures were given 7 days following the boost injection.
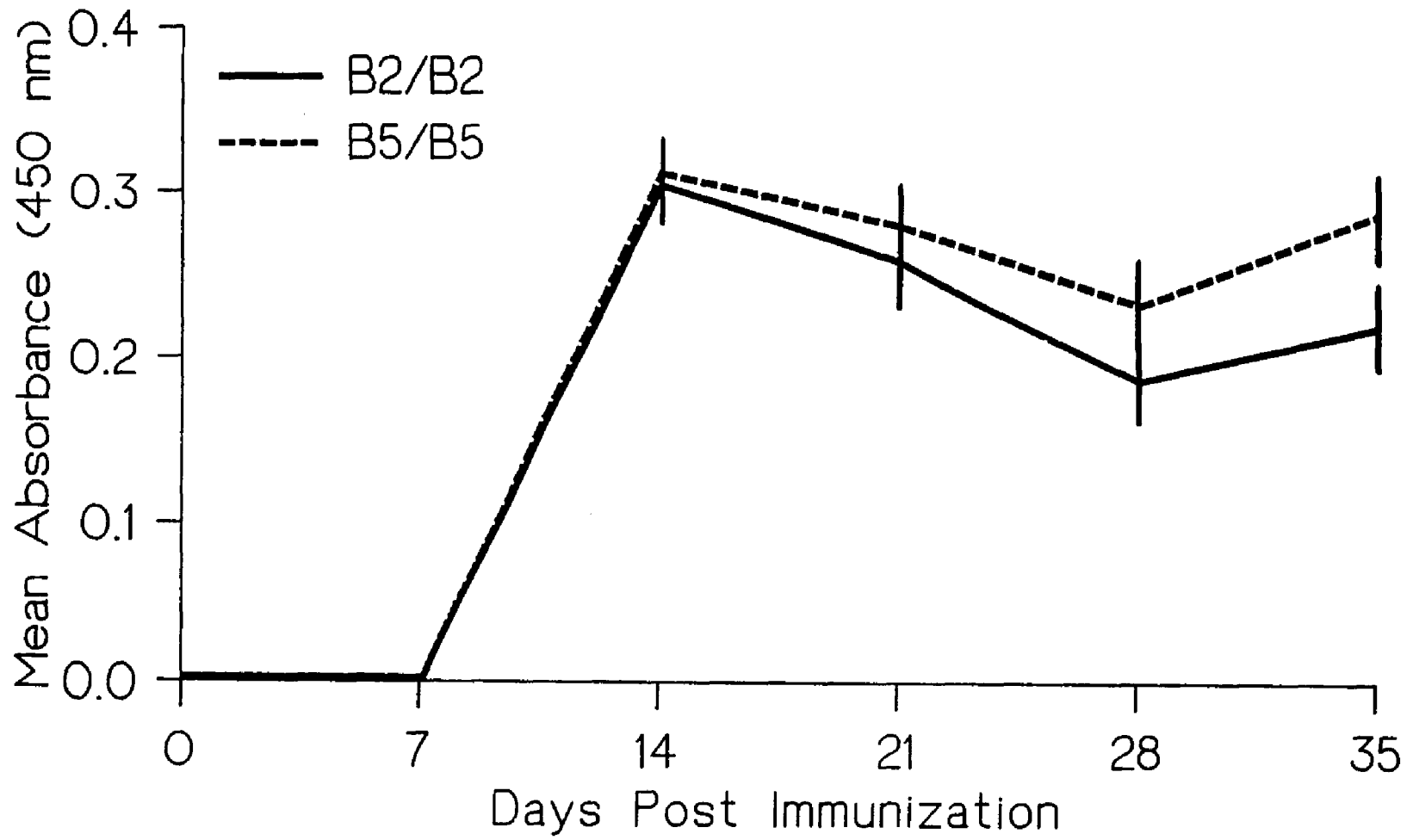
Figure 1. Experiment 1. Anti-sporozoite serum IgG titers, detected by ELISA at a 1/800 dilution and reported as Abs.$_{450}$, of $6_1.\underline{B}$ congenic chickens immunized with 2.4 ug recombinant $\underline{E}$. $\underline{tenella}$ antigen 3264 on Day 0 and boosted with 10 ug 3264 on Day 28.
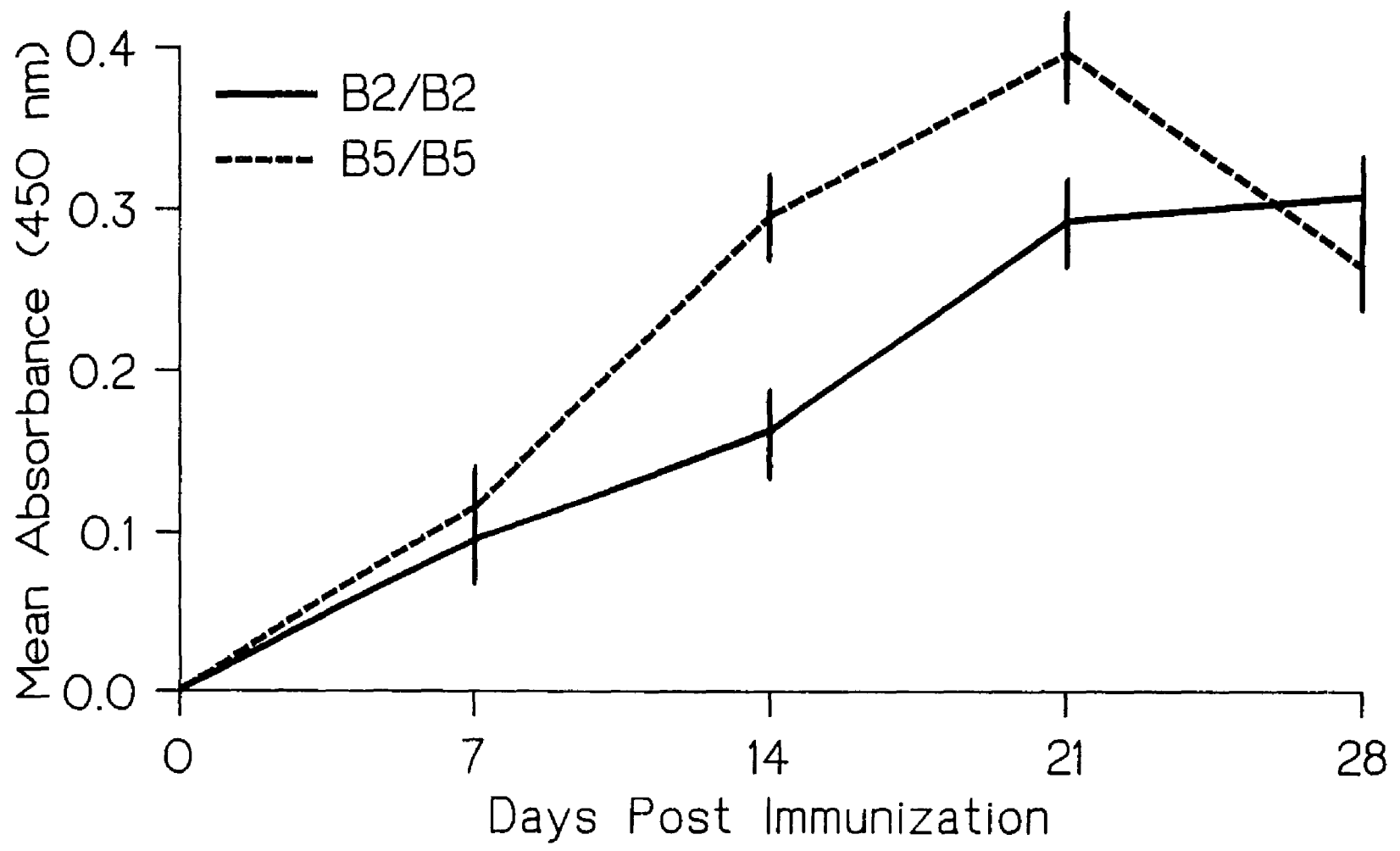
Figure 2. Experiment 2. Anti-sporozoite serum IgG titers, detected by ELISA at a 1/800 dilution and reported as Abs.$_{450}$, of $6_1.\underline{B}$ congenic chickens immunized with 10 ug recombinant $\underline{E}$. $\underline{tenella}$ antigen 3264 on Day 0 and boosted with 10 ug 3264 on Day 21.

Figure 3. Experiment 3. Anti-sporozoite serum IgG titers, detected by ELISA at a 1/800 dilution and reported as Abs.$_{450}$, of $6_1.\underline{B}$ congenic chickens immunized with 20 ug recombinant $\underline{E}$. $\underline{tenella}$ antigen 3264 on Day 0 and boosted with 20 ug 3264 on Day 14.

88
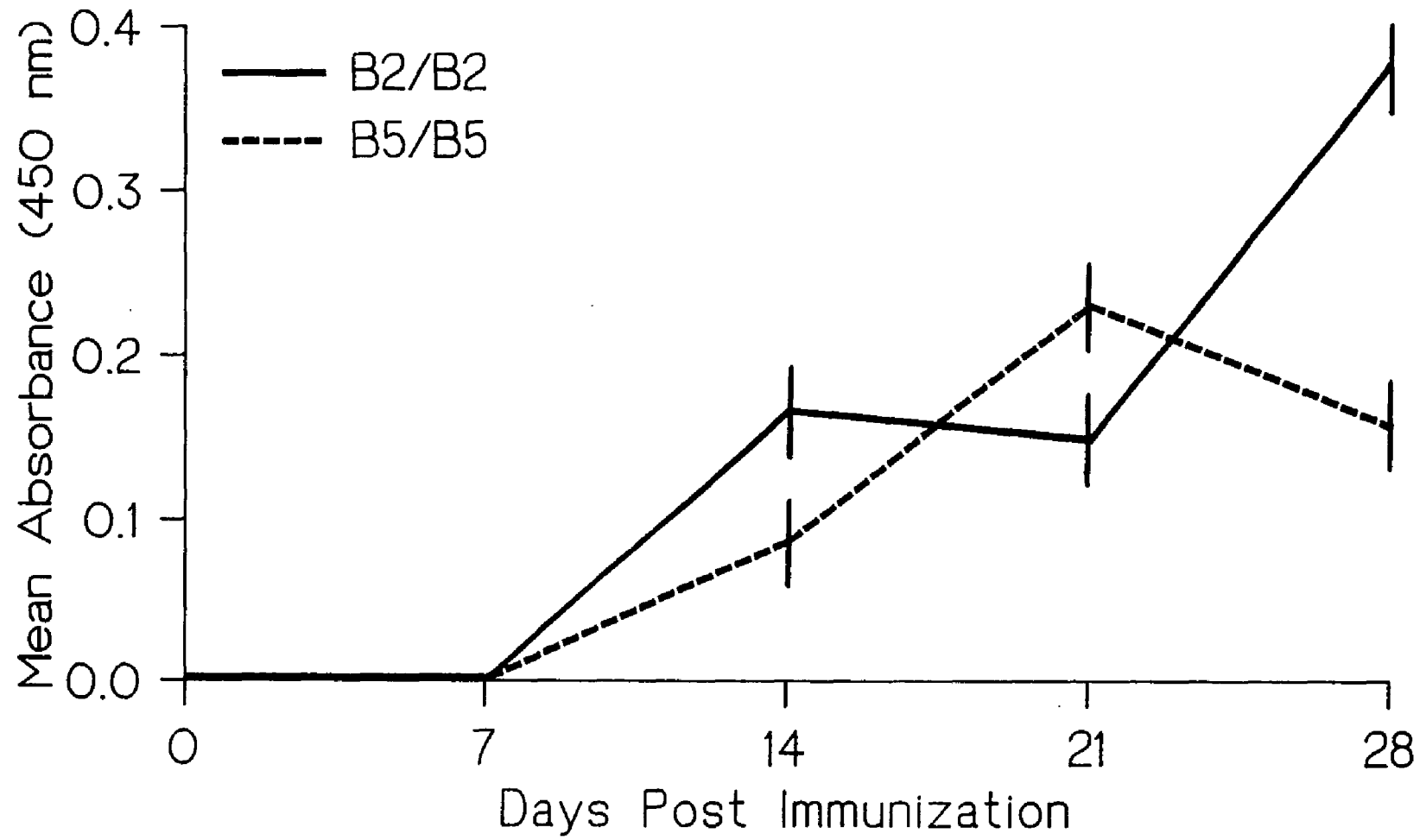
Figure 4. Experiment 2. Western blot analysis of serum from $6_1 \cdot \underline{B}$ congenic chickens immunized with recombinant $\underline{E}$. $\underline{tenella}$ antigen 3264 on $\underline{E}$. $\underline{tenella}$ sporozoite preparation. Lanes 1 and 2 at Day 14, lanes 3 and 4 at Day 21, and lanes 5 and 6 at Day 28. $\underline{B}^2\underline{B}^2$ serum is reacted in lanes 1, 3, and 5; $\underline{B}^5\underline{B}^5$ serum is reacted in lanes 2,4, and 6. Asterisk in lane 1 placed beside 15-18 kD antigen reacting with serum from $\underline{B}^2\underline{B}^2$ birds. Asterisks in lane 6 placed beside 51-53 kD and 58-60 kD antigens reacting with serum from $\underline{B}^5\underline{B}^5$ birds. Positions of M_r markers are identified on left of figure, and from top to bottom correspond to myosin, 200 kD; phosphorylase B, 97 kD; serum albumen, 68 kD; ovalbumen, 43 kD; alpha-chymotrypsinogen, 25 kD, beta-lactoglobulin, 18 kD, and lysozyme, 14 kD.

Figure 5. Experiment 3. Western blot analysis of serum from 6_1.$\underline{B}$ congenic chickens immunized with recombinant $\underline{E}$. tenella antigen 3264 on $\underline{E}$. tenella sporozoite preparation. Lanes 1 and 2 at Day 14, lanes 3 and 4 at Day 21, and lanes 5 and 6 at Day 28. $\underline{B}^2\underline{B}^2$ serum is reacted in lanes 1, 3, and 5; $\underline{B}^5\underline{B}^5$ serum is reacted in lanes 2,4, and 6. Asterisk in lane 6 placed beside 51-53 kD antigen reacting with serum from $\underline{B}^5\underline{B}^5$ birds. Positions of M_r markers are identified on left of figure, and from top to bottom correspond to myosin, 200 kD; phosphorylase B, 97 kD; serum albumen, 68 kD; ovalbumen, 43 kD; alpha-chymotrypsinogen, 25 kD, beta-lactoglobulin, 18 kD, and lysozyme, 14 kD.
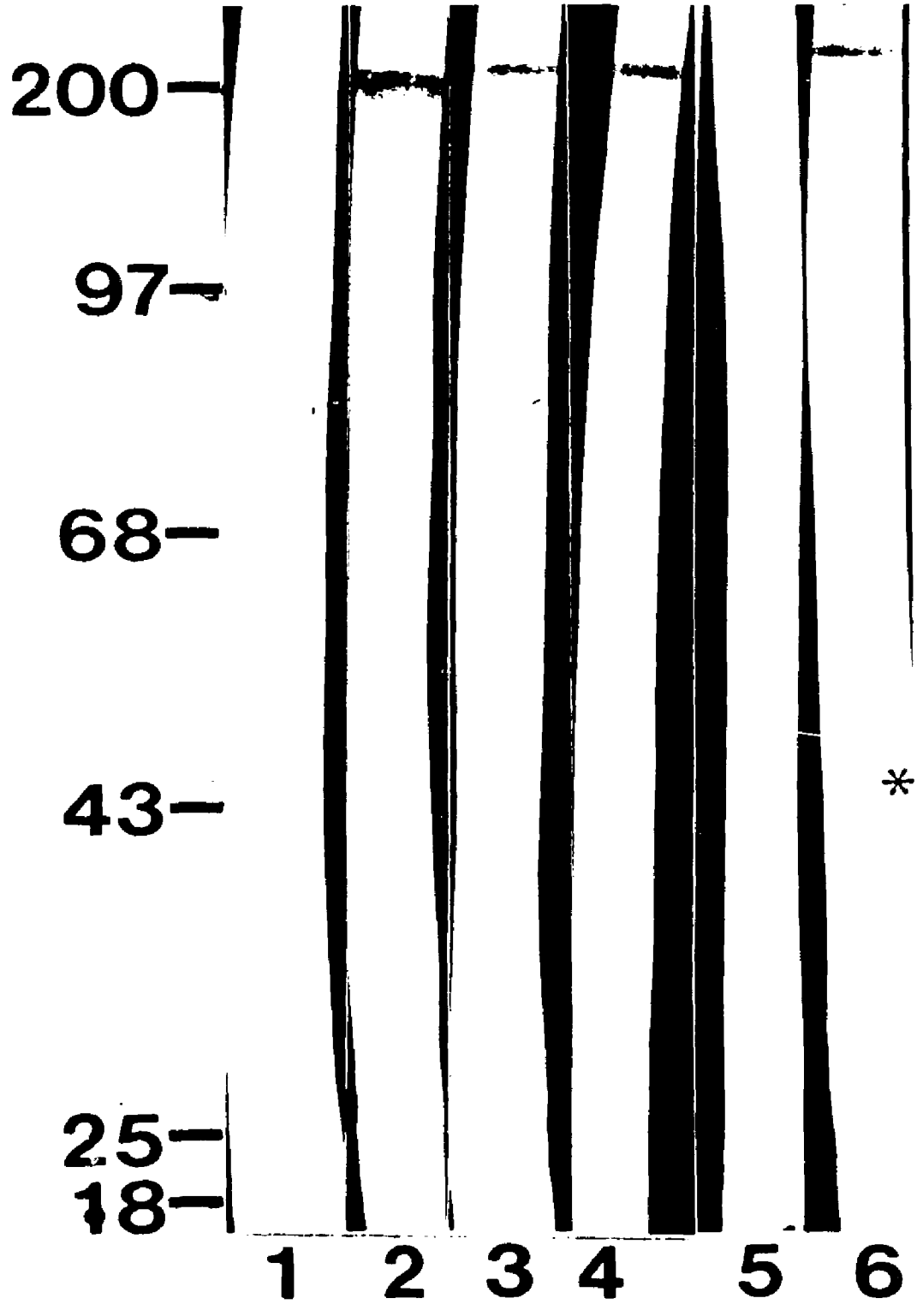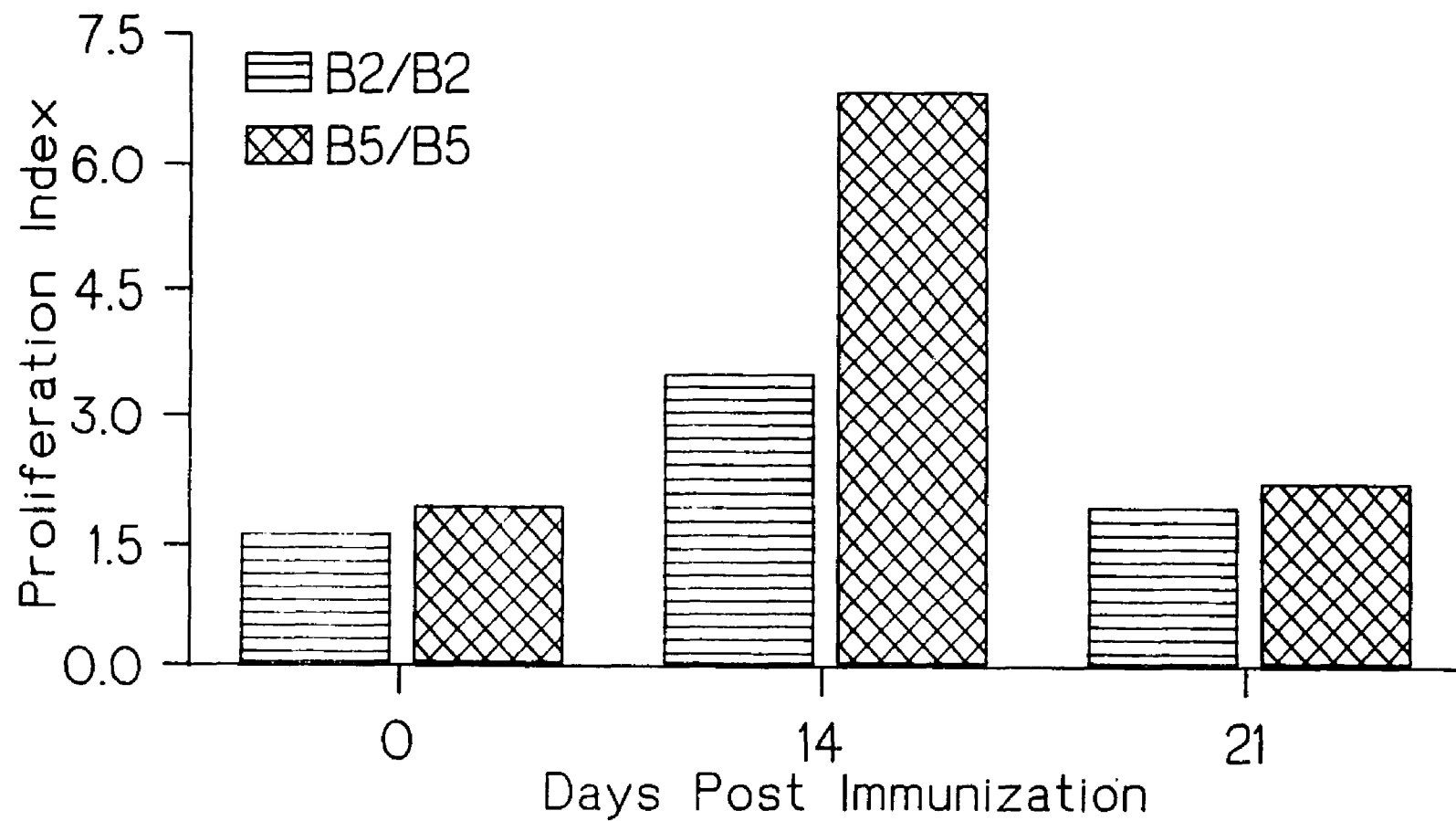
200—

97—

68—

43—   *

25—
18—

1   2   3   4   5   6

Figure 6. Experiment 2. Lymphocyte proliferation indices of peripheral lymphocytes collected from $6_1.B$ congenic chickens immunized with 10 ug recombinant E. tenella antigen 3264, and stimulated in vitro with 1 ug soluble sporozoite antigen.

CHAPTER FOUR

Comparative Analysis of _Eimeria_ _tenella_ Sporozoite and Merozoite Surface Antigens Labeled by Serum Antibodies Raised Against a Recombinant Coccidia Protein

Abstract

Antisera were raised in 5 week old $6_1 \cdot \underline{B}$ congenic chickens against a recombinant coccidial protein, designated 3264, which has been shown previously to elicit a protective immune response to <u>Eimeria</u> <u>tenella</u>. Serum was collected weekly for 4 weeks following subcutaneous immunization with 3264 emulsified in Freund's complete adjuvant. Specific antibodies were used to label air-dried <u>E</u>. <u>tenella</u> sporozoites and merozoites in an indirect fluorescent antibody (IFA) assay.

Antibodies raised against 3264 strongly labeled only the anterior portion of the sporozoite. In contrast, these same antibodies strongly labeled only the posterior portion of second generation merozoites. First generation merozoites were only slightly labeled, if at all. In addition, a single heavily labeled nodule, possibly an immune complex, was observed on the posterior surface of second generation merozoites.

Introduction

The protozoan diseases human malaria and avian coccidiosis are the subject of intense research toward the production of recombinant vaccines (Young et al, 1985; Danforth and Augustine, 1988). For both obligate intracellular protozoan parasites, cloned sporozoite antigens are being evaluated for their ability to elicit a protective immune response (Nussenzweig and Nussenzweig, 1984; Danforth, 1985). Several sporozoite surface antigens have been identified and characterized from the coccidium Eimeria tenella (McAndrew et al., 1986; Truitt et al., 1986). Two candidate proteins have been described which induce partial protection to parasite challenge in avian hosts (Danforth and Augustine, 1985; Danforth and Augustine, unpublished). Each protein elicits antibodies which react with surface and internal structures of sporozoites in an indirect fluorescent assay as well as several high molecular weight sporozoite proteins in a western blot analysis. The reactivities of these antibodies to merozoites or merozoite antigen has not been described. The purpose of the present study is to compare the different surface antigens of sporozoites and merozoites of E. tenella using antibodies raised against a recombinant coccidial protein. In addition this work may permit a further understanding of the antigenic nature of both sporozoites and merozoites.

Materials and Methods

Chickens

Five week old 6_1.B congenic chickens were used in this study. All chicks were housed coccidia-free in wire-floored brooder batteries and provided unmedicated feed and water ad libitum.

Recombinant protein

The recombinant antigen 3264 was cloned from non-sporulated and sporulated oocysts of strain L.S. #24 E. tenella (Animal Parasitology Institute, Belstville, MD) as described previously (Danforth and Augustine, 1985). A volume of 0.5 ml Freund's complete adjuvant (FCA) containing 10 ug 3264 was injected subcutaneously at the base of the neck into each bird. Serum was collected weekly from whole blood and stored frozen at -20 C until used.

IFA staining

Sporozoites were excysted by standard procedures, passed over a scrubbed nylon wool column to remove debris, centrifuged, and resuspended in phosphate-buffered saline (PBS). Merozoites were isolated from 5 day infected ceca, and purified on a DEAE-cellulose column (DE-52; Whatman, Clifton, NJ) using a pH 8.0 phosphate buffer. Purified sporozoites or merozoites at a concentration of 1×10^6/ml were fixed by air-drying to Toxoplasma titer slides. Air-dried parasites were reacted with serial dilutions of immune sera and incubated at room temperature in a humid chamber for 30 min. Slides were washed for 10 min in PBS, then

reacted with rabbit anti-chicken IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted 1:30 in PBS and incubated for 30 min in humidity. A second 10 min wash in PBS was followed by reaction with fluorescein (FITC) conjugated goat anti-rabbit IgG (Kirkegaard and Perry Laboratories) diluted 1:30 for 30 min in humidity. Slides were washed a final time in PBS, mounted with glycerol and observed under UV light microscopy.

Results

Antibodies raised against the recombinant coccidial protein 3264 produced a distinct difference in labeling pattern between sporozoites and merozoites (Figs. 1-4). Antibodies directed at sporozoites produced an intense anterior surface labeling (Fig. 1), while the same antibody preparations reacted with merozoites produced an intense posterior surface labeling on second generation merozoites only (Fig. 2). First generation merozoites, about one-half the size of the second generation, appeared only slightly labeled, if at all. In addition, either a diffuse labeling pattern (Fig. 3) or a heavily stained nodule (Fig. 2,4) was also observed on the posterior surface of second generation merozoites, a phenomenon not observed with sporozoites or first generation merozoites.

Discussion

This study investigated the antigen specificities of antisera raised in chickens against recombinant E. tenella antigen 3264. These results indicate that antigens which contain an epitope cross-reactive with recombinant antigen 3264, are expressed on sporozoites and second generation merozoites. These findings also confirm previous work which described the ability of antisera raised against a similar recombinant antigen, 5401, to label E. tenella sporozoites (Danforth, 1985).

The labelling pattern found on first and second generation merozoites had not been described previously, and these results indicate that differences in antigen expression occur between sporozoites and merozoites. Although the apical region of the sporozoite was heavily labeled with antibody, these reactive antigens do not appear to be associated with the apical complex since first generation merozoites were not labeled and second generation merozoites were labeled only posteriorly. Therefore, these antigens appear to be associated with specific stages of parasite development and not ubiquitous expression on the parasite. Although labeling appears to be surface-oriented, the possibility that these antigens are expressed and therefore labeled internally cannot be ruled out.

The absence of fluorescence labelling of first generation merozoites by antisera produced following an E. tenella oocyst infection was reported previously by

Kouwenhoven and Kuil (1976). In addition, similar fluorescence patterns were observed on both sporozoites and second generation merozoites suggesting that these stages shared common antigens not expressed on first generation merozoites. How these elicited antibodies function in the host response to parasitism is not yet understood. These antibodies' inability to label first generation merozoites may indicate that this parasite stage may be able to evade the host response. Johnson and coworkers (1979) concluded that the first generation merozoite was poorly immunogenic following studies with the attenuated WisF96 strain of E. tenella, which apparently develops through only one schizogonic cycle (McDougald and Jeffers, 1976). In contrast, further work reported that WisF96 first generation merozoites were adequately immunogenic when the WisF96 infection rate was adjusted to a rate quantitatively similar to that of the unattenuated parent strain of E. tenella (McDonald et al., 1986). In the present study, the epitope associated with 3264 does not appear to be cross-reactive with antigens expressed on the first generation merozoite and may explain why only partial protection was obtained in commercial chickens immunized with recombinant coccidial antigen 5401 (Danforth, 1985).

Finally, the significance of the localized heavily labeled nodules on the surface of certain second generation merozoites requires further study. The possibility that these nodules are assay artifacts must be considered,

however, they are numerous and found only on the posterior surface of these merozoites. The nodules could be a result of surface-antigen capping prior to air fixation, or a result of the air-drying process itself. Similar antigen clusters were not found, however, on sporozoites. These immune complexes may indicate that cross-reactive antigens are sometimes expressed in localized surface clusters. Since these specific antigens apparently are not associated with the general merozoite surface, their function in development or in stimulating the host response becomes an intriguing question.

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Figures 1-4. Indirect fluorescent antibody (IFA) antigen labelling patterns, on or in air-dried sporozoites or merozoites, produced by antisera raised against recombinant coccidia protein 3264. All X 1600. 1. Anterior labelling of sporozoites. 2. Posterior labelling of merozoites. 3. Diffuse nodule labelling of posterior merozoite surface. 4. Heavy nodule labelling on posterior merozoite surface.
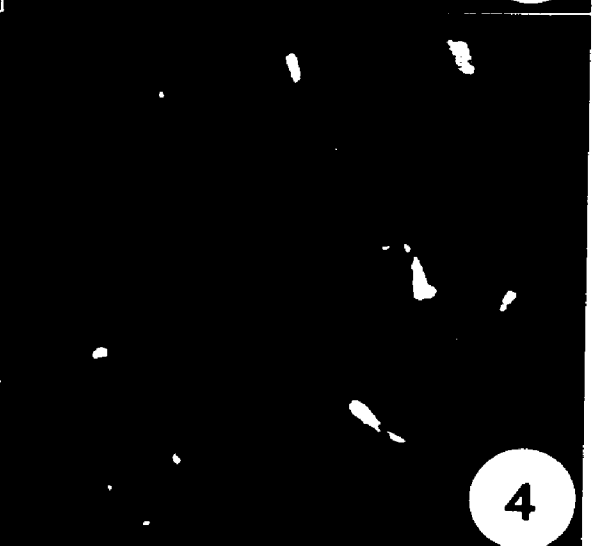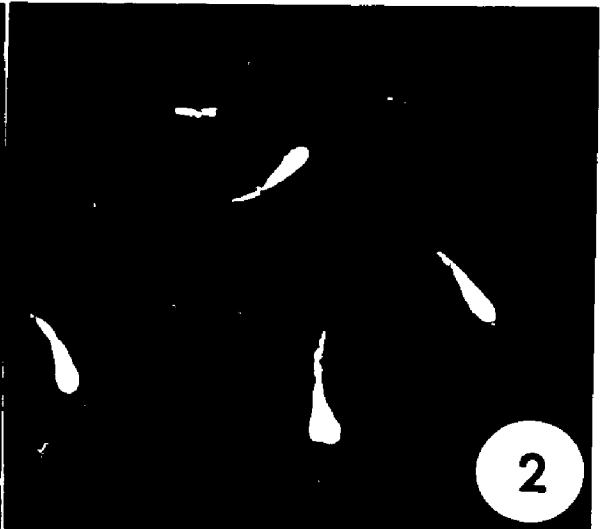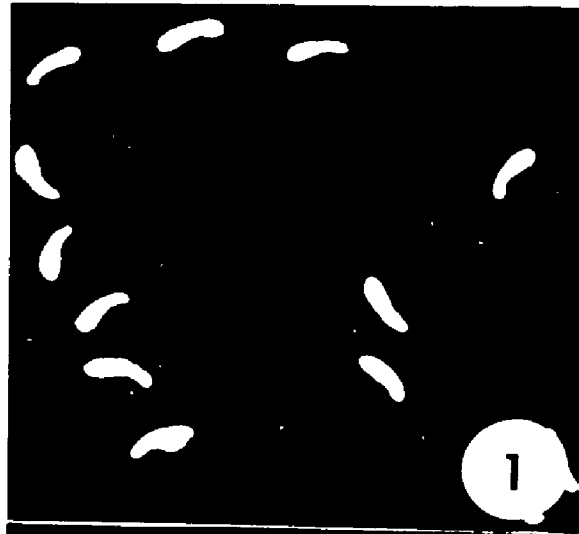
APPENDIX

IN VIVO PROPAGATION OF EIMERIA TENELLA

Infection

1. Infect 5-6 wk old birds with 50,000 to 100,000 sporulated oocysts. Harvest at 7-7.5 days.

Harvest

1. Remove cecal pouches from birds. Expel contents into blender and wash out pouch with wash bottle.

2. Blend approx. 5 min.

3. Pour contents into 750 ml wide-mouth flask containing 5 g pepsin (1:15,000 activity), and bring volume to top with rinse from blender.

4. Adjust pH to 1.0-1.8 with 10 N HCl.

5. Stir in 37 C waterbath for 2 h. (Never above 37 C.)

6. Check under scope that fecal material has been reduced.

7. Adjust pH to 7.5-8.0 with 10 N NaOH.

8. Pour contents into large jar and bring volume to top. Cover and let settle overnight.

9. Aspirate super; do not disturb sediment.

10. Swirl sediment and pour into 150 ml centrifuge tube. Rinse jar residue into a second tube.

11. Centrifuge at 1500 rpm for 10 min.

12. Aspirate of super. Combine tubes if necessary and centrifuge again.

13. Suspend pellet in 0.5% potassium dichromate. Aerate with air pump for 2-3 days. Check for degree of sporulation.

Cleaning Oocysts

1. Centrifuge dichromate suspension (1500 rpm/10 min).

2. Wash once with water.

3. Aspirate off water and add 15 ml clorox, resuspend oocysts;let stand in dark for 30 min. Shake occasionally.

4. Dilute with sterile water and centrifuge.

5. Wash twice with water.

6. Resuspend in DMEM containing 2X antibiotic (PNS).

Excystation of Sporozoites

1. Grind 7 ml of oocyst suspension in teflon tissue grinder to release sporocysts (@ 5-7 min).

2. Transfer contents to a 40 ml centrifuge tube, rinse grinder residue in to tube with Saline A. Fill tube to 40 ml line and vortex.

3. Centrifuge contents (2000 rpm/10 min) and discard super.

4. Add 20 ml excystation fluid, resuspend oocysts, and incubate at 37-41 C for 2-3 h (check ender scope). Agitate tube every 30 min.

5. Fill tube to 40 ml line with Saline A, vortex, and centrifuge. Discard super.

6. Resuspend in tris buffer.

SALINE A

    8.00 g NaCl
    0.40 g KCl
    0.35 g $NaHCO_3$
    1.00 g Dextrose

Make up in 1 L dH_2O and filter sterilize.

TRIS BUFFER FOR COLUMN

    9.00 g NaCl
    0.42 g KCL
    0.24 g $CaCl_2$
    0.20 g $KHCO_3$
    Dissolve into 1 L dH_2O.

    3.03 g Trizma-8.0 (Sigma-T-4753)
    Adjust pH to 8.0 if necessary.

Column Clean-up of Sporozoites

1. Pack 1 g scrubbed nylon fiber (Fenwal Laboratories, Deerfield, IL) into glass column or syringe. Pack tightly enough to permit a rate of flow of 2-3 drops/second.

2. Fill column with tris buffer and check rate of flow.

3. Fill column with tris buffer and allow it to drip through. Refill with tris and clamp off.

4. Wash sporozoite suspension 1X in 40 ml tris buffer. Leave in 5 ml tris.

5. Allow column to empty. Place a centrifuge tube under the aseptic filling bell to collect filtrate. Pour sporozoite suspension onto the column and allow suspension to drain into the nylon fiber. Repeat with tris rinses of tube until a volume of 40 ml has been collected.

6. Centrifuge filtrate from column. Discard super and resuspend sporozoite pellet in an appropriate medium.

    --Resuspend in PBS if doing IFA's.

    --Resuspend in Waymouth's 87/3 if infecting cells.

* Glass columns are made from 50 ml pipettes. The tip is cut off at about the 20 ml mark and discarded. A 3 1/2" length of latex tubing is fitted over the mouthpiece and connected to an aseptic filling bell.

Merozoite Clean-up

1. Remove ceca, bloody core and split down the center. Place ceca in about 400 ml of DMEM per 7 bird samples. Stir for 20 min at moderate speed (10-15 min for acervulina).

2. Centrifuge material at 300 rpm for 3 min.

3. Collect supernate and centrifuge at 1500 rpm for 10 min. Discard supernate and resuspend sediment in 50 ml DMEM.

4. Centrifuge material at 700 rpm for 8 min. (The supernate here is good for acervulina, so check.)

5. Resuspend sediment in 50 ml DMEM and centrifuge at 1500 rpm for 1 min. Collect supernate, but check sediment for amount of merozoites lost. You may need to resuspend this.

6. Wash merozoites 2-3 times in phosphate buffer, pH 8.0.

7. Pass supernate over a DE52 column (5-7 ml DE52 in a 30 ml syringe plugged with scrub nylon fiber), equilibrated with phosphate buffer, pH 8.0. Collect approximately 35 ml.

8. Centrifuge at 1500 rpm for 10 min and resuspend merozoites in media of choice.

****Note all steps must be carried out under refrigeration.

IFA PROCEDURE

1. Prepare suspension of sporozoites at 5×10^5/ml.

2. Air dry 10,000 sporozoites/20 ul on toxoplasma titer slides (Belco Glass,). Store in -80 C. When using stored slides, let come to room temperature.

3. Expose air dried sporozoites to undiluted cell culture fluid or serial dilutions of serum for 30 min at 37 C in humidity chamber.

4. Rinse and wash slides in F.A. PBS for 10 min at room temp.

5. Blot dry and expose to anti-mouse/anti-chicken fluorescein conjugate (Kirkegaard and Perry) for 30 min at 37 C in humidity chamber. Use 1/30 dilution of conjugate.

6. Rinse and wash slides in F.A. PBS for 10 min at room temp.

7. Mount with coverslips in 9 parts glycerol/1 part PBS

8. View under UV scope using 530 nm filter.

F.A. PBS
Solution A: 1.4 g Na_2HPO_4/100ml
Solution B: 1.4 g NaH_2PO_4/100 ml

Mix: 84.1 ml of A
     15.9 ml of B
      8.5 g NaCl
Dilute to 1000 ml

ENZYME-LINKED IMMUNOSORBENT ASSAY

ANTIGENS

***Preparation of Parasite Antigen:
1. Pellet sporozoites (want 2 x 10^7/ml).
   (4 x 10^7 merozoites)
2. Suspend in PBS.
3. Vortex 2 min with glass beads.
4. Draw off supernate into microfuge tubes; centr. 2 min.
5. Pool supernate and and run BIORAD total protein assay.

ELISA TEST

1. Carried out in plastic serocluster 96 well EIA plates (Costar - 3590).

2. Dilute antigen in 50 mM carbonate-bicarbonate buffer pH 9.6 containing 0.1% (w/v) sodium azide (Ag concen. to 5-10 ug/well). Allow 100 ul antigen to adsorb onto plates at least 18 h at 4 C.

3. Wash Ag coated plates with PBS and block remaining adsorptive sites by incubation with 50 ul PBS containing 3% (w/v) BSA (Sigma S-7030) for 1 h at 41 C.

4. Add 50 ul log_2 dilutions (1/50, 1/100, 1/200,etc.) of serum/culture samples to be tested (in duplicate); include positive/negative reference sera. Dilute sera in PBS with 0.05% Tween 20 (Sigma P-1379). Incubate 1 hr at 41 C.

5. Wash 3 times with PBS with 0.05% Tween 20.

6. Add 50 ul 1/1000 dilution (in PBS with Tween) goat anti-chicken/anti-mouse IgG (H+L) (Kirkegaard and Perry, 04-24-06) to each well. Incubate 1 hr at 41 C.

7. Wash 3 times with PBS-Tween 20.

8. Add 50 ul 1/1000 dilution (in PBS without Tween) peroxidase labelled rabbit anti-goat IgG (Kirkegaard and Perry)

9. Wash 3 times with PBS-Tween 20.

10. Add 150 ul substrate solution to each well. Incubate 30 min at 41 C.

9. Stop color reaction with 50 ul stop solution.

10. Read plates at 450 nm.

Preparation of Conjugate:
When received, dilute 1/10 with PBS and aliquot into 100ul volumes. Refreeze. Dilute 1/100 with PBS before use.

50 mM carbonate-bicarbonate buffer, pH 9.6 containing 0.1% sodium azide:
        sodium carbonate, anhydrous (26.5 g/250 ml)
        sodium bicarbonate (21.0 g/250 ml)
        saline (8.12 g/l NaCl)

    1) Prepare stock solutions of 1 M carbonate and 1 M bicarbonate. (bicarb. does not keep)
    2) Mix carbonate and bicarbonate 1:1 (v/v) and titrate pH to 9.6 with one of the stock solutions (carbonate increases pH, bicarb. decreases pH).
    3) Dilute to the required molarity (1:2) with saline.
    4) Add 0.1% sodium azide (1 g/l).

PBS containing 3% (w/v) BSA or 0.05% (v/v) Tween 20
        NaCl   8.0 g
        KCl   0.2 g
        Na_2HPO_4 1.15 g (0.008 M)
        KH_2PO_4 0.2 g   (potassium di-hydrogen phosphate)
        Make up into 10X stock.

Add 3% BSA or 0.05% Tween 20 to final solution.

Substrate solution:
        Buffer (10 mM citrate/phosphate buffer, pH 5)
                Sol'n A: 21.01 g/l citric acid
                Sol'n B: 35.6 g/l disodium phosphate
                Add 49 ml sol'n B to 51 ml sol'n A.
                Confirm pH.

TMB stock solution:
   Reconstitute 1-1 mg tablet TMB (Miles 98-051-1)
   in 10 ml 10 mM citrate/phosphate buffer. Swirl
   to dissolve. Store in dark up to 3 weeks.
   TMB tablets stable up to 6 months at 5 C.

Substrate solution:
   Add H_2O_2 to final concen. of 0.01-0.1%. (add
   66.7 ul to 20 ml TMB stock).
   Make up fresh each time.

Stop solution:
   11 ml H_2SO_4 (stock)
   89 ml dH_2O

SDS-PAGE

Equipment
electrophoresis apparatus
1.5 mm spacers
combs
constant current power supply

Preparation of Parasite Homegenate:
1. Pellet sporozoites/merozoites (want 2×10^7/ml).
2. Suspend in sample buffer; need 1 ml for blank or
   75 ul/well, thus adjust vol. accordingly.
3. Draw off super into microfuge tube; centr. 2 min.
4. Draw off super into test tube and place in 100 C
   waterbath for 2 min.
5. Cool, then load onto gel.
6. Use 10 ul BRL MW stds. in 65 ul sample buffer;
   follow steps 4 and 5.

Sample Buffer:
4.0 ml distilled water
1.6 ml 10% SDS
1.0 ml 0.5M Tris-HCl (pH 6.8)
800 ul glycerol
400 ul 2-beta-mercaptoethanol
200 ul 0.05% (w/v) bromophenol blue

8.0 ml

10% Separating Gel--0.375M Tris, pH 8.8
26.8 ml dH_2O
16.7 ml 1.5M Tris-HCl, pH 8.8
 670 ul 10% (w/v) SDS stock
22.2 ml Acrylamide/Bis (30% stock)
200 ul 5% ammonium persulfate (fresh daily)
 30 ul TEMED

66.6 ml total monomer (makes two 16 cm/1.5 mm gels)

   Make solution minus APS and TEMED; add last and stir.
Pipette into casting chamber quickly avoiding bubbles.
Overlay with dH_2O.

4.0% Stacking Gel--0.125M Tris, pH 6.8
12.2 ml dH2O
 5 ml 0.5M Tris-HCl, pH 6.8
100 ul 10% (w/v) SDS
 2.6 ml Acrylamide/Bis (30% stock)
100 ul 10% ammonium persulfate (fresh daily)
 20 ul TEMED
------
 20 ml total monomer (enough for two 1.5 mm gels)

        Prepare as for separating gel. Pour off water overlay.
Pipette into mold and insert comb.

Tank or Running Buffer--0.025M Tris, pH 8.3; 0.192 glycine
12.0 g Tris base
57.6 g glycine
40.0 ml 10% SDS
Bring up to 4 L; no need to check pH. Reuse the buffer in
lower chamber 4-5X's, but discard upper buffer after each
run.

        Load samples/standards onto stacking gel. Run through
stacking gel at 25 mA (@ 30-60 min). Run through separating
gel at 35 mA (@ 4-5 h). Remove gels for transfer or
staining.

Stain
250 ml methanol (50%)
 50 ml glacial acetic acid (10%)
 62.5 ml coomassie blue (0.1%); from 1% stock (stir and
filter)
Bring up to 500 ml.
Gently shake gels in stain 4-8 h.

Destain
100 ml glacial acetic acid (10%)
500 ml methanol (50%)
Bring up to 1 L.
Gently shake in several washes.

STOCK SOLUTIONS

Acrylamide/Bis (30%)
146 g acrylamide (29.2 g/100ml)
  4 g N'N'Bis-methylene-acrylamide (0.8 g/100ml)
Make to 500 ml with dH$_2$O. Filter (Whatman #1) and store in
dark, 4 C (30 days max.)

1.5M Tris-HCl, pH 8.8
54.45 g Tris base (18.15 g/100ml)
@ 150 ml dH$_2$O
Adjust to pH 8.8 with 1 N HCl. Bring to 300 ml, store at 4 C

<u>0.5M Tris-HCl, pH 6.8</u>
6.0 g Tris base
@ 60 ml dH$_2$O
Adjust to pH 6.8 with 1 N HCl. Bring to 100 ml, store at 4 C

<u>10% SDS</u>
Dissolve 10 g SDS in dH$_2$O with gentle stirring and bring to
100 ml. Store at room temp.

WESTERN BLOT ANALYSIS

A. Electrophoretic transfer:

1. Breakdown PAGE gels, remove stacking gel, and equilibrate 30 min in transfer buffer.

2. Wet filter paper, sponges, and nitrocellulose in transfer buffer.

3. Assemble blot sandwich according to western blot apparatus instructions. (I used a BioRad, Richmond, CA, Trans Blot Cell).

4. Transfer either overnight at 30V or for 3 h at 75V. Chill transfer buffer prior to transfer.

B. Immunostaining:

1. After transfer, block nitrocellulose blot with 3% gelatin in TBS, pH 7.5, for either 2 h at 37 C or overnight at room temperature.

2. Rinse 2X with distilled water.

3. Primary antibody (chicken serum):

a. Dilute in Ab buffer with Tween 20; incubate 2-6 h at room temperature on a orbital mixer.

b. Wash 2X for 15 min each with PBS-Tween 20.

4. Secondary antibody (affinity purified anti-chicken):

a. Dilute 1/1000 in Ab buffer without Tween 20; react 2-3 h at room temperature on mixer.

b. Wash 2X for 15 min each with PBS-Tween 20.

5. Third antibody (affinity purified peroxidase labeled anti-goat):

a. Dilute 1/1000 in Ab buffer without Tween 20; react 2-3 h at room temperature on mixer in dark (wrap in foil).

b. Wash 2X for 15 min each with PBS-Tween 20.

6. Develop blots using peroxidase substrate development kit (Kirkegaard and Perry, Gaithersburg, MD). Mix equal volumes of 4-chloro-naphthol and hydrogen peroxide, pour immediately onto nitrocellulose strips, and develop in dark for 10-15 min.

7. Wash with distilled water to stop development reaction. Dry and photograph as soon as possible.

Transfer Buffer, pH 8.3, 4 liters.
    6.05 g Tris
    28.83 g Glycine
    800 ml Methanol
    4 ml 10% SDS
    3200 ml DH_2O

Tris-Buffered Saline (TBS), pH 7.5, 1 liter.
    6.055 g Tris-HCL
    29.22 g NaCl
    Bring up to 1 L.

Antibody (Ab) Buffer, pH 7.5, 1 liter.
    0.994 g Na_2HPO_4
    1.794 g NaH_2PO_4
    29.22 g NaCl
    10.0 g Bovine Serum Albumen (BSA)
    Bring up to 1 L.
    Add 0.5 ml Tween 20 for buffer with Tween.

PBS-Tween 20, pH 7.5, 1 liter.
    0.852 g Na_2HPO_4
    1.932 g NaH_2PO_4
    8.766 g NaCl
    0.5 ml Tween 20
    Bring up to 1 L.