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Partial protection against *Eimeria acervulina* and *Eimeria tenella* induced by synthetic peptide vaccine

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Abstract

Coccidiosis is a major parasitic disease of poultry industry and an ideal vaccine should induce long-lasting cross-species protective immunity. Broiler chickens (Cobb 500) were inoculated with single, double or triple injections of a synthetic peptide (derived from sequences of *Eimeria acervulina* and *Eimeria tenella* antigens) homogenized in Freund's complete and incomplete adjuvants. The immune responses to the vaccine were assessed by evaluation of antibody and lymphocyte proliferation responses, and the degree of resistance of vaccinated chickens to challenge with sporulated oocysts of *E. acervulina* or *E. tenella* determined by comparison of their oocyst output with those of control chickens. The results indicated that the synthetic peptide vaccine induced a high level of antibody and cellular responses associated with partial cross-species protection against challenge with sporulated oocysts of *E. acervulina* or *E. tenella*.

Index Descriptors and Abbreviations: E., *Eimeria*; FCA and FIA, Freund's complete and incomplete adjuvants; pi, post-inoculation or post-immunization

Keywords: *Eimeria acervulina*; *Eimeria tenella*; Vaccine; Synthetic peptide; Antibody and lymphocyte proliferation responses

1. Introduction

Avian coccidiosis is one of the most economically important diseases of the poultry industry. To overcome increasing drug resistance (McDougald and Reid, 1991), cross-species immunization is being actively researched and various types of anti-coccidial vaccines have been assessed (Chapman et al., 2002; Danforth and Augustine, 1990; Kim et al., 1989; Lillehoj and Lillehoj, 2000) and the protective efficacy of some of these vaccines has been evaluated (Crouch et al., 2003; Guzman et al., 2003). The disadvantages of conventional coccidiosis vaccines (Augustine et al., 1993; Lillehoj and Trout, 1993) together with advantages of synthetic peptide vaccines (Westwood, 2001) have been encouraged research towards alternatives. The development of peptide synthesis technology in the past decade has generally enhanced the potential of epitope mapping. In a technique described by Geysen et al. (1984), a series of small overlapping peptides (derived from the sequence of a protein) are synthesized on solid supports (''pins'') and may then be used as the antigen in conventional immunological assays (ELISA). Synthetic peptide vaccines have potential as safe ''designer'' immunogens incorporating beneficial T- and B-cell epitopes and as this type of

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vaccine does not include non-contributory protein, possible side effects (Benjamini and Leskowitz, 1991) are avoided. Since peptide vaccines are made from synthetic materials, vaccine production would not require maintenance of the parasite and their stability and transport is straightforward. Perhaps because of the ability of short peptides to take up several conformations in solution, such vaccines characteristically produce responses of greater cross-reactivity than conventional antigens. For example, FMD peptides can cross the serotype barrier of protection (Doel et al., 1990). This property could usefully be exploited in avian coccidiosis where protection against several species is required and conventional immunogens produce only species-specific immunity. So far, immunization with synthetic peptide vaccines against foot-and-mouth disease in cattle (DiMarchi et al., 1986), bovine rotavirus (Ijaz et al., 1991), canine parvovirus (Langeveld et al., 1994), schistosomiasis and fascioliasis (Vilar et al., 2003), and East Coast fever in cattle (Bishop et al., 2003) has shown protective capacity.

The available methods for assessment of immunity include quantitative assessment of antibody and cellular responses, weight gain, food conversion ratio, oocyst production, and lesion scoring. Measurement of all these parameters simultaneously in the same group of chickens may be impossible. For example, scoring of lesions caused by each *Eimeria* species requires that the chickens be killed when maximum damage is present, and this is not compatible with assessment of oocyst output, which requires the challenged chickens to be kept alive until 8–10 days pi. However, percentage reduction in oocyst output following challenge with *Eimeria* species has been widely used by many investigators for assessment of immune status of chickens in experimental studies (Lillehoj et al., 1990; Rose and Mockett, 1983).

Recently, following observations on the presence of high level of cross-reactivity among antisera raised against major *Eimeria* species (Talebi and Mulcahy, 1995b), identification of conserved immunogenic band(s) in oocyst protein from five avian coccidia (Talebi, 1995) and recognition of a few non-relative but hydrophilic motifs during epitope mapping studies (Talebi and Mulcahy, 1994), we constructed some synthetic peptides as potential immunogens against avian coccidiosis. The aim of this project was to investigate the immunogenicity of a peptide, designated as CHAT-4, against *E. acervulina* and *E. tenella* infections in chickens. The immunity induced by CHAT-4 was assessed by evaluation of antibody and lymphocyte proliferation responses as well as by percentage reduction in oocyst output following challenge with sporulated oocysts of *E. acervulina* or *E. tenella*.

2. Materials and methods

2.1. Parasites

Oocysts of *E. acervulina* strain W119 were purchased from the Central Veterinary Laboratory Weybridge (Surrey, UK) and oocysts of *E. tenella* (Houghton strain) were obtained from the Institute for Animal Health, Compton Laboratory (Berkshire, England). The oocysts of each species were sporulated as previously described (Davis, 1973).

2.2. Oocysts production and oocyst/sporozoite antigen preparation

Six-week-old broiler chickens (Cobb 500) were inoculated (Mockett and Rose, 1986) with a single optimal dosage (Johnson and Long, 1989) of sporulated oocysts of the *Eimeria* species for mass production of oocysts of each species. Water-soluble oocyst/sporozoite antigens of each species were prepared by homogenizing 2 ml of a suspension containing 2×10^7/ml sporulated oocysts/purified sporozoites in PBS as previously described (Rose, 1977). Protein concentrations of the antigens were determined using the bicinchoninic acid (BCA, product Nos. 23225 and 23220, Pierce, Illinois, USA) technique based on a standard curve constructed by using bovine serum albumin (BSA, product No. A-2153, Sigma Chemical) and the assay was carried out according to the manufacturer's instructions.

Oocyst and sporozoite antigens were used for immunization of rabbits to prepare rabbit anti-oocyst/sporozoite sera.

2.3. Preparation of synthetic peptide solutions

The sequence of the synthetic peptides vaccine antigens was derived from partial published sequences of a recombinant *E. acervulina* antigen expressed in sporozoite and merozoite developmental stages (Castle et al., 1991) and *E. tenella* antigens designated GX 3264 (Bhogal et al., 1992) and GX 5401 (Danforth et al., 1989) as follows:

CHAT-4 = H2-N-L-S-N-E-Q-V-E-R-Q-L-P-P-S-E-Q-V-E-T-C-COOH.

2.4. Immunization of chickens with synthetic peptides

Sequential batches of one-day-old chickens (Cobb 500) were fed on non-medicated broiler diet *ad libitum* and raised in wire-floored cages under coccidia-free conditions. At 2 weeks of age, the chickens were leg-banded and each chicken was transferred into a separate cage. Two groups of chickens (six chickens in each) were used for each experiment. Experiment 1 was designed to assess immunity produced following vaccination with a

single dose (200 μg/bird) of vaccine 1 (prepared from homogenizing CHAT-4 in PBS with an equal volume of Freund's complete adjuvant, FCA). Three weeks later, the chickens were challenged with 1×10^4 sporulated oocysts of *E. tenella*. In Experiment 2, chickens were immunized with a single dose (200 μg/bird) of vaccine 1 and 3 weeks later with another dose (200 μg/bird) of vaccine 2 (prepared from homogenizing CHAT-4 in PBS with an equal volume of Freund's incomplete adjuvant, FIA). Two weeks after the second vaccination, the chickens were challenged with 1×10^4 sporulated oocysts of *E. tenella*. Experiment 3 was designed to evaluate the immunity produced by vaccination of chickens with a single dose (200 μg/bird) of vaccine 1 followed (3 weeks later) by two doses (200 μg/bird) of vaccine 2 at 2 weeks intervals. Two weeks after the second vaccination, the chickens were challenged with 1×10^5 sporulated oocysts of *E. acervulina*. During each experiment, the weight of each bird was recorded and blood samples were taken on day 0 (pre-immunization), weekly post-immunization (pi), before challenge, and at the end of the experiment. The collected sera were inactivated at 56 °C for 30 min and stored at −20 °C as previously described (Oz et al., 1984).

2.5. Assessment of antibody responses

Antibody capture immunoassay (ELISA) was used for determination of antibody levels of the sera as previously described (Oz et al., 1984). In brief, 96-well Nunc II immunoplates were coated with 5 μg/ml of antigen in 0.1 m carbonate/bicarbonate buffer, pH 9.6 (100 μl/well), incubated overnight at room temperature, washed five times with PBS containing 0.1% Tween 20, and dried on tissue paper. Serial 3-fold dilutions of the sera were made in dilution buffer (1% sodium caseinate, 10% sheep serum, and 0.1% Tween 20 in PBS), the immunoplates were incubated for 1 h at 37 °C, washed five times, specific binding was detected by adding 100 μl/well of 1/400 rabbit anti-chicken horseradish peroxidase conjugate and 3,3,5,5-tetramethylbenzidine, the substrate reaction was stopped after 10 min by adding 100 μl/well of 10% H_2SO_4, the plates were read in an ELSA microplate reader (Bio-Rad Model 3550) at 450 nm with 492 nm as the reference wavelength, and the antibody levels of antisera were expressed as the optical density (OD) at 450 nm.

2.6. Epitope mapping

An adaptation of the ELISA technique was used with pin technology "Pepscan" to screen reactions of sera from immunized chickens with sets of hexapeptides and to compare it with that of naturally infected chickens as previously described (Talebi and Mulcahy, 1994).

2.7. Assessment of lymphocyte proliferation responses

For evaluation of cell-mediated immunity, whole blood samples were taken from the *vena cutanea ulnaris* (wing vein) using 1 ml syringes containing 100 IU heparin sulphate on day 0, before each vaccination, before challenge, and at the end of the experiment. Lymphocyte proliferation assays were carried out using whole blood of chickens under optimal conditions as previously determined (Talebi et al., 1995). Briefly, blood samples were diluted 1 in 50 with Roswell Park Memorial Institute (RPMI) medium containing 1% sodium pyruvate, 25 mM Hepes buffer, and 1 mM L-glutamine as well as penicillin–streptomycin (100 IU/ml + 100 μg/ml, respectively). A volume of 20 μl of concanavalin-A (con-A) at a concentration of 20 μg/ml in PBS (0.4 μg/culture), a volume of 20 μl of oocyst antigen of *E. tenella* or *E. acervulina* at a concentration of 20 μg/ml in PBS (0.4 μg/culture), and a volume of 20 μl of synthetic peptide (CHAT-4) at a concentration of 20 μg/ml in PBS (0.4 μg/culture) were dispensed in triplicate into wells of a 96-well flat-bottomed Nunc tissue culture microplate. A volume of 200 μl of diluted blood from each sample was transferred into each well of the plate (for example, blood sample 1 into the wells of row A, blood sample 2 into the wells of row B, and so on). The plates were incubated for 48 h at 40 °C in a humidified atmosphere containing 5% CO_2. The cultures were pulsed with 0.2 μCi/20 μl/culture of [^{3}H]thymidine (37.0 kbq/ml, 1 mCi/ml) using an Eppendorf combitip 18 h prior to harvesting. The cells were harvested on filter mats with a Skatron Combi cell harvester, the harvested cells were dried, and disks containing cells were punched out and placed into scintillation vials. After adding 2 ml scintillation fluid (Parkard Instrument BV Chemical Operations, The Netherlands), radioactivity of individual vials was measured by disintegration per minute (DPM) using a liquid scintillation β-counter (Packard 1900 CA Liquid Scintillation Analyser, Packard Instrument Company, 2200 Warranville Rood, Downers Grove, IL 60515, USA). The stimulation index (SI) for each blood sample was calculated according to the following formula:

$$\mathrm{SI} = \frac{\text{Mean DPM of stimulated cultures}}{\text{Mean DPM of unstimulated cultures}}.$$

2.8. Assessment of oocyst output

During each experiment, faeces of each chicken were collected for 4 days starting at the 5th day pi for chickens infected with *E. acervulina* and at the 6th day pi in the case of *E. tenella*. The oocysts were harvested and counted using the McMaster method (Davis, 1973; Lillehoj et al., 1988; Long et al., 1976) with some modifications as previously described (Talebi and Mulcahy,

1995a). The immune status of the chickens used in the experiments, judged by reduction in oocyst output, was determined using the following formula described by Rose and Mockett (1983) and Lillehoj et al. (1990):

$$\%\ \text{of reduction} = 100 - 100 \times \frac{\text{No. of oocysts passed by immunized + challenged chickens}}{\text{No. of oocysts passed by control + challenged chickens}}.$$

2.9. Statistical analysis of the results

A two-tailed t test (using Microsoft Excel) was carried out to assess the significance of the results. Differences between the mean values for groups of chickens were considered significant when probability (P) values of less than 0.05 were obtained.

3. Results

In Experiment 1, some antigen-specific responses in immunized chickens (data not shown), increased slightly but after challenge with sporulated oocysts of *E. tenella*, there was no significant ($P = 0.77$) difference in mean oocyst output between immunized and control chickens ($11.1 \times 10^7$and 11.9×10^7, respectively).

In Experiment 2, as shown in Figs. 1A and B, both antibody and lymphocyte proliferation responses increased gradually following primary vaccination and were boosted following secondary injection while those of the control group remained relatively unchanged. After challenge with *E. tenella*, the mean oocyst output of vaccinated chickens was less than that of the control group. Statistical analysis of the results showed that differences in oocyst production between immunized and control chickens (mean 5.82×10^7 and 8.12×10^7, respectively) were significant ($P = 0.015$). As shown in Fig. 1A, the antibody titers of immunized chickens at 6 weeks pi (prior to challenge) to the peptide and to oocyst antigens of *E. acervulina* and *E. tenella* were high and significantly ($P = 0.015$, $P = 0.004$, and $P = 0.001$, respectively) differed from those of the control group. As shown in Fig. 1B, proliferation of lymphocytes of the immunized group in response to *E. tenella* antigen and to the peptide also was higher than that of the control group. The level of protection resulting from the vaccination protocol in this experiment against challenge with 1×10^4 sporulated oocysts of *E. tenella* was calculated 29% using the formula (Lillehoj et al., 1990; Rose and Mockett, 1983).

In Experiment 3, the kinetics of the immune response of immunized chickens were similar to those observed in Experiment 2. Analysis of the results obtained from this experiment showed a significant difference in oocyst output between immunized (8.23×10^8) and control (11.1×10^8) groups ($P = 0.03$). As shown in Fig. 2A,

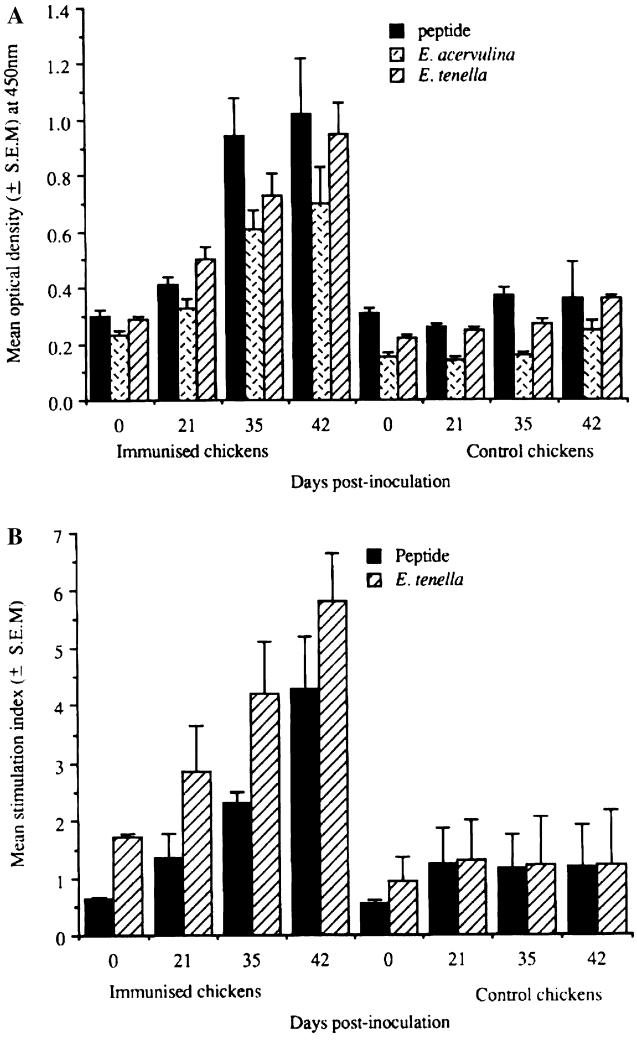

Fig. 1. Comparison of immune responses of immunized and control chickens during Experiment 2. (A) Mean antibody responses of the chicken sera (dilution 1/270) to CHAT-4, oocyst antigens of *E. acervulina* and *E. tenella*. Differences in antibody levels of immunized and control chickens were significant ($P = 0.015$ for peptide, $P = 0.04$ for *E. acervulina*, and $P = 0.001$ for *E. tenella*). (B) Mean stimulation index of lymphocytes by CHAT-4 and *E. tenella*.

antibody titers for peptide, *E. acervulina* and *E. tenella* (at 7 weeks pi, before challenge) of immunized chickens differed significantly ($P = 0.003$, $P = 0.006$, and $P = 0.001$, respectively) from those of the control group. The antibody titers of immunized chickens boosted following challenge while those of the control group did not change. As shown in Fig. 2B, the stimulation of lymphocytes from immunized chickens by peptide and *E. acervulina* antigens was also higher than that of the unimmunized group. The level of protection resulting from the vaccination (triple injections) with the peptide (CHAT-4) against challenge with 1×10^5 sporulated oocysts of *E. acervulina* was 27% using the formula (Lillehoj et al., 1990; Rose and Mockett, 1983).

Comparative epitope mapping studies of antisera from immunized and naturally infected chickens demon-

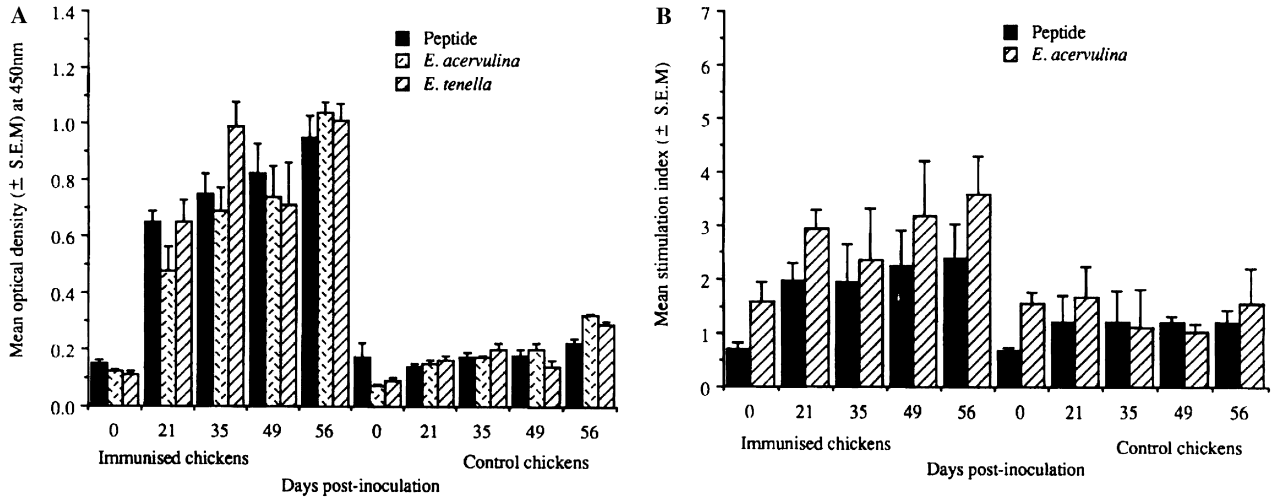

Fig. 2. Comparison of immune responses of immunized and control chickens during Experiment 3. (A) Mean antibody responses of the chicken sera (dilution 1/270) to CHAT-4, oocyst antigens of *E. acervulina* and *E. tenella*. Differences in antibody levels immunized and control chickens were significant ($P = 0.003$ for peptide, $P = 0.006$ for *E. acervulina*, and $P = 0.001$ for *E. tenella*). (B) Mean stimulation index of lymphocytes by CHAT-4 and *E. acervulina*.

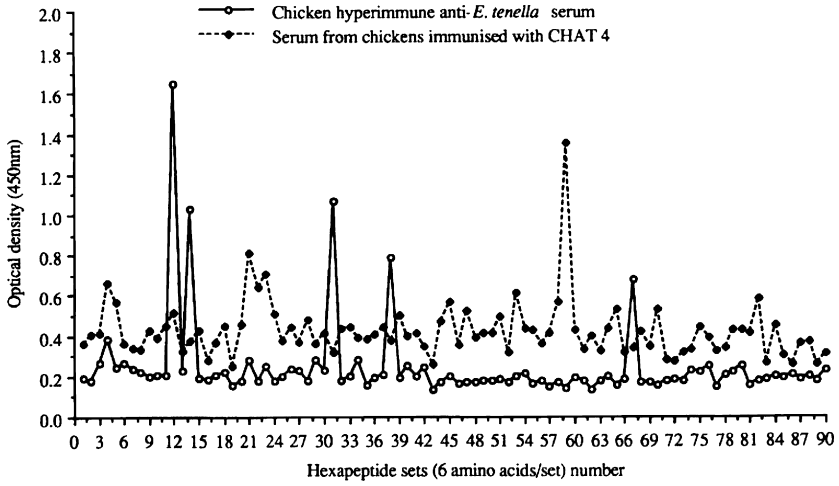

Fig. 3. Comparison of antibody responses of sera from immunized chickens with CHAT-4 (prepared on day before challenge) and naturally infected chickens using high-resolution of B-cell epitope mapping of an immunogenic sequence of *E. tenella*.

strated that reactivity of antibodies produced against the peptide differed in all aspects from those of hyperimmune chickens infected naturally with *E. tenella*. As shown in Fig. 3, a few epitopes were recognized by homologous hyperimmune sera and there was an obvious lack of response to two regions (hexapeptides Nos. 19–30 and 49–65) from the sequence. Antisera from immunized chickens had specific reactivity to these regions of the sequence and the highest reaction was with a hexapeptide containing the EQV motif (Fig. 3).

4. Discussion

In immune chickens, defense against coccidia is presumed to be dependent on the presence of sensitized lymphocytes and available local antibody in the gut mucosa (Long, 1983). Lack of complete cross-species protection following natural infection or conventional vaccination suggests that the ideal vaccine should contain conserved antigens of avian coccidia or a combination of several immunogens from *Eimeria* species to provide cross-species protection against simultaneous challenge with these parasites. The reasons that EQV motif was selected for construction of synthetic peptides were that this motif was conserved between *E. acervulina* and *E. tenella* antigenic sequences and there was no reaction against the EQV in screening of reaction of sera from naturally infected chickens. This motif occurs in a relatively hydrophilic region, suggesting that it is a potential immunogenic epitope (Talebi and Mulcahy, 1994), but because of its hydrophilicity it is likely to

be available as a B-cell epitope and the question remains as to why it is not recognized by antibodies. It was postulated that this motif represents part of a functionally important parasite structure, and is therefore flanked by highly variable immunodominant sequences to "hide" it from the immune system, or it may represent a T-cell epitope, which needs further investigation (Hopp and Woods, 1981).

The results of Experiment 1 indicated that a single injection of CHAT-4 in FCA was unable to induce an effective antibody response or reduce significantly the oocyst production following challenge with oocysts of *E. tenella* ($P = 0.77$). In Experiments 2 and 3, the high antibody levels of immunized chickens to the peptide and oocyst antigen of *E. tenella* or *E. acervulina* indicated that the peptide was able to induce a considerable antibody response following repeated immunization. In the case of Experiment 3, boosting antibody levels of immunized chickens following challenge with *E. acervulina* are a good indication of an effective secondary response to the peptide vaccine. Significant proliferation of lymphocytes from immunized chickens indicated that the peptide was also able to induce cellular immune responses but the level of blastogenic responses varied between *Eimeria* species used. Comparison of Figs. 1B and 2B showed that the degree of lymphocyte stimulation during Experiment 2 was higher than that in Experiment 3, indicating that the amino acids selected from the *E. tenella* antigenic sequence were able to induce a greater cellular response than those from *E. acervulina*. It should be emphasized that blastogenic proliferation of lymphocytes in whole blood assays is also influenced by other factors including the genetic background (Miggiano et al., 1976), breed (Lillehoj, 1986), strain (Wakelin and Rose, 1978), and age of chickens (Talebi et al., 1995). Considerable individual variation in the level of response in chickens of the same breed, strain, and age has also been reported (Sharma and Belzer, 1992). The level of partial cross-species protection obtained in this study was small, but nevertheless demonstrates the feasibility of using synthetic peptides as vaccines for the control of avian coccidiosis. As shown in Fig. 3, epitope mapping studies of the immunized sera revealed that the lack of responsiveness to some regions of the sequence (such as EQV motif) following natural infection could be overcome by immunization of chickens with short peptide vaccines. To improve the level of cross-species protection with synthetic peptide vaccines, further epitope mapping studies would be necessary to identify other conserved potentially immunogenic proteins which are not recognized by antisera from the natural host and to present them in such a way not only to overcome the lack of responsiveness but also to modulate the immune responses. An alternative strategy would be to identify immunogenic epitopes (B- and T-cell epitopes) present within antigenic sequences of each pathogenic species of *Eimeria* and to include combination of the highly reactive epitopes in candidate peptides for induction of adequate cross-species protective immunity.

5. Conclusions

Synthetic peptide vaccines may allow the species-specific immunity barrier to be overcome in the design of broad-spectrum anti-coccidial vaccines. This study demonstrated that peptides are capable of eliciting high antibody responses and relatively good proliferation of lymphocytes in chickens to *Eimeria* species, resulting in partial cross-species protection against challenge with *E. acervulina* and *E. tenella*. It also showed that lack of responsiveness to conserved epitopes could be overcome by immunization of chickens with the synthetic peptides.

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