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High-Resolution Mapping of B-Cell Epitopes within an Antigenic Sequence from *Eimeria tenella*

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Overlapping hexapeptides representing part of an *Eimeria tenella* antigenic sequence, shown to induce partial immunity to homologous challenge in chickens, were synthesized on polypropylene pins (Pepskan technique; Cambridge Research Biochemicals, Cambridge, United Kingdom). The binding to these hexapeptides of antibodies from chickens infected and rabbits immunized with five species of *Eimeria* was studied, using the coated pins as the solid phase of an enzyme-linked immunoassay. Antibody binding to most regions of the sequence was demonstrated, with peak areas of antigenicity correlating with the most hydrophilic regions. A particularly hydrophilic and antigenic area towards the N terminus of the sequence consists of a peptide motif repeated five times in the native antigen. Homologous antisera (chicken and rabbit anti-*E. tenella* antisera) differed in their pattern of reactivity from heterologous sera raised against other *Eimeria* species. While the former bound to fewer of the hexapeptides than the latter, they did so very strongly, indicating affinity maturation of the antibody response to *E. tenella*-specific sequences. No antibody reactivity to two regions of the sequence was detected. These regions occur in relatively hydrophilic areas and so are unlikely to be situated in transmembrane domains or in the interior of globular proteins. Synthetic peptides, as used in these experiments, make possible analysis of the fine specificity of immune responses and thus have a role to play in the development of novel vaccines for the control of coccidiosis.

The importance of *Eimeria* species as pathogens of domestic poultry, together with the inherent limitations of currently available control methods, has led to widespread interest in the possibility of using new vaccine technology to provide broad-spectrum protection against avian coccidiosis (1, 3, 15, 17). Undoubtedly, the mechanisms responsible for protective immunity to coccidiosis following natural infection are complex and may involve lymphokine-associated cell-mediated immunity (14, 20). The role of antibody-mediated immunity following infection is less certain (20). Chickens which have recovered from infection with one species of *Eimeria* develop antibodies which cross-react to a large extent with other *Eimeria* species (6), yet convalescent-phase immunity is largely species specific (19). Many of these infection-induced antibodies may therefore be nonprotective. However, it is quite possible for novel vaccines to induce protective immunity by mechanisms other than those operating in natural infection. It has been shown, for example, that maternal antibody induced by gametocyte antigens is capable of conferring passive protection to day-old chicks (24). Hence, characterization of *Eimeria* B-cell epitopes is directly relevant to the development of novel coccidiosis vaccines.

Synthetic peptides are powerful tools for the analysis of the fine specificity of immune responses. Furthermore, it is now recognized that even quite short peptides can be good immunogens (4), and, ultimately, synthetic peptides have the potential to be safe "designer" immunogens incorporating beneficial T- and B-cell epitopes and eliminating noncontributory or deleterious portions of antigenic proteins. This approach has been rejected by some workers concerned with protozoan vaccine development, on the grounds that protective immunity against such relatively complex organisms is likely to require recruitment of more than one effector mechanism against several antigens. Considerable progress, however, has already been made in the development of this type of vaccine for use against *Plasmodium* spp. in humans (18). Peptide technology therefore deserves attention in the quest for effective, broad-spectrum coccidiosis vaccines.

Danforth et al. (5) have characterized an antigenic sequence (GX 5401) from *Eimeria tenella* which they have shown to be capable of partially protecting chickens against *E. tenella* challenge when administered in a recombinant form. However, similar sequence, GX 3264, while protecting against *E. tenella* challenge, has no protective effect against *Eimeria acervulina* challenge (1). We have studied the reactivity of a portion of the GX 5401-GX 3264 sequence with *Eimeria*-induced antibodies at high resolution, using the Pepskan technique (Cambridge Research Biochemicals, Cambridge, United Kingdom) (12), in which overlapping peptides, in this case hexapeptides, bound to polypropylene pins are used to map B-cell epitopes.

MATERIALS AND METHODS

Production of antisera. Sporozoites of five *Eimeria* species (*E. tenella*, *E. acervulina*, *E. necatrix*, *E. praecox*, and *E. maxima*, all Weybridge strains) were purified from oocysts in the feces of monospecifically infected Cobb 500 chickens (Knocknagarm Hatcheries, Glenageary, Co. Dublin, Ireland). Briefly, oocysts were separated from the feces by flotation in saturated NaCl by the methods described by Davis (7, 8) and the collected oocysts were sporulated and purified as previously described (16), except that 30% sodium hypochlorite solution was used for purification. Sporulated oocysts were excysted and sporocysts were purified as described by Sutton et al. (22) but with Hanks' balanced salt solution in place of phosphate-buffered saline. Sporozoites were released by standard techniques (21) and purified by density gradient centrifugation in Percoll (10).

Rabbit antisporozoite sera were prepared by inoculating

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rabbits two or three times with 4×10^7 sporozoites in oil adjuvant (Freund's complete for the first inoculation and Freund's incomplete for subsequent inoculations) per rabbit. Chicken convalescent-phase anti-*Eimeria* sera were obtained from the wing vein of monospecifically infected chickens (infected 3 to 4 weeks previously with 10^4 [*E. tenella*] or 10^5 [other species] *Eimeria* oocysts). Preinoculation or preinfection and weekly postinoculation serum samples were taken from both chickens and rabbits. Antibody levels in sera were evaluated by enzyme-linked immunoassay (antibody capture assay using anti-chicken immunoglobulin or anti-rabbit immunoglobulin conjugates at 1/1,000 and tetramethyl benzidine as the substrate). Sera with peak antibody levels were stored at −20°C until used.

Epitope mapping experiments. The sequence of recombinant *E. tenella* antigen GX 3264 (1) was selected for epitope mapping analysis. This antigen represents the sequence designated GX 5401 (5) minus the first 65 amino acids, which represent highly homologous tandem repeat units (5). A 95-residue portion of the GX 3264 sequence commencing at asparagine 40 and finishing at alanine 135 was synthesized as overlapping hexapeptides (overlap 1) on polypropylene pins by a commercial procedure (Pepskan). The full sequence synthesized in this way as hexapeptides for the purpose of these experiments is as follows: N-A-E-E-L-P-G-E-E-G-G-A-G-A-G-G-A-E-G-E-T-G-L-P-G-G-E-E-G-G-A-G-G-A-G-E-G-A-G-G-E-G-G-E-V-Q-P-G-E-G-E-G-A-S-E-G-G-E-Q-V-P-E-T-P-E-T-P-E-P-E-T-P-E-A-E-R-P-E-E-Q-P-S-T-E-T-P-A-E-E-P-T-E-G-G-A. Pin 1 of the test plate, therefore, contained the sequence NAEELP, pin 2 contained AEELPG, and so on.

The first 16 N-terminal amino acids of the sequence represent a peptide repeated five times towards the N-terminal end of GX 5401.

The pins with their covalently bound peptides were used as the solid phase of an enzyme-linked immunosorbent assay (ELISA) to measure the reactivity of anti-*Eimeria* sera with each hexapeptide. ELISA procedures and disruption of antibody from the pins to allow repeated testing of multiple sera were carried out according to the manufacturer's specifications, except that the optical densities of wells following incubation with substrate were measured at 415 nm, with 492 nm as the reference wavelength. Anti-*Eimeria* sera were used at dilutions between 1/3,000 and 1/300, and anti-rabbit and anti-chicken horseradish peroxidase conjugates (Dako and Serotec, respectively) were both used at 1/1,000. Bound conjugate was detected by using azino-bis-ethylbenzthiazoline sulfonic acid (ABTS) as a substrate. Results were expressed as the optical density at 415 nm produced by the incubation of a particular antiserum with each of the hexapeptides. Pins were stored at 4°C when not in use.

RESULTS

Chicken sera. Examination of the reactivity of chicken sera with the *E. tenella* hexapeptides showed that while no reactivity was detected with sera from uninfected chickens, both homologous and heterologous convalescent-phase sera reacted with multiple epitopes along the sequence (Fig. 1). Homologous antiserum (Fig. 1b) bound to only a few of the hexapeptides but showed the greatest amplitude of response, indicating high-affinity interactions. Most of the epitopes recognized by homologous convalescent-phase chicken serum begin with the amino acids A-G (Fig. 1). This preference was not seen with heterologous chicken sera or with any of the rabbit sera studied. The heterologous chicken sera were variable in their reactivity. Little or no antibody binding was detected with anti-*E. maxima* convalescent-phase serum (Fig. 1f), while serum raised against *E. acervulina* (Fig. 1c) exhibited a high background in this assay, which is probably accounted for by a combination of nonspecific binding and low-affinity antibody-antigen reactions. Convalescent-phase anti-*E. praecox* and *E. necatrix* sera reacted quite strongly with several hexapeptides along the sequence.

Rabbit sera. Analysis of the reactivity of rabbit anti-*Eimeria* sera (Fig. 2) showed similarly that homologous serum reacted very specifically with a small number of hexapeptides, in this case just two, along the sequence (Fig. 2b). Reactivity against the hexapeptides could not be detected with normal rabbit serum (Fig. 2a) or rabbit anti-*E. acervulina* serum (Fig. 2c), while the remaining heterologous sera reacted to variable degrees with multiple hexapeptides.

Reactivity of regions of the sequence. The frequencies with which regions of the sequence react with antisera (rabbit or chicken) to each of the five species of *Eimeria* studied are shown in Table 1. There are B-cell epitopes throughout most of the sequence. Peptide sets 1 to 6 and 7 to 12 are highly reactive with all five of the *Eimeria* species studied. Pins 25 to 30 and 73 to 78 also represent highly reactive areas. The only regions of the sequence against which no antibody activity was detected are situated within areas represented by pins 55 to 60 and 67 to 72. These two nonreactive regions are shown in boldface in the following sequence: N-A-E-E-L-P-G-E-E-G-G-A-G-A-G-G-A-E-G-E-T-G-L-P-G-G-E-E-G-G-A-G-G-A-G-E-G-A-G-G-E-G-G-E-V-Q-P-G-E-G-E-G-A-S-**E-G-G-E-Q-V-P-E**-T-P-E-T-**P-E-P-E-T-P-E-A-E-R-P**-E-E-Q-P-S-T-E-T-P-A-E-E-P-T-E-G-G-A. The sequence examined in these experiments corresponds to the hydrophilic region of GX 5401 described by Danforth et al. There are just a few small hydrophobic pockets towards the N terminus. When a map of the antigenicity of the sequence is superimposed upon a plot of hydrophilicity derived by the procedure of Hopp and Woods (13) (Fig. 3), it is clear that the most hydrophilic peaks match quite closely with peak antigenicity. The two nonantigenic regions, however, also occur in relatively hydrophilic areas.

DISCUSSION

These experiments demonstrate that within a sequence from *E. tenella* shown previously to be antigenic and to elicit partially protective immune responses, most regions react with a cross-section of *E. tenella* and other *Eimeria* antisera. This is so for both rabbit and chicken sera. Sera from rabbits and chickens not exposed to antigenic stimulation with *Eimeria* antigens do not react with the peptides studied, indicating that the reactions measured with immune sera are specific.

When the reactivities of homologous and heterologous antisera are compared, it is clear that the former react with fewer sequences and also that the background reactivity at comparable serum dilutions is lower than that for heterologous sera. These features are seen with antisera generated in both rabbits and chickens and are consistent with the development of high-affinity antibodies by homologous immunization. Heterologous immunization is likely to induce antibodies of lower specificity and lower affinity, resulting in a lower signal-to-noise ratio and a "fuzzy" pattern of reactivity.

The antibodies in sera raised against *E. acervulina* (both rabbit and chicken) reacted less well with the hexapeptides in this study than did those in sera raised against *E. necatrix* and *E. praecox*, for example. Although there is no definitive evidence to suggest that the protection against *E. tenella* challenge observed with antigen GX 3264 is mediated by an antibody, nevertheless, a correlation between antibody re-

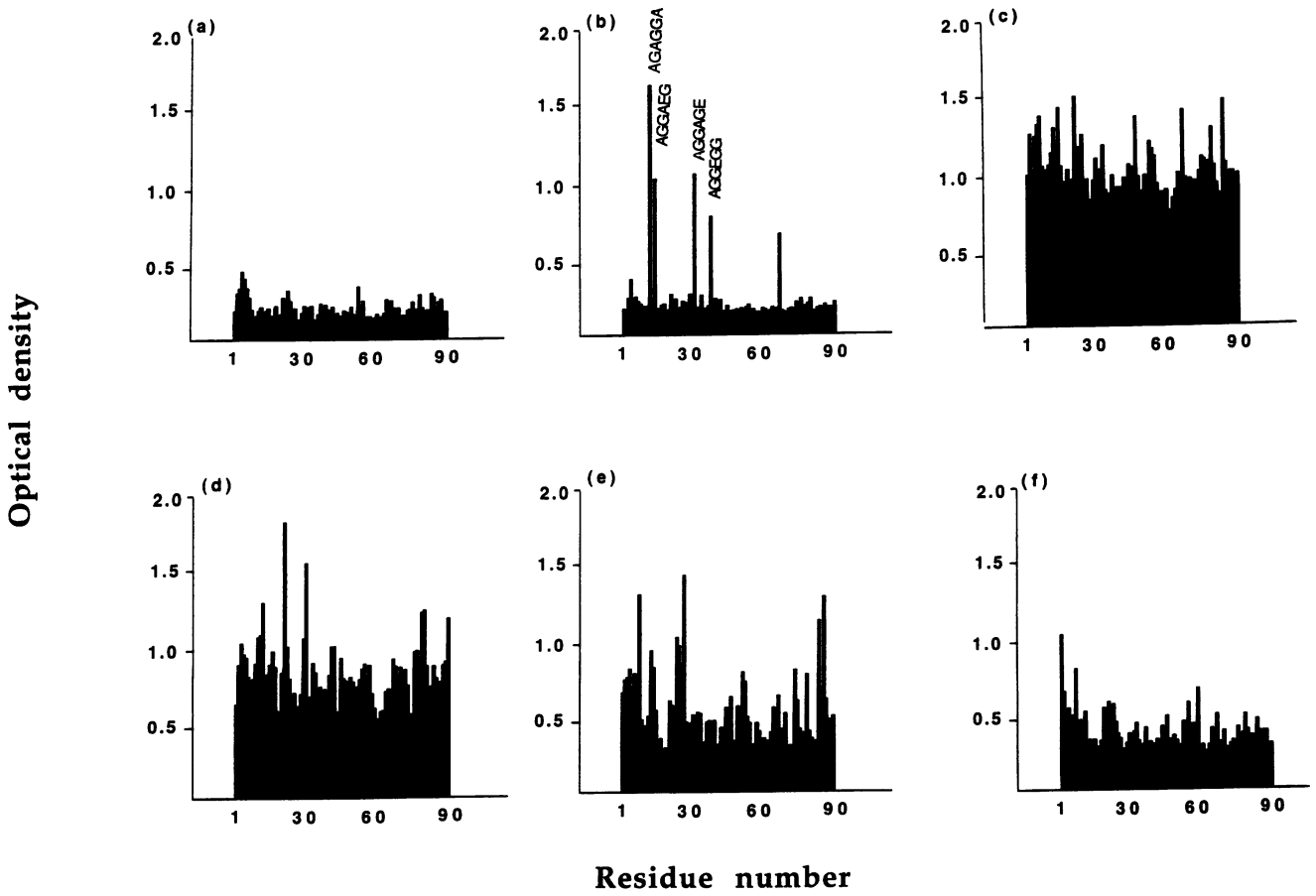

FIG. 1. Reactivity of chicken sera with an *E. tenella* antigenic sequence. The sera were incubated with 90 overlapping hexapeptides from the sequence which had been synthesized on polypropylene pins (pin numbers are indicated on the *x* axes). Following washing to remove unbound antibody, specific reactivity was detected by sequential addition of horseradish peroxidase-labelled anti-chicken immunoglobulin and ABTS as a substrate. The reactivities of normal chicken serum (a) and of sera from chickens which had recovered from infection with *E. tenella* (b), *E. acervulina* (c), *E. praecox* (d), *E. necatrix* (e), and *E. maxima* (f) are shown. Sera were used in the assay at dilutions ranging from 1/300 to 1/3,000, the dilution in each case being chosen to optimize the signal-to-noise ratio. Also shown in panel b are the hexapeptides commencing with amino acids A-G, against which most antibody-binding activity in the homologous chicken antiserum was detected.

sponse and protection has been observed with this antigen (11). Therefore, the failure of this antigen to protect against *E. acervulina* (1) might not be reflected in its potential to protect against some other *Eimeria* spp.

While the occurrence of antibodies cross-reacting between various species in the absence of heterologous protection has often been interpreted to mean that antibody is not relevant to protection from challenge, it is pertinent to consider that qualitative aspects of antibody production are also relevant to protective capacity. Antibodies which react with low avidity in the general area of an important epitope may be nonprotective (2) or may even, in some circumstances, block other effector mechanisms, leading to antibody-dependent enhancement of infection (9). The difference between homologous and heterologous sera in the type of signal obtained in these assays is clear and serves to point out that a lack of heterologous protection in the presence of heterologous antibody does not necessarily indicate that antibodies are not relevant in protective immunity to coccidia. Workers interested in the identification and evaluation of vaccine candidate antigens often use antibody titers as their sole criterion. However, as knowledge of immunological effector mechanisms and the technology for measuring antibody isotypes and affinity improve, it is likely that the quality of antibody induced by a particular antigen will be seen to be as important as the level of antibody production in determining protective capacity. While we have not as yet performed any direct measurements of the avidities of various sera in these assays, such measurements are often technically feasible with peptide antigens and provide valuable information during screening of candidate vaccine epitopes and during evaluation of immunization protocols.

The clear preferential reactivity of homologous chicken antisera, and no others, with hexapeptides commencing with A-G is difficult to explain but may indicate that structures with such conformations are expressed particularly during invasion or replication.

Almost all of the amino acids within the study sequence form part of B-cell epitopes recognized by one or more chicken or rabbit antisera. The antigenicity of each region of the sequence broadly coincides with the occurrence of hydrophilic peaks. Such regions normally lie on the exposed surface of proteins, and hydrophilicity is widely regarded as a good predictor of B-cell immunogenicity (13). There is a particularly striking co-occurrence of antigenic and hydrophilic peaks around hexapeptides 1 to 12, 25 to 30, and 73 to 78. The first of these peaks occurs within the multiple-repeat domain R1 to

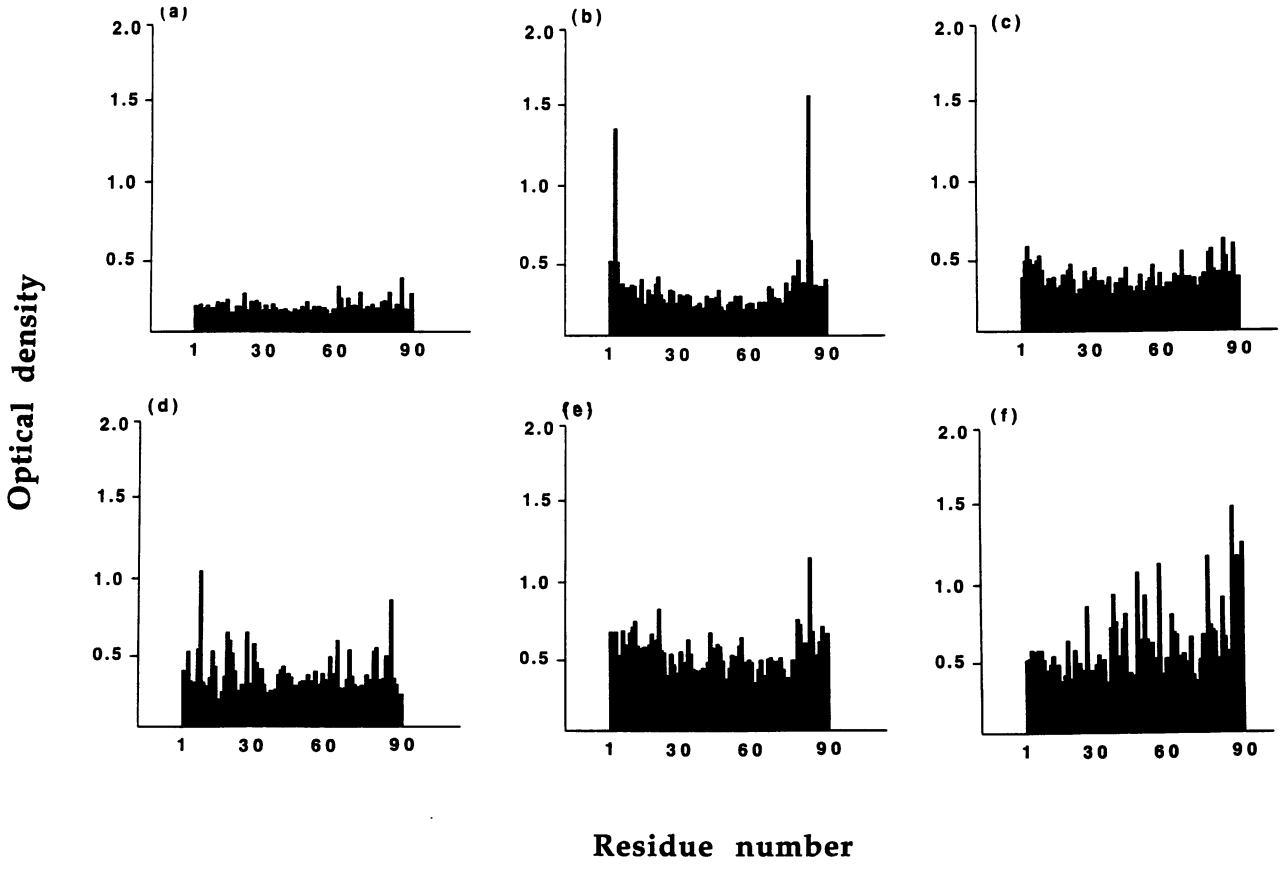

FIG. 2. Reactivity of rabbit sera with an *E. tenella* antigenic sequence. The reactivities of normal rabbit serum (a) and of sera from rabbits immunized with *E. tenella* (b), *E. acervulina* (c), *E. praecox* (d), *E. necatrix* (e), and *E. maxima* (f) sporozoites are shown. Sera were used at optimal dilutions ranging from 1/300 to 1/1,000.

R5 (5) of GX 5401. These repeated regions, therefore, are likely to account for a large proportion of the antibodies induced by GX 5401 as an immunogen. Given the tandem repeat nature of this region, it is possible that it may be used as a smokescreen by the parasite to focus the B-cell response of the host on a region of little functional significance to the parasite.

The antigenic peaks seem to be shifted slightly to the right of residues of maximum hydrophilicity, indicating perhaps that B-cell epitopes are preferentially formed with the most hydrophilic amino acids towards the N terminus.

Since many of the antibody specificities in convalescent-phase chicken serum reacting with the sequence are induced by more than one species of *Eimeria*, and since protective immunity following infection is species specific, it must be concluded that such antibodies are not capable of mediating heterologous resistance to challenge. As outlined above, however, the limitations of these antibodies may be due in part not to an overall ineffectiveness of antibody in protective immunity against coccidiosis, but to a failure of antibodies induced by infection to react with sufficiently high affinity with heterologous species. The use of synthetic antigens may allow this barrier to be overcome in the design of broad-spectrum coccidiosis vaccines.

Synthetic immunogens may also provide for immune recognition of sites not recognized as epitopes when presented as components of natural antigens. There are two regions of the

TABLE 1. Reactivities of hexapeptides along the mapped sequence with rabbit and chicken sera raised against five *Eimeria* species[a]

Pins	Reactivity with serum against:				
	E. tenella	*E. praecox*	*E. maxima*	*E. necatrix*	*E. acervulina*
1–6	+++	++	+	++	+
7–12	+	++++	+	+++	++
13–18	+			+	
19–24		++			
25–30		++++	+	++	++
31–36	++				
37–42	+	++	+		+
43–48		+			
49–54				+	
55–60					
61–66		+		+	
67–72					
73–78			+	++	++
79–84	++			+	
85–90		+	+++	+	+

[a] The 90 pins bearing the overlapping hexapeptides are grouped in sets of six in the first column. Each hexapeptide within the set reacting with a serum raised against an *Eimeria* species is denoted by a +. Thus, a score of +++, for example, in a particular category indicates that three of the hexapeptides within the set react with antiserum of that specificity. In many cases, the scores reflect the broad cross-reactivity of the overlapping peptides.

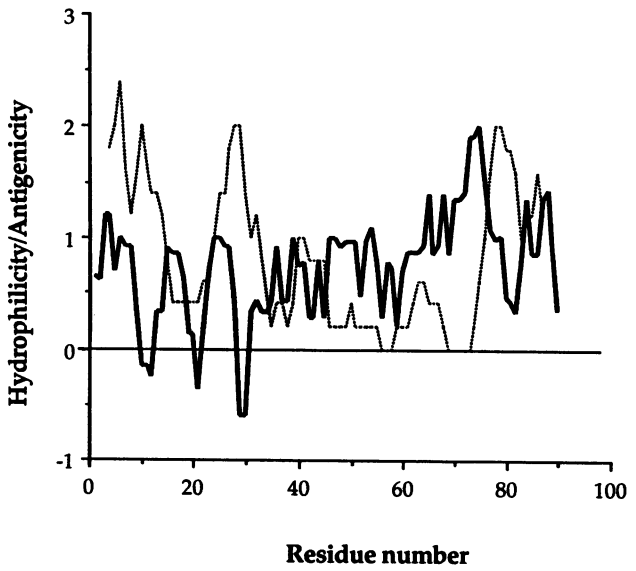

FIG. 3. Hydrophilicity profile of the *E. tenella* sequence, together with an estimation of the antigenicity of corresponding regions of the sequence. The hydrophilicity profile (solid line) was derived by the method of Hopp and Woods (13), using a window of six amino acids. Each point on the graph therefore represents the mean hydrophilicity value for six residues. Hydrophilic amino acids appear above the baseline. The antigenicity profile (dotted line) was derived by assigning a value to each hexapeptide on the basis of the number of antisera which elicit antibodies against that peptide. The values were multiplied by 0.2 and rounded off in order to be displayed in a format compatible with the hydrophilicity data.

antigen mapped in these experiments which were not recognized by any conventionally prepared chicken or rabbit antisera. These occur in relatively hydrophilic regions of the sequence, so they are unlikely to be unavailable as B-cell epitopes by virtue of being located in transmembrane domains or in the interior of a globular protein. We have not as yet evaluated the T-cell-stimulatory activity of peptides within the sequence, and it is possible that they may represent T-cell epitopes. Synthetic peptide technology provides a powerful tool for epitope mapping and for the design of novel immunogens.

The ideal vaccine for the control of avian coccidiosis would be one in which broad-spectrum immunity is conferred with a single dose of antigen. *Eimeria* parasites, although host specific, nevertheless may possess proteins in the apical complex which are highly conserved (23) and which are necessary for host cell recognition and attachment. There is scope, therefore, for the targeting of this recognition process by vaccine technologists and for the eventual production of a broad-spectrum anti-*Eimeria* vaccine by these means.

Conclusions. Peptide vaccines have been shown to be capable of eliciting protective immunity in several diseases, including malaria, which, like the disease under discussion here, is caused by apicomplexan pathogens. The targeting of potential host cell recognition sites holds out the prospect of the development of vaccines capable of conferring broad-spectrum immunity. In our studies of antibody recognition of an *E. tenella* immunogenic sequence, we have shown that most regions of this sequence are recognized by antibodies from rabbits and chickens exposed to homologous and heterologous *Eimeria* antigens. The highly immunogenic nature of the tandem repeat region within these sequences indicates that the screening and identification of putative vaccine candidate molecules on the basis of their capacity to react with immune sera may not necessarily identify the most-relevant epitopes. Synthetic peptides are a powerful tool for analyzing the fine specificity and the quality of antibody responses and therefore are useful in the rational design of novel vaccines. The use of peptides as antigens and as immunogens is a valuable strategy in the development of vaccines for the control of apicomplexan parasites.

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