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Characterization and Immunoprotective Properties of a Monoclonal Antibody against the Major Oocyst Wall Protein of *Eimeria tenella*

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The oocyst wall of *Eimeria* spp. consists of a 10-nm-thick outer lipid layer and a 90-nm-thick inner layer of glycoprotein which has been described previously to be composed of a single major protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and ^{125}I labelling of oocyst wall fragments and of delipidated intact oocysts revealed a molecule of approximately 12 kDa as the major protein component of the oocyst wall of *Eimeria tenella*. An immunoglobulin M monoclonal antibody (C11B9F3) was produced against this 12-kDa oocyst wall protein sliced from a preparative SDS-polyacrylamide gel. Its reactivity by immunofluorescence against oocyst wall fragments and sporozoites or by immunoperoxidase assays of infected tissue sections was stage restricted to gametocytes and oocysts but pan-specific against all seven *Eimeria* spp. of chickens. Immunogold labelling revealed that the antibody bound to epitopes on the inner face of the oocyst wall. In chicks passively immunized with C11B9F3, oocyst output was significantly ($P < 0.01$) reduced by 42 to 54% after homologous *E. tenella* infection and by 35% after heterologous *Eimeria maxima* infection compared with that of control groups. The results demonstrate the presence of a highly conserved, low-molecular-weight antigen on the oocyst wall and the gametocytes of *Eimeria* spp. which is a candidate for inclusion in a pan-specific, transmission-blocking vaccine against avian coccidiosis.

A number of studies have reported the elaboration of monoclonal antibodies (MAbs) against different *Eimeria* spp. (2–4, 18, 22). Most of these have been generated against sporozoites and merozoites, although some which define antigens associated with the sexual stages of *Eimeria tenella* (10) and *Eimeria maxima* have been described (24). These MAbs vary in their degree of stage specificity and species specificity. Attempts to develop MAbs against the oocyst wall protein of *Eimeria* spp. have not been reported before, although MAbs which recognize an epitope present on the oocyst wall (12), are species specific, and have been used successfully in immunodiagnosis (16) have been produced against *Cryptosporidium parvum*. The initial objective of this study was to seek species-specific MAbs which recognized epitopes associated with the oocyst wall of *Eimeria* spp. and which might be useful for the identification of intact oocysts.

Oocysts are the most resistant stage of the eimerian life cycle. Earlier studies by Stotish et al. (19) showed that the wall consists of a 10-nm-thick outer lipid layer covering a 90-nm-thick inner layer of glycoprotein which produces a single band of 10-kDa molecular size on sodium dodecyl sulfate (SDS)-polyacrylamide gels. In this study, we have confirmed that the oocyst wall is composed of a single major protein which, in our hands, has a molecular size of approximately 12 kDa by SDS-polyacrylamide gel electrophoresis (PAGE). This report describes the characterization of a MAb produced against the 12-kDa protein of *E. tenella*. The MAb identified an antigen expressed only by gametocytes and oocysts but which was represented in all seven chicken *Eimeria* spp. In view of the interest in exploiting gametocyte antigens of *E. maxima* to provoke transmission-blocking immunity (23), we have investigated the protective properties of this MAb.

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MATERIALS AND METHODS

***Eimeria* spp.** *E. tenella* (Houghton) was originally supplied by M. E. Rose and has been maintained in this laboratory by serial passage in 5- to 10-day-old chicks in plastic film isolators since 1982. The Weybridge (W) strains of *Eimeria necatrix*, *Eimeria brunetti*, *Eimeria praecox*, and *Eimeria mitis* were kindly supplied by M. A. Taylor. *Eimeria maxima* and *Eimeria acervulina* were single oocyst-derived lines isolated from naturally infected chickens in Bangladesh (9).

Delipidation of oocysts. Sporulated oocysts of *E. tenella* were incubated with 2% sodium hypochlorite in 16% sodium chloride (Milton) for 20 min at room temperature. After centrifugation at 700 × *g* (3 min), the oocyst-rich scum was taken off and washed repeatedly in excess water by centrifugation at 1,600 × *g* (5 min each) to remove the hypochlorite. The oocysts were delipidated as described earlier (11, 19), with minor modification. Briefly, 2×10^6 sporulated oocysts of *E. tenella* were incubated in 2 ml of methanol-chloroform (1:2) at room temperature (10 min), or in 0.1 M KCl-methanol-chloroform (1:2:1) at 4°C (overnight), and then washed three times in sterile phosphate-buffered saline (PBS; pH 7.2) by centrifuging at 1,600 × *g* (5 min each).

Preparation of oocyst wall fragments. Oocyst wall fragments of *Eimeria* spp. were prepared by the method of Stotish et al. (19). Purified oocysts (10^7/ml) were suspended in PBS containing 0.003 M phenylmethylsulfonyl fluoride and disrupted with 425- to 600-μm-diameter glass beads in a Whirlimix (5 min). After separation from the glass beads, the suspension was centrifuged at 10,000 × *g* (10 min) at 4°C in a high-speed centrifuge (MSE Scientific Instruments, West Sussex, England) and washed twice in distilled water by centrifuging as described above. After five washes in 1.0 M sucrose solution (2,500 × *g*, 15 min each), the pellet was rewashed five times in distilled water (10,000 × *g*, 10 min each). The final pellet was stored in PBS containing 0.003 M phenylmethylsulfonyl fluoride at −20°C.

Extraction of oocyst wall protein. Oocyst wall protein was extracted from oocyst wall fragments by boiling (15 h) and from intact oocysts by incubation at room temperature (6 h) in 0.025 M dithiothreitol–8.0 M urea (19). After centrifugation (10,500 × *g*, 10 min), the supernatants were stored in aliquots at −20°C.

Labelling of oocyst or oocyst wall fragments by ^{125}I. Oocysts (2.5×10^6) or oocyst wall fragments (an equivalent amount) were labelled with ^{125}I (250 μCi of Na^{125}I; Amersham) by the iodogen method (7). Extracts of the labelled preparations were made as described above; the labelled oocysts were either disrupted and the oocyst walls were boiled, or the oocysts were incubated at room tem-

perature in the dithiothreitol–urea solution without breaking. The supernatants were collected after centrifuging at 10,500 × *g* (10 min). The extracts were analyzed by SDS-PAGE in a 12.5% gel under reducing conditions. The gel was dried under vacuum and then exposed to Fuji X-ray film at −70°C.

Production of MAbs. Mice were injected with the 12-kDa protein sliced from a SDS–12.5% polyacrylamide preparative gel. The 12-kDa band from the gel was turned to powder by repeated freezing and grinding, suspended in 300 to 500 μl of PBS to which an equal volume of complete or incomplete Freund's adjuvant was added, and agitated in a Whirlimix to make an emulsion. BALB/c mice were injected subcutaneously with 0.2 ml of antigen suspended in Freund's complete adjuvant at multiple sites. Three booster injections of antigens in Freund's incomplete adjuvant on days 14, 21, and 46 were given as before. A final booster intraperitoneal injection of 0.2 ml of antigen without adjuvant was given 3 days before fusion. The preparation of splenic lymphocyte suspensions and fusions with NS-1 myeloma cells were performed by standard techniques (5, 6). Secreting hybridomas were detected by an immunofluorescent antibody test (IFAT) against oocyst wall fragments (see below) and cloned by limiting dilutions. Isotyping of the antibody was performed with a commercial kit (Serotec Ltd., Kidlington, Oxford, United Kingdom) as described in the manufacturer's instruction. An immunoglobulin M (IgM) MAb raised against *Campylobacter* spp. (kindly provided by Marcel Hommel) was used as a control MAb. MAb culture supernatants were used neat unless otherwise stated.

IFAT. A two-step IFAT was carried out as described previously (21) on acetone-fixed purified oocyst wall fragments or sporozoites with a 1:200 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Sigma Chemical Co. Ltd., Poole, Dorset, England). In addition to the control MAb against *Campylobacter* spp., the NS-1 myeloma cell supernatants were used as a negative control.

Immunoblotting. Western blotting (immunoblotting) was performed by use of the Trans-Blot cell operating instructions (Bio-Rad Laboratories, Richmond, Calif.), with minor modifications, on proteins separated by SDS-PAGE under reducing conditions.

Immunoperoxidase staining. Immunoperoxidase staining of cecal tissue sections from chicks 5 and 7 days postinfection with 10^3 oocysts of *E. tenella* and *E. maxima* was done by the method of Jones et al. (8), with minor modification. Deparaffinized and rehydrated sections were treated with 1% H_2O_2 (30 min) to quench endogenous peroxidase, washed three times (15 min each) in 0.05 M Tris-buffered saline (pH 7.6), and then incubated (20 min) with 5% normal sheep serum. MAb culture supernatants were applied neat and incubated overnight at 4°C in a humid chamber. After washing (as described above), sections were covered with peroxidase-conjugated anti-mouse immunoglobulin (Sigma; 1:1,500 dilution in Tris-buffered saline) and incubated (90 min) in a moist chamber at room temperature. After washing as before, the sections were treated with 0.067% 3,3′-diaminobenzidine in 0.01 M Tris-HCl buffer (pH 7.6) containing 0.08% H_2O_2 for 3 to 5 min in the dark. Following a 5-min wash in running tap water, the sections were counterstained in Mayer's hematoxylin, blued in tap water, dehydrated in graded alcohol, and mounted in DPX mountant (BDH Ltd., Poole, Dorset, England) after clearing in xylene.

Immunogold electron microscopy. Sodium hypochlorite-treated washed oocysts of *E. tenella* (2×10^6) were disrupted in 0.5 ml of PBS by use of 425- to 600-μm-diameter glass beads. After two washes in PBS (each at 10,500 × *g*, 5 min), the oocyst wall pellet was incubated with 100 μl of neat MAb supernatants (1 h, room temperature). After two washes in PBS, the pellet was incubated (1 h) with 100 μl of gold-conjugated anti-mouse IgM (Zymed Laboratories; 1:20 in PBS). After two washes in PBS, 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) was trickled onto the pellet, and the pellet was left overnight at 4°C. The pellet was washed twice in 0.1 M cacodylate buffer and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (1 h, room temperature). After three washes in 0.1 M cacodylate buffer, the pellet was dehydrated in 70, 90, and 100% (three changes) ethanol (1 h each). The pellet was placed in propylene oxide for 30 min, propylene oxide-resin at the ratio of 1:1 for 2 h and at 1:2 overnight, and finally, in two changes (4 h each) of Epon-araldite resin and polymerized at 80°C (48 h). Ultrathin 130-nm sections were cut with a diatome diamond knife on a Sorwall MT 6000 ultramicrotome and examined with a Philips CM-10 transmission electron microscope after staining with 2% uranyl acetate for 20 min and Reynold's lead citrate for 2 min.

Passive immunization of chicks. Two-week-old Hysex cockerels were infected per os with 100 sporulated oocysts of *E. tenella* or *E. maxima*. The MAb supernatants (test and control), concentrated by centrifugal filtration at 3,000 × *g* over a 10-kDa cutoff filter (Centriprep-10 filter; Amicon Ltd., Stonehouse, Gloucestershire, England) to 550 μg of protein per ml, were inoculated intravenously into treated and control groups, respectively, at 0.3 ml per bird per day for 5 days from day 4 postinfection. A third group of birds served as uninfected sentinels for adventitious infection. Birds were caged individually, and the daily oocyst output per bird was counted from day 6 to 9 postinfection, a 4-day period (and 6 to 12 days postinfection in experiment 3 to ensure that counts did not recrudesce). Briefly, feces collected per 24-h period per bird were homogenized in a known volume of saturated salt solution (NaCl), and oocysts were counted after flotation in a McMaster counting chamber. Group mean oocyst counts were compared by Student's *t* test.

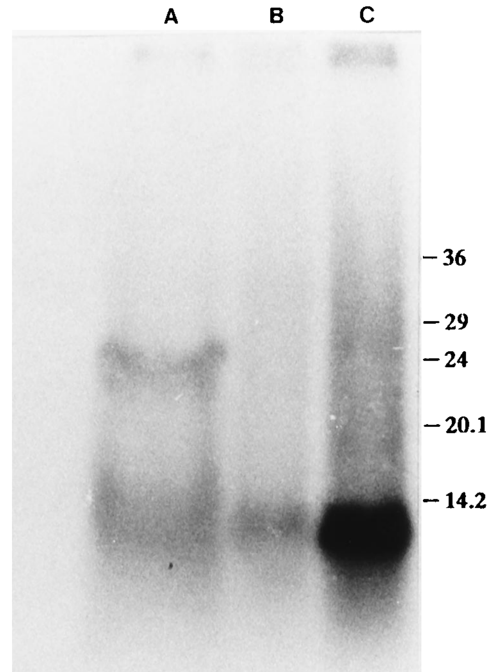

FIG. 1. Autoradiograph of ^{125}I-labelled *E. tenella* oocyst wall protein after separation by SDS-PAGE. (A) KCl-methanol-chloroform-delipidated oocysts, labelled intact and extracted at room temperature; (B) KCl-methanol-chloroform-delipidated oocysts, labelled intact and then broken and extracted by boiling; (C) oocyst wall fragments, labelled and extracted by boiling. Numbers on the right indicate molecular sizes in kilodaltons.

RESULTS

Extracts of oocyst wall fragments produced a single major protein band of approximately 12 kDa in Coomassie blue-stained gels (results not shown). The ^{125}I labelling experiments also revealed a single major protein component of 12 kDa, although an additional band at 24 kDa was seen in preparations from intact oocysts. The yield of protein was much greater from the labelled oocyst wall fragments than from oocysts labelled intact (Fig. 1).

Following fusion, one hybridoma producing antibodies against oocyst wall fragments of *E. tenella* was found (Fig. 2). The resulting MAb, designated C11B9F3, was of the IgM isotype and cross-reacted strongly in the IFAT with oocyst wall fragments of the other six chicken *Eimeria* species. However, it did not bind to sporozoites of *E. tenella*. Repeated attempts by IFAT to detect antibody binding to intact oocysts, with or without hypochlorite treatment or delipidated by a number of lipid solvents, were unsuccessful. No fluorescence was observed when oocyst wall fragments were incubated with the control MAb.

In tissue sections stained by the immunoperoxidase method, oocysts and macrogametocytes showed a dark-brown uniform staining and the wall-forming bodies were not apparently stained (Fig. 3). The staining was more intense in mature macrogametocytes and immature oocysts than in the early macrogametocytes and the microgametocytes. Schizonts and merozoites of *E. tenella* and *E. maxima* were not stained, but the antibody cross-reacted with oocysts and gametocytes of *E. maxima* (results not shown). No staining of any parasite stage was seen when sections were incubated with the control MAb.

Immunogold labelling of oocyst wall fragments of *E. tenella* revealed antibody binding to epitopes on the inner surface of

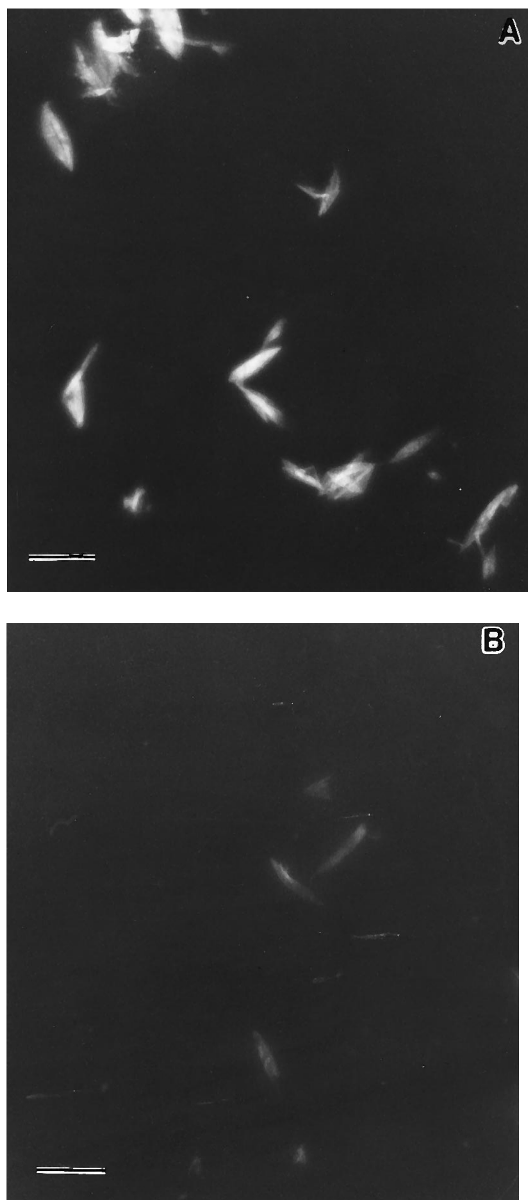

FIG. 2. Immunofluorescence staining of oocyst wall fragments of *E. tenella*. (A) Oocyst wall fragments incubated with a specific MAb, C11B9F3; (B) oocyst wall fragments incubated with control NS-1 supernatants. Bar, 10 μm.

the wall of the oocyst (Fig. 4). No binding of gold particles was seen when the oocyst wall fragments were incubated with the control MAb. However, MAb C11B9F3 failed to recognize any protein on immunoblotting of oocyst wall extracts, although polyclonal serum from the mouse which provided the spleen cells for fusion strongly recognized a 12-kDa band from the oocyst wall extract (results not shown).

In passive transfer experiments (Table 1), MAb C11B9F3 provided partial protection against homologous challenge manifest as a reduction ($P < 0.01$) of 40 to 54% in total oocyst output compared with that of the control groups in three experiments, despite a wide variation between individual counts within the same group. Moreover, a reduction ($P < 0.01$) in oocyst output was also conferred against heterologous infection with *E. maxima*. There was no evidence from daily oocyst output figures that treatment delayed oocyst excretion, and peak oocyst counts were observed for all cases either on day 7 or 8. In experiment 3, oocyst counts declined progressively from peak counts on day 7; 90% of the oocyst output on days 6 to 12 was excreted between days 6 to 9. No oocysts were detected in the feces of sentinel birds.

DISCUSSION

This report describes the development and characterization of a MAb against the major oocyst wall protein of *E. tenella*. The single protein band of approximately 12 kDa from oocyst wall preparations observed by SDS-PAGE under reducing conditions is in agreement with the findings of Stotish et al. (19), in which the authors identified a single major 10-kDa protein that was thought to constitute 70% of the total polypeptide present in the oocyst wall. The apparent difference in molecular size is presumably due to minor technical differences and error associated with measurement at these sizes on gels; in addition, Stotish et al. (19) noted that the polypeptide was somewhat heterogeneous in molecular size, possibly because of the random cleavage of smaller peptide fragments during solubilization. The additional band of 24 kDa in the preparations from the ^{125}I-labelled unbroken oocysts extracted at room temperature suggests that the protein is present as a dimer or multimer and the extreme solubilization procedures then reduce it to a single 12-kDa polypeptide. This is, to our knowledge, the first published surface labelling study of eimerian oocysts, and the simplicity of the protein profile is in marked contrast to that generated with *Cryptosporidium* sp. oocysts in which a complex array of proteins was revealed on reduced gels (20).

Iodogen labelling experiments demonstrated that the major oocyst wall protein could be labelled in intact oocysts which had been delipidated by treatment with KCl-methanol-chloroform but that the yield of proteins was much greater if the oocyst wall was broken up prior to labelling (Fig. 1). Therefore, this latter method was used to generate amounts of the 12-kDa protein in preparative gels to immunize mice. Despite the recognition of the 12-kDa band on Western blots by serum from the mouse immunized to generate it, the MAb C11B9F3 did not react in immunoblots, indicating the destruction of the epitope during the blotting procedure. The cross-reactivity of the MAb with oocyst wall fragments of other chicken *Eimeria* species indicates the presence of common epitopes among them and precludes its use for species identification. Moreover, we were unable to demonstrate binding of the MAb to intact oocysts, in spite of delipidation treatments which permitted ^{125}I labelling of the major oocyst wall protein in intact oocysts. This suggested that the target epitope(s) was located on the internal face of the protein layer, and ultrastructural immunolocalization studies confirmed this.

The immunocytochemical studies showed that MAb C11B9F3 reacted strongly and uniformly over the entire macrogametocyte and was not more concentrated over the wall-forming bodies. The less-intense staining of the mature oocyst is presumably due to the increasing impermeability of the oocyst wall as the oocyst matures. The results thus suggest the presence of common epitopes on the inner oocyst wall and the macrogametocyte, which are well conserved between all chicken *Eimeria* species.

Passive transfer of the MAb had a significant effect in protecting birds in terms of a reduction in the number of oocysts produced after infection with *E. tenella*. Reduction in total oocyst output of a similar magnitude was reported when chicks infected with *E. maxima* were injected with a MAb produced

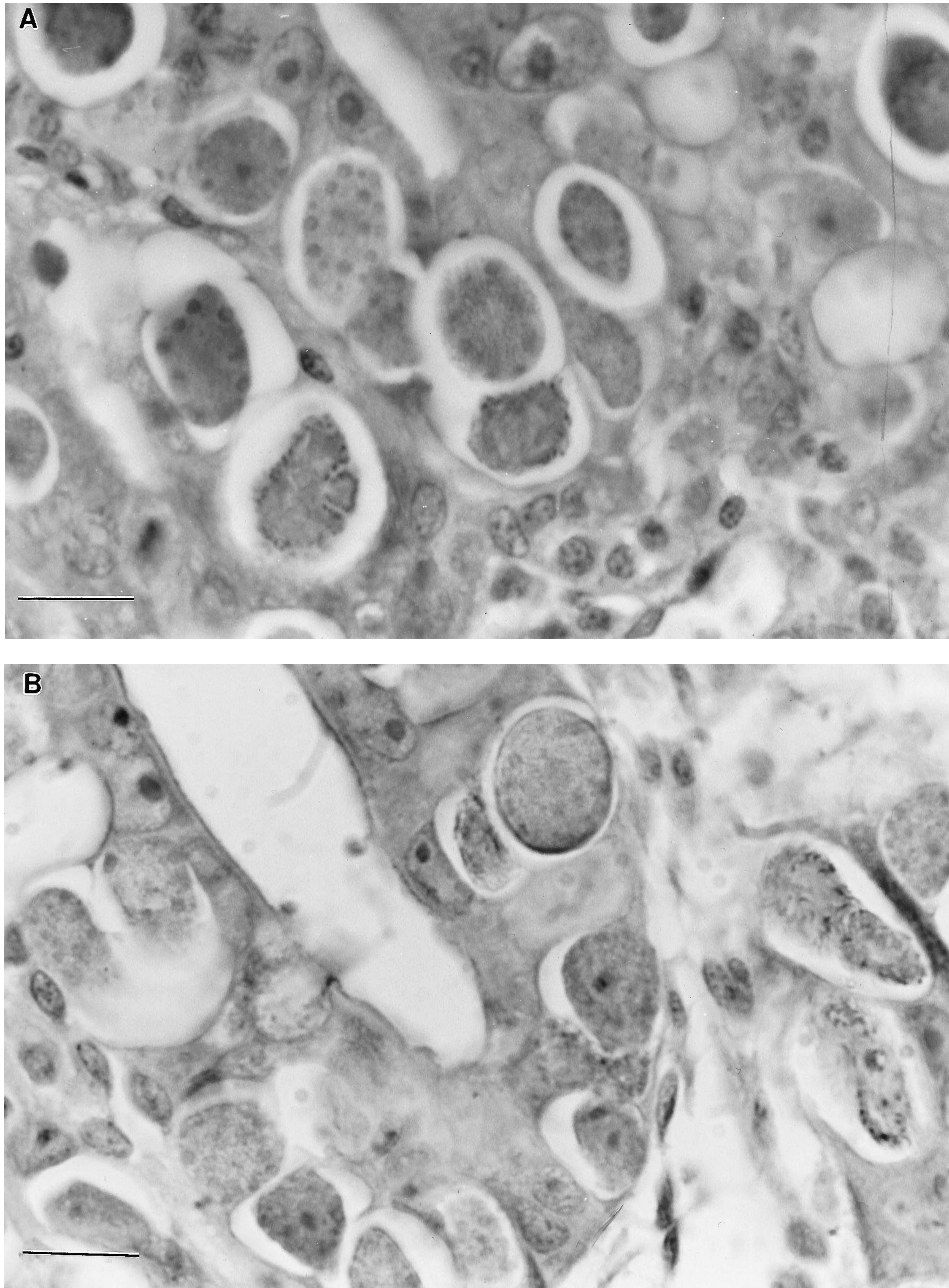

FIG. 3. Immunoperoxidase staining of *E. tenella* in sections of ceca 7 days postinfection with 10^3 oocysts. (A) Section incubated with the specific MAb C11B9F3; (B) section incubated with the control MAb. Bar, 20 μm.

against purified gametocyte antigens of *E. maxima* (24). Such antibodies would have no effect in reducing the pathogenic effects produced by the asexual stages of the parasites, but a reduction in oocyst output, were it to be achieved in farm situations, would reduce the degree of environmental contamination, which is an important factor in disease pathogenesis (25). The mechanism by which the antibody inhibits oocyst production is not understood. It may block an important re-

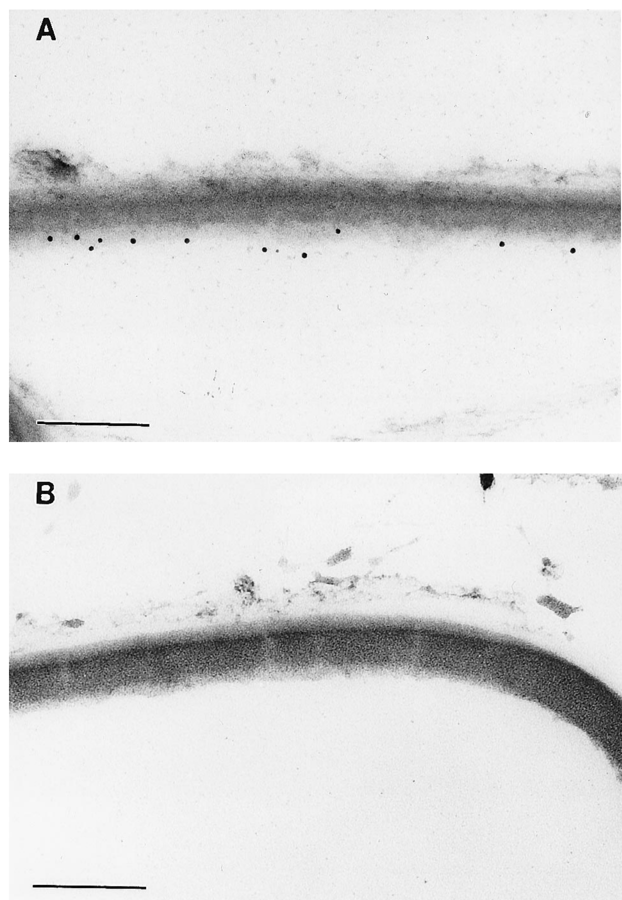

FIG. 4. Transmission electron micrograph of *E. tenella* oocyst wall fragments after immunogold labelling. (A) Section incubated with the specific MAb C11B9F3 showing deposition of gold particles on the inner surface of the oocyst wall; (B) section incubated with the control MAb. Bar, 0.2 μm.

ceptor present on the macrogametocyte surface, directly prevent the growth of the gametocyte (23) or inhibit fertilization (10).

Wallach and coworkers have demonstrated that MAbs directed against gametocyte antigens of *E. maxima* protect birds passively and that the relevant affinity-purified antigens could be used to immunize hens and, in so doing, reduce oocyst output in their chicks following homologous infection (23, 24). Accordingly, they have proposed this transmission-blocking immunization as one approach towards immunoprophylaxis of avian coccidiosis (25). *E. maxima* was chosen for such studies because earlier research had demonstrated some protection by serum transfer to birds with *E. maxima* but not with *E. tenella* (13, 14), although serum from birds after multiple infection with *E. tenella* does protect chick embryos (15). *E. tenella* is arguably the most important of all seven *Eimeria* species infecting chickens because of its pathogenicity and prevalence, and the current report indicates that antibody of very defined specificity directed against gametocyte or oocyst determinants can confer protection in *E. tenella*. Although, classically, immunity to *Eimeria* spp. in the fowl is described as species specific (22), there is evidence that there is a degree of heterologous species cross-protection conferred by infection (see reference 17) or recombinant antigens (1). It has recently been shown that the maternal transfer of antibodies induced against *E. maxima* by infection in breeding hens confers partial protection against *E. tenella* in their progeny, and this is associated with a high degree of cross-reactivity of maternally derived IgG to antigens of the two species in enzyme-linked immunosorbent assay and Western blot analysis (17). The current report adds further support to the possibility of inducing transmission-blocking immunity by the use of shared antigens to generate pan-specific protection, and it demonstrates one such conserved, low-molecular-weight antigen which is a potential vaccine candidate.

TABLE 1. Oocyst counts postinfection after passive immunization of chicks with MAb C11B9F3 against the oocyst wall protein of *E. tenella*[a]

Expt	Species	Group oocyst count (10^6)[b]		% Reduction	Level of significance (P)[c]
		C11B9F3	Control MAb		
1	*E. tenella*	5.20 ± 2.28	11.28 ± 1.78	54	<0.001
2	*E. tenella*	2.63 ± 0.93	4.52 ± 1.67	42	<0.01
3	*E. tenella*	10.11 ± 2.88	17.40 ± 5.35	42	<0.01
4	*E. maxima*	1.31 ± 0.26	2.01 ± 0.46	35	<0.01

[a] Chicks were infected with 10^2 oocysts per bird.
[b] Oocyst output per chick (mean ± standard error) on days 6 to 9 (inclusive) postinfection (except for experiment 3, which is the output on days 6 to 12 postinfection). Number of chicks per group, 10 (except 8 for experiment 1).
[c] Determined by Student's *t* test.

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