Monoclonal Antibody Produced against Partially Purified Sporozoite Antigen Inhibits Invasion of Cells by Sporozoites of Avian Eimeria Species (43296)

P. C. AUGUSTINE[1]

United States Department of Agriculture, Agricultural Research Service, Livestock and Poultry Sciences Institute, Beltsville, Maryland 20705

Abstract. **Previous studies showed that molecules of *in vitro*-cultured primary turkey kidney cells bound to 23-, 40-, and 60- to 65-kDa antigens of sodium dodecyl sulfate-solubilized sporozoites of *Eimeria adenoeides*. Similar binding to antigens of three other species of avian Eimeria, *E. tenella*, *E. acervulina*, and *E. meleagrimitis*, is now reported. Strips containing the most avidly bound sporozoite antigen (~40 kDa) were excised from the sodium dodecyl sulfate-polyacrylamide gels on which *E. adenoeides* antigens had been electrophoretically separated. The strips were homogenized and injected into mice to produce hybridoma cell lines. Twelve cell lines secreting monoclonal antibodies (McAb) that reacted with *E. adenoeides* sporozoites were detected. One of these McAb, H11C3, reacted with structures in the anterior tip of sporozoites of *E. adenoeides* and five other species of avian Eimeria. When included in the inoculation medium, this McAb significantly inhibited invasion of cultured kidney cells by sporozoites of *E. adenoeides* and *E. tenella*. In contrast, when the sporozoites were pretreated with McAb H11C3 and then washed free of the antibody, no inhibition of invasion was observed.**

[P.S.E.B.M. 1991, Vol 198]

Rigid site specificity for invasion is exhibited by the avian Eimeria in both natural host and foreign host birds (1, 2), suggesting that definitive interactions (recognition) between host cells and sporozoites are associated with the invasion process. In cell culture systems, however, sporozoites of all of the species of avian Eimeria that have been tested invade a number of different types of animal cells (3), suggesting that in addition to unique factors dictating site specificity for invasion by each species, there are also common factors in the mechanism of cellular invasion. The nature of these factors is unknown. Modification of the surface of cultured avian kidney cells by exposure to compounds having differing biological activities (enzymes, cations, lectins, etc.), or pretreatment of sporozoites with monoclonal antibodies (McAb) before inoculation into cultures, has been shown to significantly reduce the number of intracellular sporozoites (4). Because these compounds and McAb are relatively specific in their activities, it has been proposed that cellular invasion was inhibited by removal, blockade, or neutralization of specific sporozoite and host cell molecules (either structural or secreted) that mediate the invasion process. Recently, modified Western blot procedures identified sporozoite antigens having molecular masses of approximately 23, 40, and 60-65 kDa to which components of a homogenate of cultured kidney cells bound (5). Although conclusive evidence was not available at the time, it was speculated that these sporozoite antigens might constitute sporozoite receptor or recognition molecules for interaction with, and subsequent invasion of, the cultured host cells. The present study was conducted to explore this speculation, that is, to examine whether the sporozoite antigens that were bound by host cell molecules are involved in cellular invasion by avian Eimeria sporozoites.

[1] To whom requests for reprints should be addressed at Building 1040, Room 102, PDL, LPSI, BARC-East, USDA, ARS, Beltsville, MD 20705.

Received December 3, 1990. [P.S.E.B.M. 1991, Vol 198]
Accepted May 9, 1991.

0037-9727/91/1981-0606$3.00/0

Materials and Methods

Binding Assay. Sporozoites of *Eimeria adenoeides*, *E. tenella*, *E. meleagrimitis*, and *E. acervulina*

were subjected to the same electrophoresis, transblot, and binding assay procedures as reported earlier (5). Briefly, sporozoites were disrupted in sample buffer contained 2.3% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, and 5% (w/v) dithiothreitol in 62 m*M* Tris chloride buffer (pH 6.8). Samples containing 4–5 μg of sporozoite protein were applied to 0.75-mm gels (11% polyacrylamide, with an acrylamide to bisacrylamide ratio of 33:1) and subjected to SDS-polyacrylamide gel electrophoresis (Mighty Small II Vertical Slab Unit; Hoefer Scientific, San Francisco, CA) at 80 V for 3.5 hr at 4°C. Molecular weight standards (Biorad Laboratories, Richmond, CA), which included myosin (mol wt 200,000), phosphorylase *b* (mol wt 97,400), bovine serum albumin (mol wt 66,200), ovalbumin (mol wt 42,699), carbonic anhydrase (mol wt 31,000), soybean trypsin inhibitor (mol wt 21,500), and lysozyme (mol wt 14,400), were subjected to electrophoresis along with the sporozoite proteins. The separated proteins were transferred from the gel onto nitrocellulose sheets (Mini Transfer; Hoefer Scientific) at 200 mA for 4 hr with a Tris-glycine-SDS transfer buffer (pH 7.9) containing 20% methanol. The nitrocellulose strips were incubated sequentially in i) a homogenate of cultured turkey kidney (PTK) cells (5) or Tris-chloride buffer (pH 6.8) containing 10% fetal bovine serum and approximately 1 m*M* CHAPSO for 6 to 7 hr at 41°C, ii) rabbit anti-PTK serum or normal rabbit serum (1/200 in antibody buffer) for 4 hr at 22 ± 2°C room temperature, and iii) peroxidase-conjugated goat anti-rabbit IgG (H + L chain specific; ICN Immunobiologicals, Lisle, IL; 1/500 in antibody buffer) for 2 hr at room temperature. The color reaction was initiated with 4-chloro-1-naphthol and terminated with distilled water. The nitrocellulose strips were washed twice with PBS + Tween 20 between each step.

Production and Characterization of Monoclonal Antibodies. Aliquots of *E. adenoeides* sporozoites in sample buffer were subjected to SDS-polyacrylamide gel electrophoresis, as described above, except that 1.5-mm gels were used. Based on the migration of the standards, strips of gel containing proteins having molecular masses of approximately 35 to 45 kDa were excised. One such gel strip was applied to the top of a second gel, subjected to electrophoresis for a second time, and stained with Coomassie blue dye. Other similarly excised gel strips were washed in three changes of phosphate-buffered saline (pH 7.2), ground in a tissue grinder, and injected intravenously into mice on three occasions at 2-week intervals. A fourth intravenous injection, containing disrupted *E. adenoeides* sporozoites (approximately 30 μg of protein in phosphate-buffered saline) was given 1 week after the last injection of gel-derived protein. Three days later, the spleen cells from the immunized mice were fused with a mouse myeloma cell line (P3-X63-Ag8) (6). Resulting hybridoma cell lines were screened by indirect fluorescent antibody procedures on air-dried and methanol-fixed *E. adenoeides* sporozoites to identify cell lines producing antisporozoite McAb. Antibody-producing hybridomas were cloned by limiting dilution techniques (6) and expanded in Dulbecco's minimal essential medium containing 10% NCTC 109, 20% fetal bovine serum, 1% L-glutamine, and 1% antibiotic/antimycotic. The location of the antigens recognized by the McAb was determined by indirect fluorescent antibody on air-dried sporozoites of *E. adenoeides*; the cross-reactivity of the McAb was determined by indirect fluorescent antibody on sporozoites of *E. tenella*, *E. meleagrimitis*, *E. acervulina*, *E. maxima*, and *E. mivati*. The subclasses of selected McAb were determined using a mouse monoclonal subisotyping kit (HyClone Laboratories, Logan, UT). The molecular weights of the antigens recognized by the clones were determined by Western blot analysis on SDS-solubilized *E. adenoeides* sporozoites (5).

Cell Cultures. Primary turkey kidney cells were prepared as reported earlier (7). Cultures were grown in 24-well tissue culture plates on glass coverslips (12 mm^2) seeded with 1×10^4 cell clumps. The growth medium was Dulbecco's minimal essential medium containing 10% fetal bovine serum, 1% L-glutamine, and 1% antibiotic/antimycotic. Cultures were incubated in a 10% CO_2 and 90% air atmosphere, and used 3–4 days after they were prepared.

Quantitation of Invasion. Individual or pooled supernatant fluid (supernatants) from cloned hybridoma cell line H11C3 were tested for their ability to inhibit cellular invasion. Sporozoites of *E. tenella* and *E. adenoeides* were preincubated for 15 min at room temperature in culture supernatants that gave strongly positive reactions on air-dried sporozoites at a dilution of 1/64, and 2-, 7-, and 16-fold dilutions of the supernatants. Control sporozoites were preincubated in supernatants from cultures of P3-X63-Ag8 myeloma cells. The suspensions of sporozoites were inoculated into cell cultures along with the preincubation media, held at 41°C for 45 min, and fixed with 10% buffered formalin. In parallel studies, sporozoites were similarly preincubated in culture supernatant containing H11C3 or in P3X control supernatant. A portion of the sporozoites from each treatment group was inoculated onto cultures at the end of the preincubation period, as described above; the remaining sporozoites in each treatment group were washed free of the preincubation media (two washes in Dulbecco's minimal essential medium) and resuspended in culture medium containing no antibody. These sporozoites were held at 4°C for 16 hr and then inoculated into cultures. In each experiment, from 2.9 to 3.5×10^5 sporozoites were inoculated into each cell culture. Culture confluency (percentage of the coverslip occupied by cells) and the total

number of intracellular sporozoites in 20 equally spaced microscopic fields at ×650 magnification were determined (8). Invasion was expressed as the number of intracellular sporozoites per 10 mm^2 cells at ×650 magnification (8). The data were subjected to analysis of variance and Duncan's multiple range procedures and were expressed as the means ± SE.

Results

Binding Assay. Binding of PTK cell components to antigens of sporozoites of *E. tenella*, *E. meleagrimitis*, and *E. acervulina* was similar to that reported earlier for *E. adenoeides* (5) (Fig. 1). For each species, the most intense binding was to an approximately 40-kDa band, with lighter binding to antigens of molecular masses of approximately 23 and 65–69 kDa. Evidence of binding to higher molecular masses antigens (>100 kDa) was observed occasionally, but because similar binding was sometimes seen on control strips, it was considered to be, at least in part, nonspecific, and was not examined further at this time.

Monoclonal Antibodies. Strips excised from gels yielded a partially purified sample of sporozoite antigens having molecular masses of approximately 35 to 45 kDa (Fig. 2). From mice immunized with these antigens, almost 300 hybridomas were produced, 12 of which secreted antibodies that reacted with refractile bodies, surface, or structures in the anterior tip of air-dried and methanol-fixed *E. adenoeides* sporozoites.

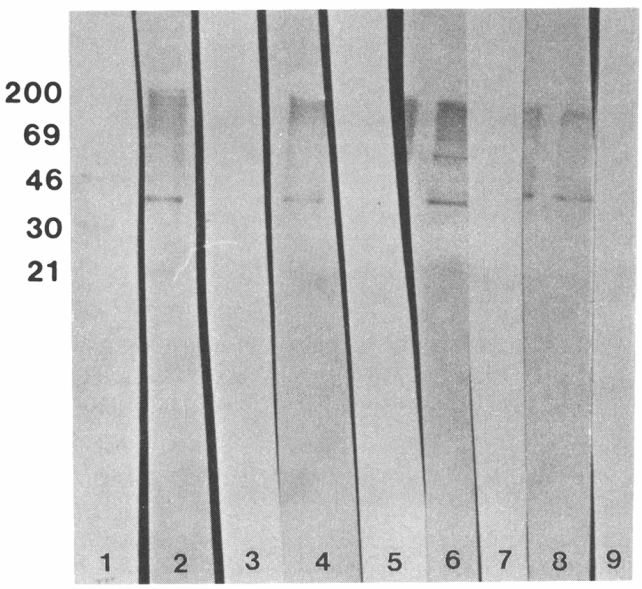

Figure 1. Modified Western blot analysis of sporozoites of *E. acervulina* (Lane 2), *E. tenella* (Lane 4), *E. adenoeides* (Lane 6), and *E. meleagrimitis* (Lane 8) showing binding of host cell homogenate to sporozoite antigens. Lane 1 contains molecular mass standards, and lanes 3, 5, 7, and 9 are control strips for each species of Eimeria. The sporozoite antigens were electrophoretically separated and exposed to cell homogenate or Tris buffer (controls) and then to rabbit anticell membrane serum. Binding of cell homogenate was detected with peroxidase-conjugated antirabbit IgG.

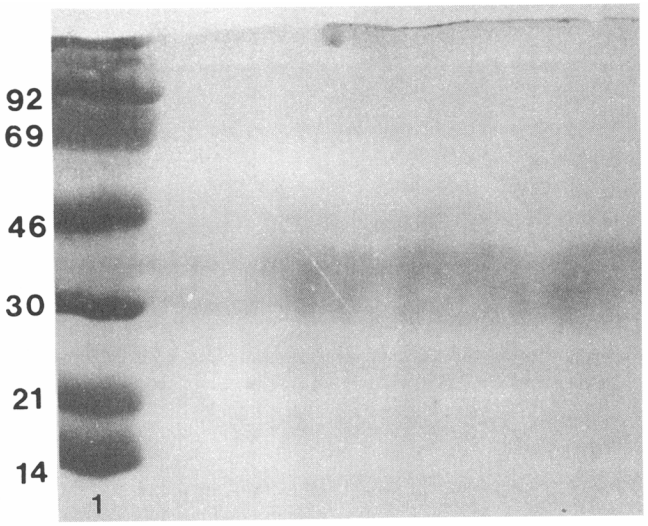

Figure 2. Preparative SDS polyacrylamide gel stained with Coomassie blue dye. The antigen applied to the gel and electrophoretically separated was a strip excised from a second gel containing sporozoite antigens of approximately 35 to 45 kDa. Lane 1 contains molecular mass standards.

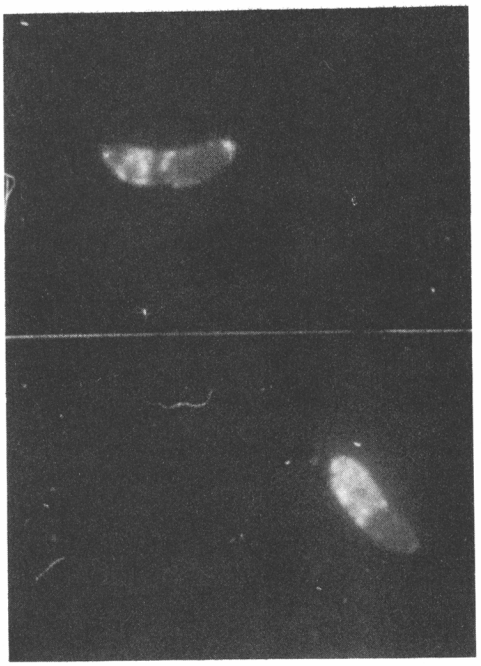

Figure 3. Indirect immunofluorescent antibody reaction of McAb H11C3 on air-dried sporozoites of *E. adenoeides*. The sporozoites were exposed to the McAb and then to fluorescein-conjugated rabbit antimouse IgG (original magnification ×800).

One of the tip McAb, H11C3 (Fig. 3), belonged to class IgG1, and cross-reacted with sporozoites of *E. tenella*, *E. meleagrimitis*, *E. acervulina*, *E. maxima*, and *E. mivati*. Western blots of *E. adenoeides* sporozoites using McAb H11C3 showed slight reactivity in the area of 40-kDa antigens and stronger reactivity with antigens having molecular masses of approximately 28 kDa and

between 60 and 100 kDa (Fig. 4). No reactivity was observed on control strips.

Invasion. When sporozoites of *E. adenoeides* or *E. tenella* were preincubated in culture supernatants containing McAb H11C3 (titer > 1/64) and inoculated, along with the incubation medium, into cell cultures, there was a significant inhibition of cellular invasion (Table I). Preincubation of sporozoites in the culture supernatants diluted by 1/2 or 1/7 also significantly reduced the number of intracellular sporozoites, but to a lesser degree (Table I, Experiment 3), whereas incubation in supernatants diluted by 1/16 had little inhibitory effect (Table I, Experiment 4). When *E. tenella* sporozoites were preincubated in McAb H11C3, washed free of the antibody, held overnight at 4°C, and inoculated into cell cultures in the absence of the McAb, there was no inhibitory effect on invasion (Table I, Experiment 5b). The McAb produced no significant changes in the confluency or gross morphology of the PTK cell cultures at the light microscope level.

Discussion

Using the assay developed previously for *E. adenoeides* (5), it was shown that components of a homogenate of PTK cells bound to antigens of sporozoites of *E. tenella*, *E. meleagrimitis*, and *E. acervulina* having the same molecular weights as those bound in sporozoites of *E. adenoeides*. In each species, the most intense binding of host cell components was to the 40-kDa antigen, and lesser binding was to antigens having molecular masses of 23 kDa and 60–65 kDa.

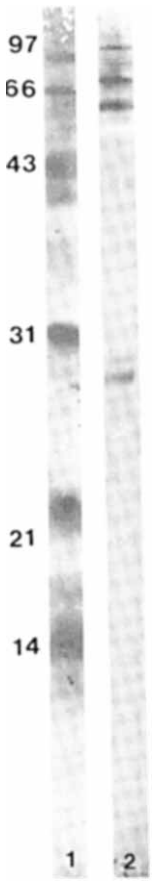

Figure 4. Western blot analysis of SDS-solubilized *E. adenoeides* sporozoites that were probed with McAb H11C3 and then exposed to peroxidase-conjugated rabbit antimouse IgG (Lane 2). Lane 1 contains molecular mass standards.

Because the 40-kDa sporozoite antigen appeared to bind the most host cell material, it was selected for producing McAb. Injections of gel shown to contain primarily sporozoite antigens in the 35- to 45-kDa range, followed by a single injection of homogenized sporozoites, gave rise to 12 hybridoma cell lines that secreted antibodies reacting with structures of air-dried sporozoites. Several of the McAb reacted with the refractile bodies, a finding that was not unexpected because refractile body antigens having molecular masses of approximately 43 kDa have been demonstrated (9). Two of the McAb recognized granules in the anterior tip of the sporozoites; one of these reacted exclusively with *E. adenoeides* sporozoites and the other, McAb H11C3, cross-reacted with air-dried sporozoites of five other species of avian Eimeria. The same distinct reaction with anterior granules and slight reactivity with the sporozoite surface were observed in all six species. Western blots probed with McAb H11C3 were somewhat of an enigma. Although three of the four injections of antigen which lead to the production of this McAb contained primarily antigens having molecular masses between 35 kDa and 45 kDa, only slight reactivity was seen on Western blots with antigens in this range. The reason for this is unknown. Loss of native configuration or binding capacity, with accompanying loss of recognition by appropriate antibodies, has been observed after SDS treatment of some antigens (10, 11). Moreover, extended exposure to methanol during electrophoresis has been shown to destroy the ability of some antigens of Eimeria sporozoites to react on immunoblots (9). Either of these phenomena could have caused the lack of reactivity of H11C3 with antigens in the 40-kDa range. That the McAb reacted with antigens well outside of the molecular mass range of the injected antigens may be explained by the presence of immunodominant epitopes, in low concentration in antigens in the 40-kDa range and in greater concentrations in antigens outside that range. Because the cell line producing McAb H11C3 was cloned by limited dilution techniques, the reactivity outside of the 40-kDa range was probably not caused by the presence of more than one antibody species.

Sporozoites of the avian Eimeria invade the same cell types *in vitro* (3); therefore, it is not unreasonable to speculate that some elements of the invasion process are similar among the species. Moreover, invasion is initiated by contact of the anterior tip of the sporozoite

Table I. Invasion of Cultured Primary Turkey Kidney Cells by Sporozoites of *Eimeria tenella* and *E. adenoeides*

Experiment	McAb (dilution)	n^b	Intracellular sz/10 mm² cells[c]	Percentage of inhibition of invasion[d]
1	0	6	36.05 ± 3.34	
	H11C3	6	1.12 ± 0.17*	97
2	0	6	51.43 ± 3.87	
	H11C3	6	0.47 ± 0.10*	99
3	0	4	23.02 ± 3.20	
	H11C3 (1/2)	4	8.29 ± 1.11*	64
	H11C3 (1/7)	4	9.75 ± 0.92*	58
4	0	6	52.52 ± 6.10	
	H11C3 (1/2)	6	13.80 ± 1.51*	74
	H11C3 (1/16)	6	45.48 ± 4.77	13
5a	0	5	11.04 ± 1.43	
	H11C3 (1/2)	5	1.05 ± 0.51*	90
5b	0	5	11.11 ± 0.62	
	H11C3 (1/2)	5	11.50 ± 0.61	—

[a] Table represents invasion of cultured primary turkey kidney cells by sporozoites (sz) of *Eimeria tenella* (Experiments 1, 3, 4, and 5) and *E. adenoeides* (Experiment 2) that were pretreated for 15 min with McAb H11C3 and inoculated into the cultures along with the preincubation media or washed free of the antibody (Experiment 5b).

[b] Number of coverslips quantified.

[c] Data are expressed as mean ± SE; means followed by asterisks are significantly different from the control mean of the parameter in that experiment.

[d] The percentage of inhibition was calculated using the following equation:

$$\% \text{ inhibition} = 100 - \frac{\text{no. sz/10 mm}^2 \text{ cells with antibody} \times 100}{\text{no. sz/10 mm}^2 \text{ cells without antibody}}$$

with the host cell membrane (12). Based on these observations, McAb H11C3 was selected for studies on cellular invasion because (i) it reacted with six species of avian Eimeria and (ii) it recognized antigens in the anterior tip of the sporozoite. Although the McAb was originally produced against *E. adenoeides*, because of the availability of parasites, most of the invasion studies were conducted with *E. tenella* sporozoites. When sporozoites of either *E. adenoeides* or *E. tenella* were preincubated in supernatants containing a high titer of McAb H11C3 and then inoculated into cell cultures along with the McAb, their ability to invade cells was inhibited by 97–99%, as compared with controls. As the H11C3 supernatants were diluted by factors of 2, 7, and 16, the inhibition of invasion decreased proportionally. Although the nature of the inhibitory action is not known at this time, it clearly is not lethal toxicity. Phase contrast examination of cell cultures immediately before they were fixed showed no decrease in refractility or flexing and gliding motions among the antibody-treated sporozoites, as compared with the control sporozoites. Moreover, when suspensions of *E. tenella* sporozoites were preincubated in McAb H11C3, washed free of the antibody, and held overnight in antibody-free medium, they invaded cultured cells as effectively as did control sporozoites similarly treated with medium from P3X cultures. As mentioned earlier, it has been hypothesized that there are interactions between the sporozoites and host cells that are involved in cellular invasion by the avian Eimeria, and that specific recognition sites or molecules may modulate the process (4). The data presented here suggest that a 40-kDa sporozoite antigen, recognized by McAb H11C3, may contribute to this interaction.

The author gratefully acknowledges the excellent technical assistance of Michael A. Hickey.

1. Joyner LP. Host and site specificity. In: Long PL, Ed. The Biology of the Coccidia. Baltimore: University Park Press, pp35–62, 1982.
2. Augustine PC, Danforth HD. Avian *Eimeria*: Invasion in foreign host birds and generation of partial immunity against coccidiosis. Avian Dis **34**:196–202, 1990.
3. Doran DJ. Cultivation of coccidia in avian embryos and cell cultures. In: Hammond DM, Long PL, Eds. The Coccidia: *Eimeria, Isospora, Toxoplasma*, and Related Genera. Baltimore: University Park Press, pp183–252, 1973.
4. Augustine PC. The *Eimeria*: Cellular invasion and host-cell parasite interactions. In: Yvore P, Ed. Coccidia and Intestinal Coccidiomorphs. Tours, France: INRA Publications, pp205–215, 1989.
5. Augustine PC. Molecular interactions of cultured turkey kidney cells with specific antigens of *Eimeria adenoeides* sporozoites. Proc Soc Exp Biol Med **191**:30–36, 1989.
6. Danforth HD. Development of hybridoma-produced antibodies directed against *Eimeria tenella* and *E. mitis*. J Parasitol **68**:392–397, 1982.
7. Augustine PC, Doran DJ. Development of *Eimeria meleagrimitis* Tyzzer from sporozoites and merozoites in turkey kidney cell cultures. J Protozool **25**:82–86, 1978.
8. Augustine PC. Effect of polyions, Ca^{++}, and enzymes on penetration of cultured cells by Eimeria meleagrimitis sporozoites. J

Parasitol **66**:498–501, 1980.
9. Augustine PC, Danforth HD, McAndrew SJ. Monoclonal antibodies reveal antigenic differences in refractile bodies of avian *Eimeria* sporozoites. J Parasitol **74**:653–659, 1988.
10. Tsang CW, Peralta JM, Simons AR. Enzyme-linked immunoelectrotransfer blot techniques (EITB) for studying the specificities of antigens and antibodies separated by gel electrophoresis. In: Colowick SP, Kaplan NO, Eds. Methods in Enzymology, New York, London: Academic Press, Vol **92**: pp377–391, 1983.
11. Bers G, Garfin D. Protein and nucleic acid blotting and immunobiochemical detection. Biotechniques **3**:276–288, 1984.
12. Long PL, Speer CA. Invasion of host cells by coccidia. In: Taylor AER, Muller R, Eds. Parasite Invasion. London: Blackwell Scientific Publications, pp1–16, 1977.