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Ultrastructural observations of development of *Eimeria tenella* in a novel established avian-derived cell line

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Abstract Transmission electron microscopy was used to study the in vitro development of *Eimeria tenella* in a novel established avian-derived cell line (designated CEV-1/F7) used for antigen production in chicken immunization studies. Sporozoites of *E. tenella* were inoculated onto cell monolayers and the cells were fixed at 24-h intervals. Large numbers of intracellular sporozoites were seen at 24 h postinoculation (p.i.), and trophozoites were identified at 24–48 h p.i. Immature schizonts, some with budding merozoites, were seen by 48 h p.i. At 72–96 h p.i., immature and mature schizonts and extracellular merozoites were observed. No merozoite invasion occurred, but immature second-generation schizogony was seen in parasitophorous vacuoles of first-generation schizonts. No further development occurred and degeneration of most schizonts was seen by 120–144 h p.i. The results confirmed synchronous development of *E. tenella* until 48 h p.i., followed by asynchronous development and ultrastructural degeneration with increased incubation time.

Introduction

The increased drug resistance that avian coccidia have exhibited to anticoccidial compounds has led to numerous attempts to produce a coccidial vaccine for use in the poultry industry (see review by Danforth et al. 1993). These vaccine approaches have used wild-type and attenuated *Eimeria* species, precocious strains, and recombinant antigens to elicit protective immunity (Reid 1975; Shirley and Millard 1986; Lee 1987; Danforth et al. 1993). A recent report (Clare et al. 1993) has described the use of a novel avian-derived cell line that is highly permissive to invasion and development of *Eimeria tenella* to produce cell-culture antigens for use in bird vaccination trials. Light microscopy studies identified the development through first-generation schizogony in this cell line but did not determine if further coccidial development occurred. Also, it was not known if aberrant development or degeneration of the parasite was occurring during the time the medium was collected as a source of antigen. The lack of knowledge about parasite development in this cell line could have an important bearing on the time at which the antigen should be collected after coccidial inoculation to insure that the proper immune response was elicited in chickens. In this study, we report on the in vitro ultrastructural development of *E. tenella* within this cell line.

Materials and methods

Parasite source and preparation

Oocysts of *Eimeria tenella* (strain LS 65) were produced by routine passage through Peterson Arbor Acre broilers. Parasites were sporulated and cleaned of fecal material, and a sporozoite inoculum was obtained by use of a previously described technique (Dulski and Turner 1988) that was modified by Clare et al. (1993).

In vitro development

The cell line used in this study was originally derived from a tissue mass in the intestine of SPAFAS (COFAL-24) 20-day-old

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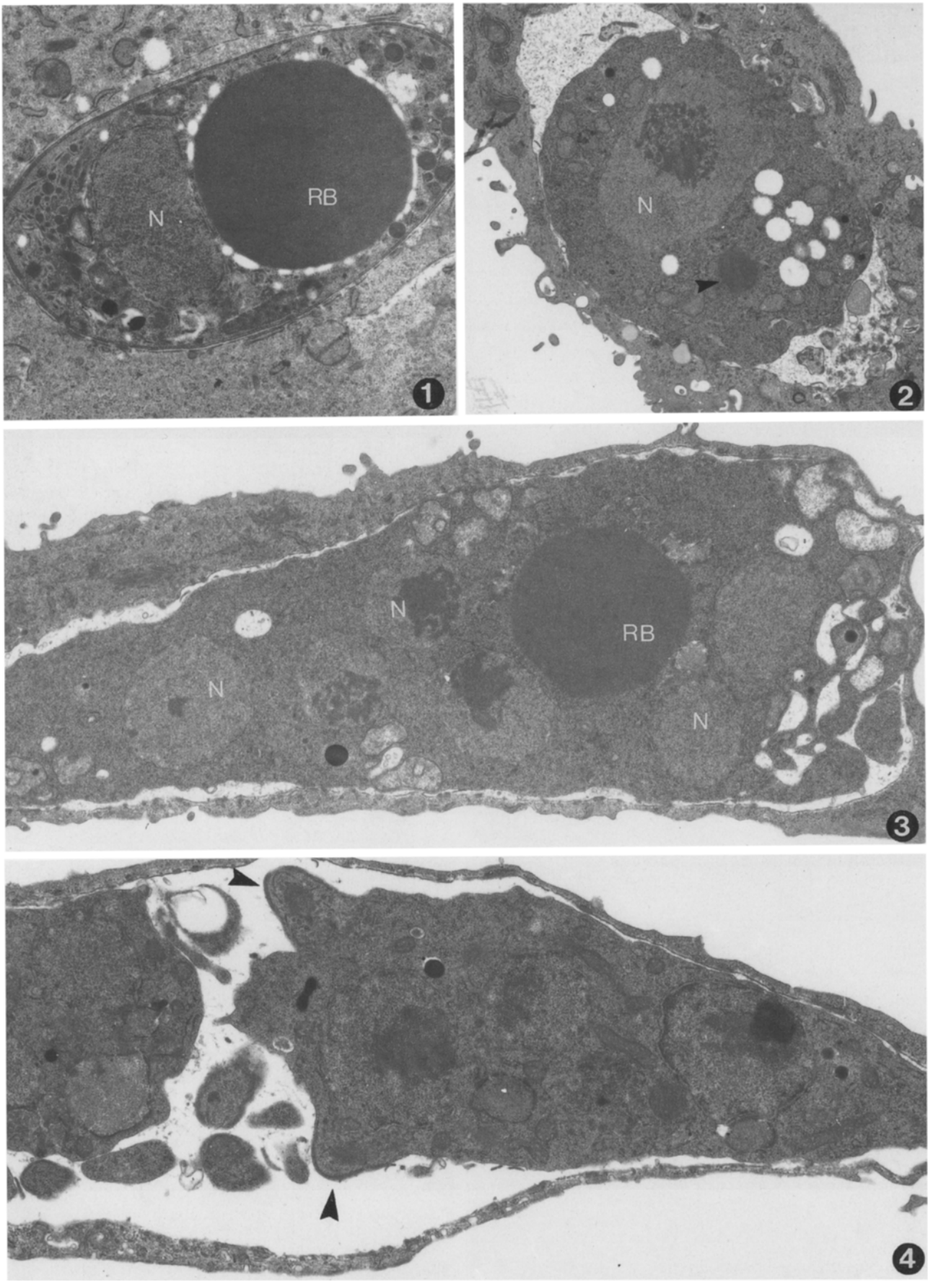
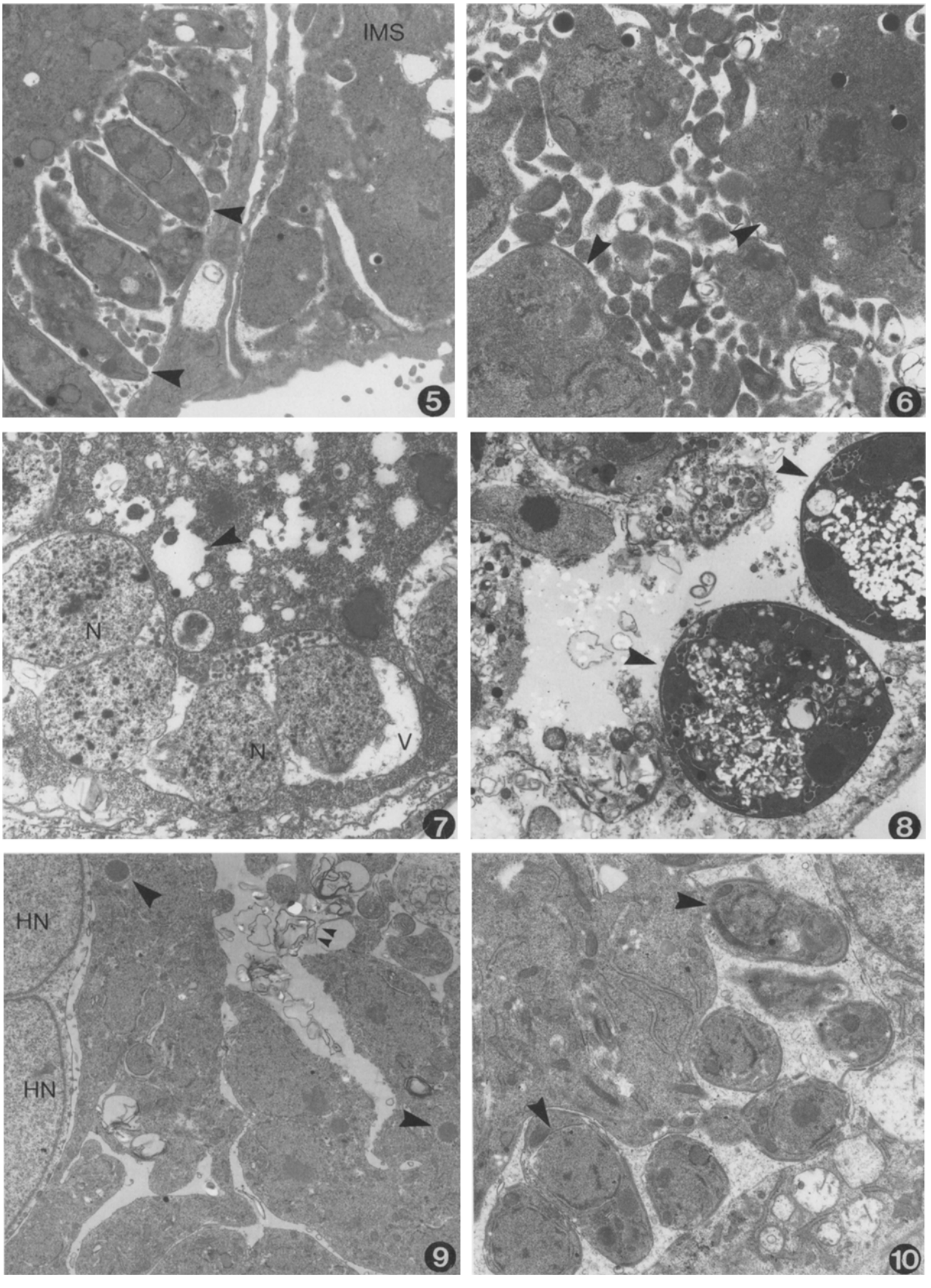
Fig. 1 Transmission electron micrograph of an intracellular sporozoite of *Eimeria tenella* at 24 h p.i. onto CEV-1/F7 cells. Note the prominent nucleus (*N*) and the large refractile body (*RB*). ×12,500. **Fig. 2** Trophozoite seen at 48 h p.i. within a well-defined parasitophorous vacuole. Note the presence of refractile body material (*arrowhead*) and the large nucleus (*N*) with a nucleolus. ×8,700. **Fig. 3** Immature meront seen at 48 h p.i. with numerous nuclei (*N*) and a large refractile body (*RB*). ×11,000. **Fig. 4** Immature schizont seen at 48 h p.i. with budding merozoites (*arrowheads*). ×10,000



Figs. 1–4



Figs. 5–10

chick embryo, which, following crisis, transformed from an epithelial to a fibroblastic morphology. These cells have subsequently been subcultured through over 100 passages. One clone of this cell line (designated CEV-1/F7, ATCC number CRL10495) was used to seed tissue-culture flasks (25 cm^2) at a density of 1.0×10^5 cells/ml suspended in Medium 199 with 1% fetal calf serum. After an overnight incubation at 40.5° C in an atmosphere containing 5% CO_2 that allowed for a 50% confluency of cells, 3 ml of medium containing approximately 1.0×10^6 excysted sporozoites/ml was added to the flasks. At 4 h post-inoculation, fresh medium was placed in the flasks. Infected cells were fixed at 24-h intervals (24–144 h) for 2 h in 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer and washed twice in 0.1 *M* cacodylate buffer. A rubber policeman was used to remove the cells gently from the tissue flasks. The cells were transferred to 1.5-ml ultracentrifuge tubes, postfixed for 1 h in 2% OsO_4 in 0.1 *M* cacodylate buffer, and stored in the same buffer at 4° C. All specimens from each incubation period were then dehydrated, prestained in 0.1% uranyl acetate, and embedded as previously described (Barta et al. 1987; Danforth and Anderson 1989). Tissue was sectioned, stained with uranyl acetate and lead citrate, and viewed in a Philips 201C electron microscope.

Results

Intracellular sporozoites were found in large numbers in the CEV cells at 24 h postinoculation (p.i.). The ultrastructure of these sporozoites was nearly identical to that described by numerous authors (see review by Chobotar and Scholtyseck 1982) and will not be repeated in this paper (Fig. 1). Synchronous development of *Eimeria tenella* was seen in the CEV-1/F7 cells at 24–48 h p.i. Trophozoite stages within a defined parasitophorous vacuole (PV) were present at 24–48 h p.i. and were characterized by a large, single nucleus with a prominent nucleolus and a single refractile body (Fig. 2). Immature first-generation schizonts that contained multiple nuclei and, in most specimens, a large, single refractile body, were first seen at 48 h p.i. (Fig. 3). Approximately 20% of these schizonts had budding merozoites, most of which contained a conoid and rhoptry anlagen (Fig. 4).

A more asynchronous type of parasite development coupled with a degeneration of first- and second-generation schizonts was seen in the CEV cells at 72–96 h p.i. Immature schizonts, some of which were budding merozoites, and mature first-generation schizonts were found in large numbers by 72 h p.i. (Fig. 5). In approximately 20% of the mature first-generation schizonts, the merozoites were rounded in appearance, had undergone nuclear division, and were initiating a second-generation schizogony within the original first-generation schizont PV (Fig. 6). A few (approximately 5%) of the first-generation schizonts were undergoing ultrastructural degeneration by 72 h p.i. (Fig. 7). In these specimens, vacuolization occurred around the nuclei and in the cytoplasm of the schizont.

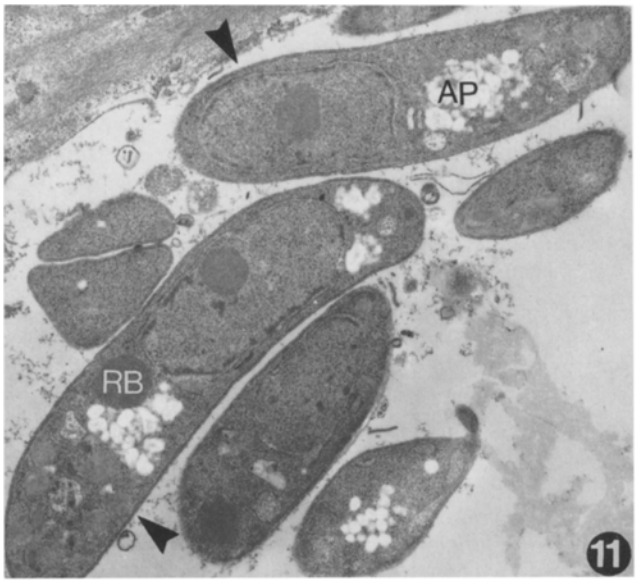
At 96 h p.i., degeneration was seen in most (approximately 90%) of the second-generation schizonts within the first-generation schizont PV (Fig. 8). These second-generation schizonts showed two types of degeneration that could occur within the same PV: (1) a loss of plasma membrane coupled with a complete loss of schizont ultrastructure or (2) a condensing of the schizont cytoplasm that contained large numbers of amylopectin granules; pycnotic nuclei were present within these condensed specimens. Most (approximately 80%) of the large, immature first-generation schizonts were also undergoing ultrastructual degeneration at 96 h p.i. (Fig. 9). Membranous swirls were seen in the cytoplasm of these schizonts, the PV membrane was disrupted, and no merozoite was budding from the membrane-bound invaginations. Apparently normal schizonts with budding merozoites and large numbers of extracellular merozoites were also present at 96 h p.i. (Figs. 10, 11). None of these extracellular merozoites had invaded host cells.

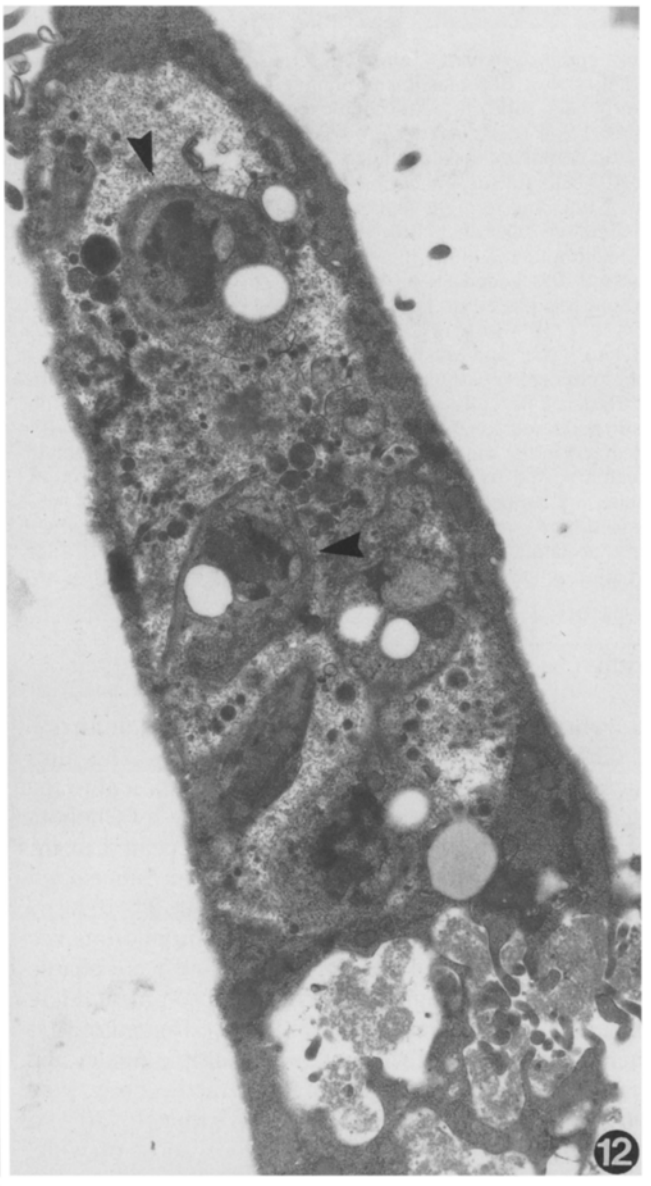
Most (approximately 90%) of the schizonts seen at 120–144 h p.i. were in some form of continued degeneration, although a few nondegenerating mature first-generation schizonts and extracellular merozoites were observed during this period. Some of the smaller mature schizonts, which had not undergone degeneration at 96 h p.i., contained merozoites that were vacuolated and were degenerating within the PV (Fig. 12). In other CEV cells, remnants of small meronts that contained a few condensed nuclei but lacked a PV were seen (Fig. 13). Larger immature meronts had numerous nuclei but little cytoplasm, if any (Fig. 14), and some specimens showed a complete degeneration of the meront with scattering of nuclei among the vacuolated cytoplasm and membrane ghosts (Fig. 15). No further development of the parasite was seen during this period.

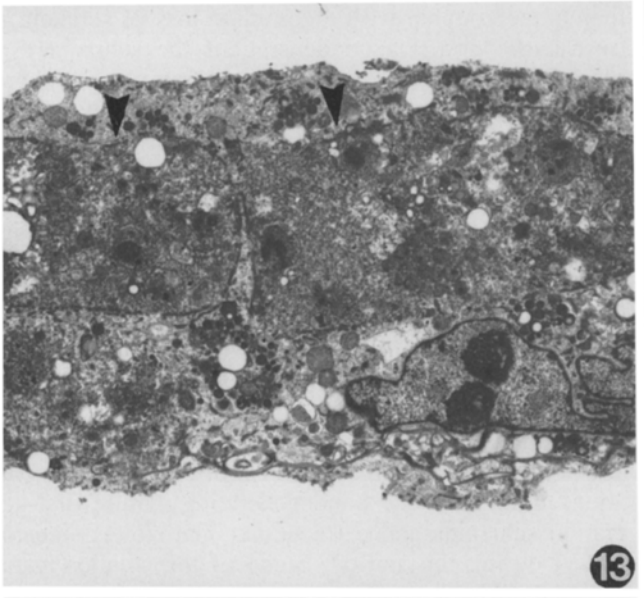
Discussion

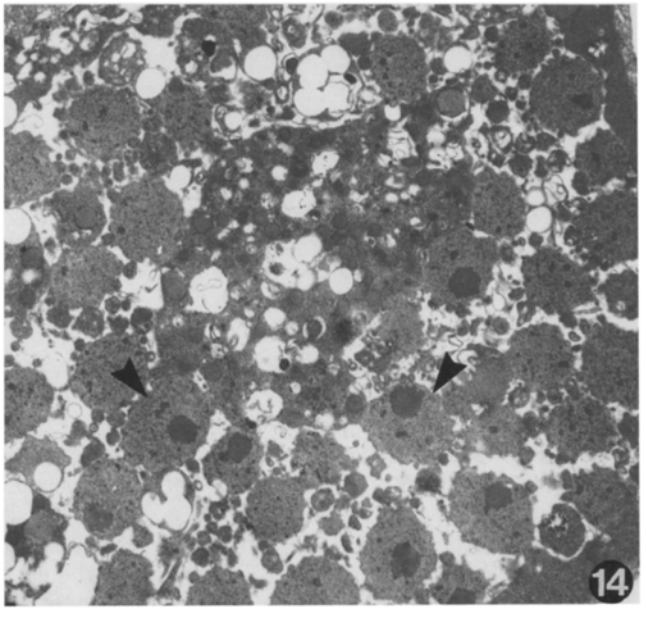
An important parameter in successfully propagating *Eimeria* species in vitro is the choice of the host cell. It is well documented that *E. tenella* will grow in a number of primary and established cell lines (see review by Doran 1982; Strout and Schmatz 1990), but until the development of the CEV cell lines, no attempt had been made to grow this species in an established avian-derived cell

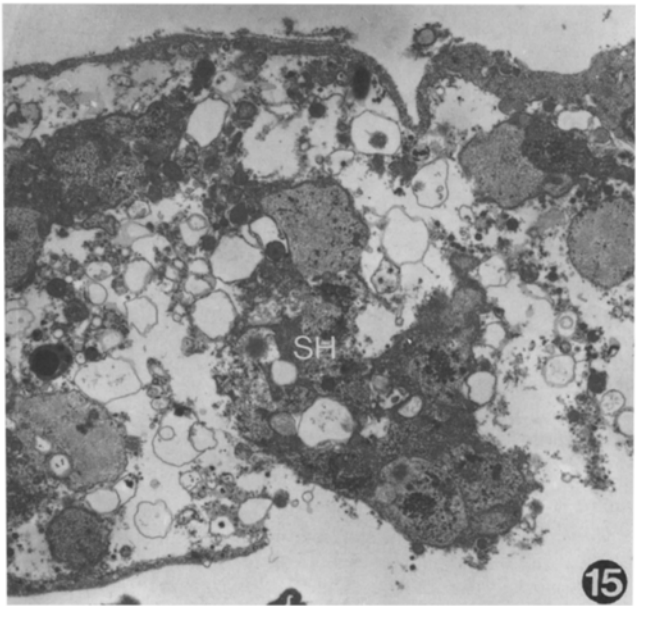
Fig. 5 Budding and immature schizonts (*IMS*) of *E. tenella* seen at 72 h p.i. Note the budding merozoites (*arrowheads*). ×6,800. **Fig. 6** Second-generation schizogony within the parasitophorous vacuole of first-generation schizonts seen at 72 h p.i. Note the second-generation schizonts derived from first-generation merozoites that have rounded up (*arrowheads*) and are attempting to bud off merozoites. ×12,000. **Fig. 7** Degenerating first-generation schizont seen at 72 h p.i. Note the vacuolization (*V*) around the nuclei and within the schizont cytoplasm (*arrowhead*). ×12,000. **Fig. 8** Degenerating second-generation schizonts (*arrowheads*) within the parasitophorous vacuole of a first-generation schizont, seen at 96 h p.i. Note that degenerating schizonts have amylopectin granules and condensed nuclei, whereas other degenerating first-generation merozoites have not undergone merogony. ×6,000. **Fig. 9** Degenerating immature first-generation schizont seen at 96 h p.i. Note the two host cell nuclei (*HN*), the refractile body material (*large arrowheads*), and the loss of schizont membrane in some areas of schizont invagination and presence of membrane swirls (*small double arrowheads*). ×6,000. **Fig. 10** First-generation schizont seen at 96 h p.i. Note the budding of merozoites (*arrowheads*). ×9,000











Figs. 11–15

line. Although CEV-1/F7 cells are highly permissive to *E. tenella* sporozoite invasion and first-generation schizont development, which results in extensive merozoite development (Clare et al. 1993), it appears that these parasites are inhibited from further development.

Results obtained in this study show that a synchronous development of *E. tenella* in the CEV-1/F7 cell line occurs at up to 48 h p.i. with the appearance of immature first-generation schizonts. Asynchronous development then becomes more predominant, as immature, budding and mature first-generation schizonts were seen at 72–96 h p.i. Most of the developing schizonts and the mature merozoites showed no ultrastructural abnormality at up to 72 h p.i., although no invasion by the merozoites was seen at 72–96 h p.i. There was, however, an initiation of second-generation schizogony at 72 h p.i., with the first-generation merozoites rounding up within the first-generation PV, undergoing nuclear division, and attempting to bud off merozoites. This type of schizogony has not previously been described for *E. tenella* either in vitro or in vivo. Previous investigators have reported that the merozoites bud either radially from the periphery or from blastophore subdivisions and invaginations of the schizont during the two to three schizogonic generations seen with this parasite (see review by Doran 1982). Because little work, if any, has been done with established avian cell lines, it is not known if this is a form of aberrant development that occurs exclusively with in vitro development within the CEV-1F7 cell line.

Degeneration of first-generation meronts was first observed as early as 72 h p.i. The number of degenerating first- and second-generation meronts increased in number by 96 h p.i., and most of the meronts observed at 120–144 h p.i. showed ultrastructural degeneration. Since this cell line is being used to produce coccidial antigen for bird vaccination trials, it is apparent from the present study that collection of cell-culture medium that has the least amount of degenerative coccidial antigen should not extend beyond 72 h p.i.

Exactly why merozoites do not invade host cells or why first- and second-generation schizogony is not supported during the later incubation periods is unknown. The Lilly 65 strain of *E. tenella* used in inoculating the CEV-1/F7 cells has consistently produced the best results for in vitro growth (Strout and Schmatz 1990). Frequent changes of growth media have not improved or increased the development of the parasite (Clare et al. 1993). The extracellular merozoites observed in the present study did not appear to have undergone ultrastructural degeneration. As suggested by Clare et al. (1993), it may be that the CEV-1/F7 cells lack a specific receptor for the merozoite invasion or that the intensive parasite development seen within the first 72 h p.i. somehow alters the metabolism of the parasites such that they cannot invade or undergo further development.

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Fig. 11 Extracellular merozoites of *E. tenella* seen at 96 h p.i. Note the presence of amylopectin granules (*AP*) and refractile body (*RB*) material. ×12,500. **Fig. 12** Degenerating small first-generation schizont seen at 120 h p.i. Note the degenerating vacuolated merozoites (*arrowheads*) within the parasitophorous vacuole. ×14,000. **Fig. 13** Degenerating small, immature schizonts (*arrowheads*) seen at 144 h p.i. ×4,500. **Fig. 14** Large degenerating schizont containing numerous nuclei (*arrowheads*) and little cytoplasm, seen at 144 h p.i. ×5,600. **Fig. 15** Schizont (*SH*) that has undergone complete degeneration, seen at 144 h p.i. ×4,500

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