Establishment of a Turkey Cecal Cell Line and Development of Turkey Coccidia within the cells (43734)

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Abstract. **Cells were dispersed from cecal tissues of 1- to 2-day-old turkeys using a mixture of collagenase and dispase to enrich for epithelial cells. The initial culture produced from these cells (TCC) appeared to be heterogeneous, but, as the cells were cultured through 25 passages, they assumed a more fibroblastic appearance. Cellular invasion of the TCC by two species of turkey coccidia, *Eimeria adenoeides* and *Eimeria meleagrimitis*, was not enhanced, as compared with invasion in turkey kidney cells (TKC), the cell culture system standardly used to study the avian coccidia *in vitro*. However, early development by one of the species, *E. meleagrimitis*, was markedly increased in the TCC (Passages 6 through 19) over that in TKC. Thirty to 42% of the parasites that invaded the TCC developed beyond the sporozoite stage, as compared with 5% development in TKC. Mature first-generation schizonts were observed within 24 hr postinoculation in the TCC, but not until 48 hr in the kidney cells. There was no evidence that development of the second generation was initiated in either the TCC or kidney cells.** [P.S.E.B.M. 1994, Vol 206]

There have been numerous attempts to obtain the complete *in vitro* development of species of avian coccidia, using a variety of primary cultures and cell lines (1). Although most of the attempts were unsuccessful, they did show that greater success toward completion of the life cycle was obtained when avian rather than mammalian cells were used. For example, Doran (2), followed shortly by Strout (3), reported the first successful *in vitro* cultivation of a species of avian coccidia, *Eimeria tenella*, through the complete life cycle in chick kidney cells. The development of *E. tenella* in this system closely paralleled that in the host chicken, both in the time of appearance and the morphology of the stages.

Subsequently, Augustine and Doran (4) reported the complete development of a turkey coccidium, *E. meleagrimitis*, in cultures of turkey kidney cells. The asexual generations developed in cultures inoculated with sporozoites and the sexual generations formed in cultures inoculated with merozoites isolated from infected turkeys. Complete development throughout the life cycle from sporozoites to oocysts was not accomplished.

In the host birds, the avian coccidia are primarily intestinal parasites. However, most of the studies on the *in vitro* cultivation of these parasites have been conducted in mammalian or avian kidney cells. There are very few reports on the cultivation of the coccidia in avian intestinal cells because of the difficulty of producing usable primary cultures and the absence of suitable intestinal cell lines. Therefore, the goals of the current study were (i) to develop a cell line from turkey cecal tissues and (ii) to compare the invasion and development of two species of turkey coccidia, *Eimeria adenoeides* and *E. meleagrimitis*, in the cell line with development in turkey kidney cell cultures.

Materials and Methods

Turkey Kidney Cell Cultures. Turkey kidney cells (TKC) were dispersed for cultivation as reported previously (4). The cells were then suspended in Medium 199 containing 20% fetal bovine serum (FBS) and

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10% dimethyl sulfoxide (DMSO) at 2 to 10 × 10^6 cells/vial, frozen slowly by incubation for 30 min each at 4°C and −70°C, and then stored at −135°C. This stock of cells was used to prepare TKC cultures throughout the study. For preparation of TKC cultures, the frozen cells were thawed quickly in a 40°C water bath and resuspended in 15 ml of Medium 199 containing 20% FBS. After 3 min, the cell suspension was adjusted to 50 ml with Medium 199 (without serum) and centrifuged for 4 min at 180*g*. The cells were resuspended in Medium 199 containing 5% FBS, 1.3% L-glutamine, and 2% 100× penicillin/dihydrostreptomycin (pen/strep) (complete M199). The cell suspension was plated on glass coverslips in 24-well Linbro plates at 1.5 to 2.0 × 10^4 cells/well, and incubated at 41°C to the desired confluency (2 to 3 days).

Turkey Cecal Cell Cultures. Primary cultures of turkey cecal cells (TCC) were prepared from 1- to 2-day-old Beltsville medium white turkeys by a modification of a procedure for culturing mouse intestinal cells (5). Briefly, the ceca were excised, flushed twice with 30 ml Hank's Balanced Salt Solution (HBSS) containing 2% pen/strep and 2% fungizone, and slit longitudinally. The opened ceca were minced into 2- to 3-mm pieces and washed eight times in HBSS (the washing involved shaking the cecal pieces vigorously in 50 ml of the medium for 1 min, allowing the pieces to settle for 1 min, and replacing the supernatant with fresh HBSS). The pieces of tissue were then finely minced with scalpels, suspended in 20 ml HBSS containing 6000 U of collagenase and 2 mg of dispase, and stirred in a trypsinizing flask for 30 min. The tissue suspension was vigorously pipetted (30×) and transferred to a 50 ml tube. The pieces of tissue were allowed to settle, the supernatant was decanted, and the tissue was resuspended in 20 ml HBSS. Following centrifugation at 180*g* for 4 min, the supernatant was decanted and the tissue was resuspended in fresh HBSS (this step was repeated six times, or until the pellet was well-defined and the supernatant was clear). The pellet was resuspended in Medium 199 containing 4500 mg/l glucose, 5% FBS, 5 μg/ml heparin, 2.5 μg/ml insulin, 10 ng/ml epidermal growth factor, 2% pen/strep, 0.2% gentamycin, and 0.2% fungizone. The cells were plated heavily in six-well dishes coated with collagen or fibronectin (Collaborative Biomedical Products, Bedford, MA), uncoated plastic petri dishes (100 mm diameter), or flasks (75 cm^2). After 24 hr, the medium and nonadherent cells were gently removed from the culture dishes and fresh medium was applied. When the cell layer covered 30%–40% of the culture dish, the cells were dissociated with PBS (pH 7.6) containing 0.04% trypsin and 0.53 m*M* EDTA, washed once in PBS containing 1% FBS, and replated in complete M199. For subsequent passages, the cells were similarly dissociated from the culture dishes when the cell layer had attained 70%–80% confluency (% of coverslip covered by cells).

Parasite Invasion and Development. Both TCC and TKC cultures were grown in 24-well Linbro plates on 12-mm glass coverslips in complete M199. Sporozoites of *E. adenoeides* and *E. meleagrimitis* were suspended in complete M199 (containing 1% instead of 5% FBS) and inoculated into cultures of TCC and TKC at 3 × 10^5 sporozoites/well. Passages 6–19 of the TGC were used. For invasion, cultures were fixed after 2 hr in buffered formalin. The number of intracellular sporozoites and the percent confluency was determined for each coverslip. The mean number of sporozoites/10 mm^2 of cells was calculated for each coverslip (6) and differences between cell types were tested for significance at the 0.05 level by Student's *t* test. In the remaining cultures, the inoculation medium was replaced with complete M199, and coverslips were fixed at intervals from 18 to 72 hr postinoculation (PI). In some experiments, the medium was replaced with fresh complete M199 at 48 hr PI. Development in each cell type at each interval was determined. A total of seven experiments were done with each cell type, with two or three coverslips/coccidial species/interval.

Results

Turkey Cecal Cell Cultures. Primary cultures of turkey cecal cells initially consisted of single cells, clumps of cells, and microexplants of tissue from which cells spread outwards and were heterogeneous in appearance. Attachment of the cells to untreated culture dishes and flasks was as efficient as to collagen and fibronectin-treated flasks (data not shown), so untreated flasks were routinely used. The initial growth of the cells was quite slow. For first passage, done at 1.5 to 2 weeks when the cell layer was approximately 30%–40% confluent, the cells were split 1:2. The cells continued to multiply slowly during the first two to three passages. However, after the fifth passage, the rate of growth increased gradually until it was similar to that of the kidney cells. For example, inoculation of 24-well Linbro plates with 1.2 × 10^4 cells/well of either TCC or TKC provided cultures that were 50%–60% confluent in 3 days. The TCC has been cultured through 25 passages and the cells have gradually assumed a fibroblastic appearance. When 100% confluent, the cell culture retains its monolayer characteristics and rolls off the culture dish. There are no focci of increased cell growth that indicate the loss of contact inhibition.

Invasion. Cellular invasion in TCC and TKC did not differ significantly ($P > 0.05$). The mean numbers (±SE) of *E. adenoeides* sporozoites in the TCC and TKC were 8.27 ± 1.6 and 9.74 ± 2.1, respectively. The mean numbers of *E. meleagrimitis* sporozoites in TCC and TKC were 7.18 ± 1.40 and 5.12 ± 1.01, respectively.

Development. The early development of *E. meleagrimitis* in TCC markedly exceeded that in the TKC. In the TCC, by 24 hr PI, half of the sporozoites had enlarged, and mature first-generation schizonts, containing 10–12 merozoites/schizont were apparent (Fig. 1A). By 38 hr PI, 20% of the sporozoites had developed further and there were numerous free first-generation merozoites (Fig. 1B). Peak development (30%–42%) occurred between 48 and 68 hr PI (Fig. 1C). By 72 hr PI, the numbers of parasites were markedly reduced and consisted primarily of sporozoites and immature schizonts. There was little evidence of reinvasion of cells or further development by the first-generation merozoites.

In TKC, the overall development of *E. meleagrimitis* did not rise above 5%, although the stages that did develop were morphologically similar to those in TCC. By 24 hr PI, approximately half of the sporozoites had enlarged and there were a few immature schizonts containing 8 to 10 nuclei. By 48 hr PI, a few

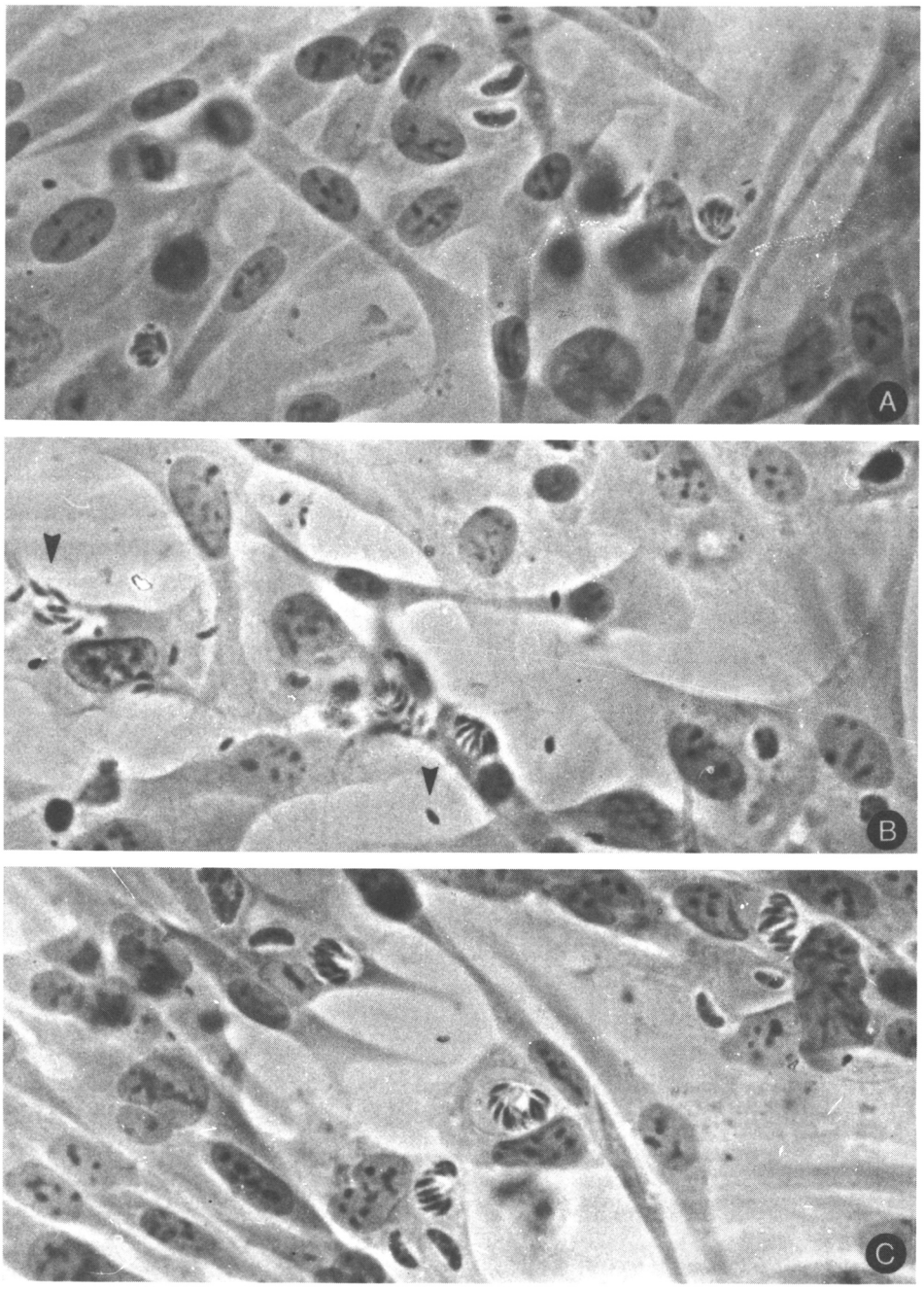

Figure 1. Cultures of turkey cecal cells inoculated with sporozoites of *Eimeria meleagrimitis* and fixed at 24 (A), 38 (B), and 48 hr (C) postinoculation. Arrows in (B) indicate free merozoites. Cultures were fixed in buffered formalin and stained with hematoxylin and eosin (magnification ×2700).

mature first-generation schizonts, containing 8 to 10 merozoites, were observed. Free merozoites were not seen.

In contrast, in six of the seven experiments, development of *E. adenoeides* in TCC did not differ from development in TKC. In both cell types, at 24 and 48 hr PI, some sporozoites were enlarged and a few immature schizonts, containing six nuclei/schizont, were seen. In one experiment, mature schizonts, containing 30–34 merozoites had developed by 72 hr PI (Fig. 2).

Discussion

The method for culturing the mouse intestinal epithelial cells (5) was also satisfactory for preparation of TCC. Dissociation of cells by collagenase/dispase yielded a mixture of single cells and clumps of cells or tissues, which were capable of attaching to plastic flasks. Within 5 to 7 days, most of the single cells had disappeared or were vacuolated. However, a monolayer of cells grew outwards from the attached clumps and these constituted the original cells from which the cell line was derived. The cell line was established and has been propagated under conditions that favor epithelial cell growth (5), but the cells have not yet been cloned or characterized. Therefore the makeup of the cell line is not known at this time.

Invasion of the TCC cultures (Passages 6–19) by sporozoites of *E. adenoeides* and *E. meleagrimitis* did not differ significantly from that in TKC cultures. This was surprising in light of fact that the intestinal cell line was derived from cecal cells, and one of the coccidial species, *E. adenoeides,* preferentially invades the ceca in the turkey (7). There are several possible explanations for these findings. First, the intestinal cells may have dedifferentiated in culture and no longer expressed the specific putative characteristics (secretory compounds, receptor sites, etc.) that are recognized for invasion by the coccidia. Second, in cell culture, the sporozoites are delivered to the surface of the cells by gravity and thus may require no specific cell stimulus to attract them to the appropriate cell for invasion. Third, cells that are normally invaded in the intestine may represent so small a portion of the total population of TCC that they do not significantly influence the overall efficiency of invasion. Finally, certain environmental conditions in the intestine (pH, enzymes, etc.) which are absent in the TCC may play an active role in cellular invasion.

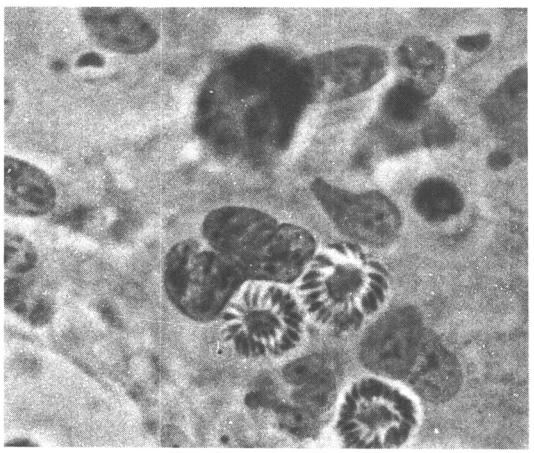

Figure 2. Culture of turkey cecal cells inoculated with sporozoites of *Eimeria adenoeides* and fixed at 72 hr postinoculation. The culture was fixed in buffered formalin and stained with hematoxylin and eosin (magnification ×2700).

The early development by one of the species, *E. meleagrimitis,* was markedly enhanced in the TCC. In a previous study (4) and in the present one, development by *E. meleagrimitis* sporozoites in TKC was approximately 5%, and, although a few immature first-generation schizonts were observed at 24 hr PI, mature ones were not seen until 48 hr PI. In the TCC cultures, there was an 8-fold increase in the rate of development. Moreover, mature first-generation schizonts appeared by 24 hr PI and free merozoites were abundant by 38 hr PI. Although appearing early and in moderately good numbers, the first-generation schizonts in the TCC contained only 10–12 merozoites as compared with 80–100 merozoites/schizont in the bird (8). In fact, the culture-derived schizonts more closely resembled the description of second-generation schizonts in the turkey than the description of the first generation. The enhancement in development in TCC did not extend to the second generation. Although there were numerous free, extracellular first-generation merozoites, there was little evidence for reinvasion by these merozoites and no appearance of second-generation schizonts.

Surprisingly, there was little development by the cecal coccidium, *E. adenoeides,* although this species normally invades and develops in cecal cells in the turkey (7, 9). It may be that *E. adenoeides* is more fastidious in its growth requirements than *E. meleagrimitis* and thus will not develop in cells maintained under the conditions of this study, regardless of their origin. Alternatively, Doran (2) hypothesized that islets of tissue or areas of dense growth are preferred areas for development in culture. The TCC grow as a monolayer with few areas of heavier growth; thus the structural characteristics of the cultures may have deterred the development of the species.

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