INFECTION AND IMMUNITY, Nov. 1991, p. 4042–4048
0019-9567/91/114042-07$02.00/0

Vol. 59, No. 11

X-Irradiation of *Eimeria tenella* Oocysts Provides Direct Evidence that Sporozoite Invasion and Early Schizont Development Induce a Protective Immune Response(s)

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Received 11 March 1991/Accepted 28 August 1991

Sporulated oocysts of the protozoan parasite *Eimeria tenella* were attenuated by exposure to various doses of X-radiation to inhibit intracellular replication and thus determine whether sporozoites alone can induce a protective immune response. Exposure to doses greater than 15-kilorads had a significant effect on development, as indicated by the absence of oocyst production in chickens infected with parasites treated with 20 or 30 kilorads of radiation. Infection with nonirradiated or 15-kilorad-exposed parasites led to either normal or reduced oocyst shedding. Equivalent protection was afforded chickens inoculated with a minimum immunizing dose of either nonirradiated or 20-kilorad-irradiated *E. tenella* oocysts. Immunofluorescence staining of cecal tissue from chickens inoculated with 10^7 nonirradiated or 20- or 30-kilorad-irradiated oocysts with stage-specific monoclonal antibodies showed no significant difference in sporozoite invasion between treatment groups. Normal merogonic development was observed at appropriate times (48, 60, 72, and 96 h) postinfection in chickens inoculated with nonirradiated oocysts. In contrast, irradiated parasites exhibited minimal merogonic development at 48 h postinfection. Furthermore, no merogonic stages were observed at times of otherwise peak merozoite development (60, 72, and 96 h) in cecal tissue from chickens inoculated with irradiated parasites. Infection of chicken cells with irradiated or nonirradiated parasites in vitro corroborated these findings and indicate that events early after sporozoite invasion induce a protective immune response against this parasite.

Coccidiosis in chickens is caused by protozoa of the genus *Eimeria*. The parasites have a rather complex life cycle, involving both asexual and sexual stages, that is completed by shedding of unsporulated oocysts. Oocysts sporulate in the environment and are then capable of infecting naive chickens to begin the life cycle once again. Although resistance to coccidiosis is established by previous infection, protection is species specific. Successful development of a subunit vaccine against coccidiosis will depend upon a thorough understanding of the protective immune response that is elicited during a primary infection. It remains unclear what effector cell(s) is involved and which developmental stage(s) of the parasite induces and is targeted by the host immune system. As indicated by adoptive transfer (21), immune suppression (13, 21), and lymphocyte depletion (22) studies, immune T cells play a major role in preventing reinfection. Recent in vitro and in vivo work has shown that gamma interferon may play a role in controlling development of the parasite (11, 24, 25). On the basis of studies utilizing drugs that inhibit merogonic development, several researchers have hypothesized that first-generation meronts of *Eimeria tenella* are responsible for inducing resistance to coccidiosis (4, 15, 16, 20) and that sporozoites play a minor role. This point remains controversial, as other studies, using similar anticoccidial agents (8), have found that merogonic development is not required and sporozoites themselves are capable of eliciting a protective response. Consistent with the latter hypothesis is the demonstration that resistance to challenge appears to be directed against sporozoites (12, 23) or sporozoite-infected host cells (17, 23).

Attempts to identify the targeted developmental stage for several other protozoa, including *Plasmodium* (5, 18, 29, 30), *Trypanosoma* (27), and *Babesia* (19) species, by using X-radiation have implicated initial invasive forms of the parasite. Our recent work using X-irradiated oocysts of a related coccidium, *E. acervulina*, suggest that sporozoite invasion of intestinal host cells and subsequent metabolism therein are sufficient to establish resistance (9). It remains to be determined whether this is a general mechanism for all *Eimeria* parasites. Two distinctive features of coccidial parasites is tropism for specific regions of the intestine and the absence of cross-protection elicited by each species. The purpose of the present study was to evaluate the immunizing potential of X-irradiated oocysts of *E. tenella*, a coccidial species that invades the lower intestine, and resolve the conflicting data on the role of sporozoites in inducing a protective immune response against this coccidian parasite.

MATERIALS AND METHODS

Parasites and attenuation procedure. *E. tenella* oocysts (Protozoan Diseases Laboratory strain 28) were obtained by both cecal harvest and fecal collection, sporulated in vitro, and purified by using standard procedures (28). The oocysts were subjected to 0 to 30 kilorads of X-radiation with a ^{137}Cs Gammator M radiation source at 1.25 kilorads/min. Samples of oocysts exposed to the various radiation levels were excysted by using standard methods (26) to determine whether X-irradiation had any effect on sporozoite release.

Effect of irradiation on oocyst production. Susceptible outbred chickens (five per group) were inoculated with 10^4 oocysts that had been exposed to 0, 15, 20, or 30 kilorads of radiation. Ceca were harvested at 7 days postinoculation and

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processed for tissue oocyst enumeration to determine the effect of irradiation on oocyst development. In brief, ceca from each group were homogenized in a Waring blender in phosphate-buffered saline. Oocysts in the cecal extract were banded by centrifugation in 1 M sucrose and counted on a McMaster chamber by using standard techniques (9). The detection limit for this technique has been estimated to be about 10^3 oocysts per chicken (unpublished data).

Vaccination with irradiated oocysts and assessment of protection. Outbred Sexsal chickens (10 per group) 1 or 7 days old were inoculated with *E. tenella* oocysts that had been exposed to 0, 20, or 30 kilorads of X-radiation. Animals received either one dose of 2×10^4 oocysts on day 1 or four doses of 2×10^4 oocysts (8×10^4 total) on days 1, 5, 8, and 12. Previous experimentation had shown that 2×10^4 nonirradiated oocysts was the minimum immunizing dose required to obtain significant levels of protection against homologous challenge (unpublished data). Two groups of animals were sham inoculated as controls. All animals, except one group of sham-inoculated chickens, were challenged with 2×10^4 *E. tenella* oocysts on day 19. All chickens were sacrificed on day 26 and examined for the presence of intestinal lesions by using standard procedures (10). Weight gain during the infection period was measured for each animal and compared between treatment groups by using analysis of variance and Duncan's multiple range test.

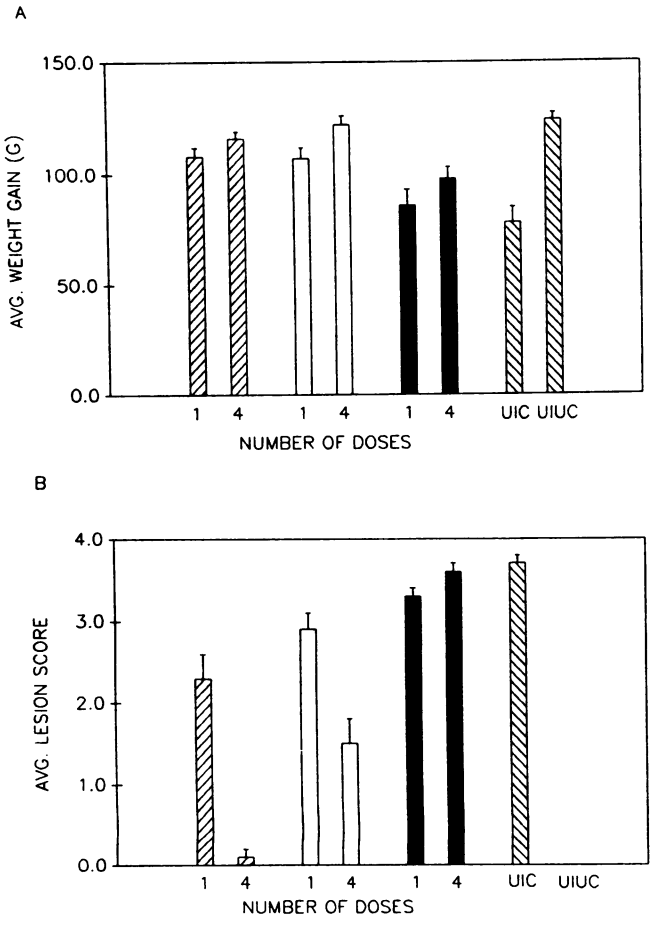

FIG. 1. Protection against weight depression (A) and intestinal lesions (B) in 1-day-old chickens inoculated with one or four doses of nonirradiated (▨), 20-kilorad-irradiated (□), or 30-kilorad-irradiated (■) *E. tenella* oocysts (2×10^4 per dose). UIC, unimmunized, challenged controls; UIUC, unimmunized, unchallenged controls. Values represent means of 15 determinations ± standard deviation. AVG., average.

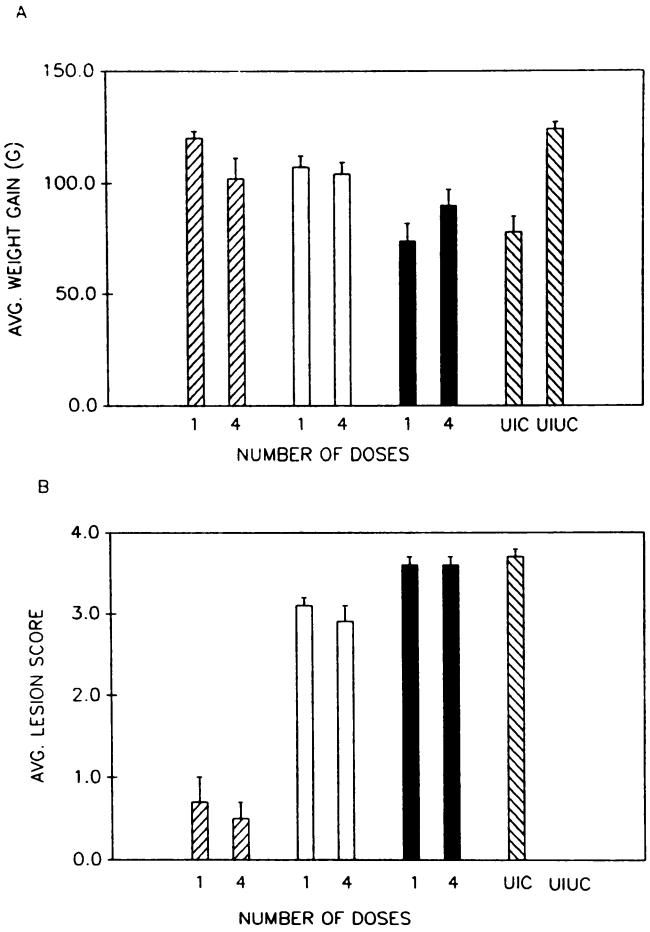

FIG. 2. Protection against weight depression (A) and intestinal lesions (B) in 7-day-old chickens inoculated with one or four doses of nonirradiated (▨), 20-kilorad-irradiated (□), or 30-kilorad-irradiated (■) *E. tenella* oocysts (2×10^4 per dose). UIC, unimmunized, challenged controls; UIUC, unimmunized, unchallenged controls. Values represent means of 15 determinations ± standard deviation. AVG., average.

Assessment of in vivo development of irradiated and nonirradiated parasites. Susceptible 1-week-old outbred chickens were inoculated with high numbers of sporulated *E. tenella* oocysts (10×10^6 oocysts per chicken for 6-, 24-, and 48-h tissue samples; 3×10^5 oocysts per chicken for 60- and 72-h tissue samples; and 4×10^4 oocysts per chicken for 96-h samples) that had been exposed to 0, 20, or 30 kilorads of radiation. These inoculation levels were chosen to ensure that high numbers of intracellular sporozoites and merogonic stages would be found and that the absence of either stage would not be due to an insufficient number of parasites in the original inoculum. Tissue samples were taken by removing a 1-cm cross-section of the cecum from duplicate chickens sacrificed at 6, 24, 48, 60, 72, and 96 h postinfection. The cecal tissue was fixed as previously described (7) and embedded in paraffin. Tissue cross-sections (5 μm) were deparaffinized in xylene and immunostained by indirect immunofluorescence with either sporozoite-specific monoclonal antibody (MAb

1209 or merozoite-reactive MAb 1207 by following described procedures (1, 7). No less than eight cross-sections were examined for each treatment group. Means and standard deviations of intracellular sporozoites and merogonic stages per cross-section were calculated for each treatment group.

Assessment of in vitro development of irradiated and nonirradiated parasites. Sporozoites were obtained from irradiated (20 and 30 kilorads) and nonirradiated (0 kilorads) *E. tenella* oocysts and used to infect primary chicken kidney (PCK) cell monolayers grown in vitro on glass coverslips by using previously described methods (1). Sporozoite invasion and intracellular development were assessed by hematoxylin-eosin staining of formalin-fixed cultures 24 and 72 h postinfection by following standard procedures (14). To assess intracellular sporozoite metabolism, infected PCK monolayers were harvested at 24, 48, 72, and 96 h, fixed in ice-cold methanol for 2 min, and then washed several times with phosphate-buffered saline, pH 7.2. Intracellular metabolism was assessed by indirect immunofluorescence staining of sporozoite-infected PCKs with a MAb (C34F1) that recognizes an 8- to 10-kDa metabolic protein (unpublished data). Although the greatest proportion of sporozoites proceed to the first and second merogonic stages, previous research has shown that a significant number of *E. tenella* sporozoites do not develop in vitro and yet continue to produce this low-M_r metabolic antigen (unpublished data).

TABLE 1. Numbers of intracellular sporozoites and merogonic stages in intestinal tissues of chickens inoculated with *E. tenella* oocysts[a] exposed to 0, 20, or 30 kilorads of X-radiation, as detected by immunofluorescence staining with stage-specific MAbs[b]

Time (h) postinfection	Radiation dose (kilorads)	Mean no. of intracellular asexual stages[c] (±SD)	
		Sporozoites	Merogonic stages
6	0	27 ± 9	ND
6	20	26 ± 5	ND
6	30	24 ± 5	ND
24	0	20 ± 4	ND
24	20	24 ± 6	ND
24	30	20 ± 4	ND
48	0	18 ± 5	21 ± 5
48	20	17 ± 6	1 ± 1
48	30	18 ± 3	0
60	0	0	26 ± 3
60	20	0	0
60	30	0	0
72	0	0	120 ± 18
72	20	0	0
72	30	0	0
96	0	0	TNTC
96	20	0	0
96	30	0	0

[a] Chickens were inoculated with 10×10^6 oocysts each for 6-, 24-, and 48-h tissue samples; 3×10^5 oocysts each for 60- and 72-h tissue samples; and 4×10^4 oocysts each for 96-h samples.

[b] Tissue sections were stained by indirect immunofluorescence with either a MAb specific for sporozoites (MAb 1209) or a MAb reactive with merogonic stages (MAb 1207).

[c] The values reported are mean numbers of asexual stages detected in 8 to 10 tissue cross-sections. ND, not done; TNTC, too numerous to count.

RESULTS

Effect of irradiation on oocyst production. Cecal oocyst numbers at 7 days postinfection were high (3.1×10^6 per chicken) in groups receiving nonirradiated oocysts and decreased by 2 orders of magnitude (3.0×10^4 per chicken) in animals inoculated with oocysts that had been exposed to 15 kilorads of X-radiation. Oocysts were not detected ($<10^3$) in chickens inoculated with parasites that had been exposed to higher radiation doses (20 and 30 kilorads). In vitro excystation of oocysts exposed to 10, 15, 20, and 30 kilorads of X-radiation showed no appreciable difference in release of motile sporozoites (unpublished data).

Protection against coccidiosis with X-irradiated oocysts. Inoculation of 1-day-old chickens with nonirradiated or 20-kilorad-irradiated *E. tenella* oocysts conferred significant ($P < 0.05$) protection against homologous challenge compared with groups immunized with 30-kilorad-treated oocysts or unimmunized controls, as indicated by weight gain during the infection period (Fig. 1A). The weight gains during this period for animals immunized with nonirradiated or 20-kilorad-irradiated oocysts were similar ($P > 0.05$) to those of the unchallenged controls (Fig. 1A). There was a slight improvement in the average weight gain in chickens immunized four times with both 20-kilorad-irradiated and nonirradiated oocysts over those inoculated once with these oocysts (Fig. 1A). This phenomenon was also observed in the group that received four doses versus one of 30-kilorad-treated oocysts.

Immunization of 7-day-old chickens with nonirradiated and 20-kilorad-irradiated oocysts also conferred significant levels of protection against weight depression compared with unimmunized or 30-kilorad-irradiated controls (Fig. 2A). Chickens immunized once with either oocyst preparation (0 or 20 kilorad treated) exhibited weight gains similar ($P > 0.05$) to those of the nonchallenged controls. However, weight gains were lower in groups that received four doses of either irradiated or nonirradiated oocysts (Fig. 2A).

In general, protection against intestinal lesions was not as marked as weight gain. There was a significant difference ($P < 0.05$) in lesion score values between 1-day-old chickens inoculated with 20-kilorad-exposed oocysts and groups that received either nonirradiated or 30-kilorad-irradiated oocysts, being higher than the former and lower than the latter (Fig. 1B). No protective effect ($P > 0.05$) against intestinal lesions were noted in 7-day-old chickens inoculated with 20-kilorad-treated oocysts (Fig. 1B). As expected, significant protection ($P < 0.05$) was observed in groups that received nonirradiated oocysts (Fig. 1B). As with weight gain, chickens immunized four times with nonirradiated or 20-kilorad-irradiated oocysts exhibited greater protection against lesions than the respective animals immunized once with these oocyst preparations (Fig. 1B).

Assessment of in vivo development of irradiated and nonirradiated parasites. There was no significant difference ($P < 0.05$) in the level of invasion of chicken epithelium between nonirradiated and 20- and 30-kilorad-irradiated sporozoites as measured by immunofluorescence staining of infected tissue at both 6 and 24 h postinoculation (Table 1). Sporozoite numbers decreased by about 30% at 48 h and were negligible at later times (60, 72, and 96 h). Mature first-generation schizonts and small clusters of second-generation trophozoites were detected by 48 h postinfection in chickens inoculated with nonirradiated parasites (Table 1). A maximum of three and a total of five immature first-generation schizont stages (as indicated by the presence of a large refractile body) were seen in 10 tissue cross-sections from chickens inocu-

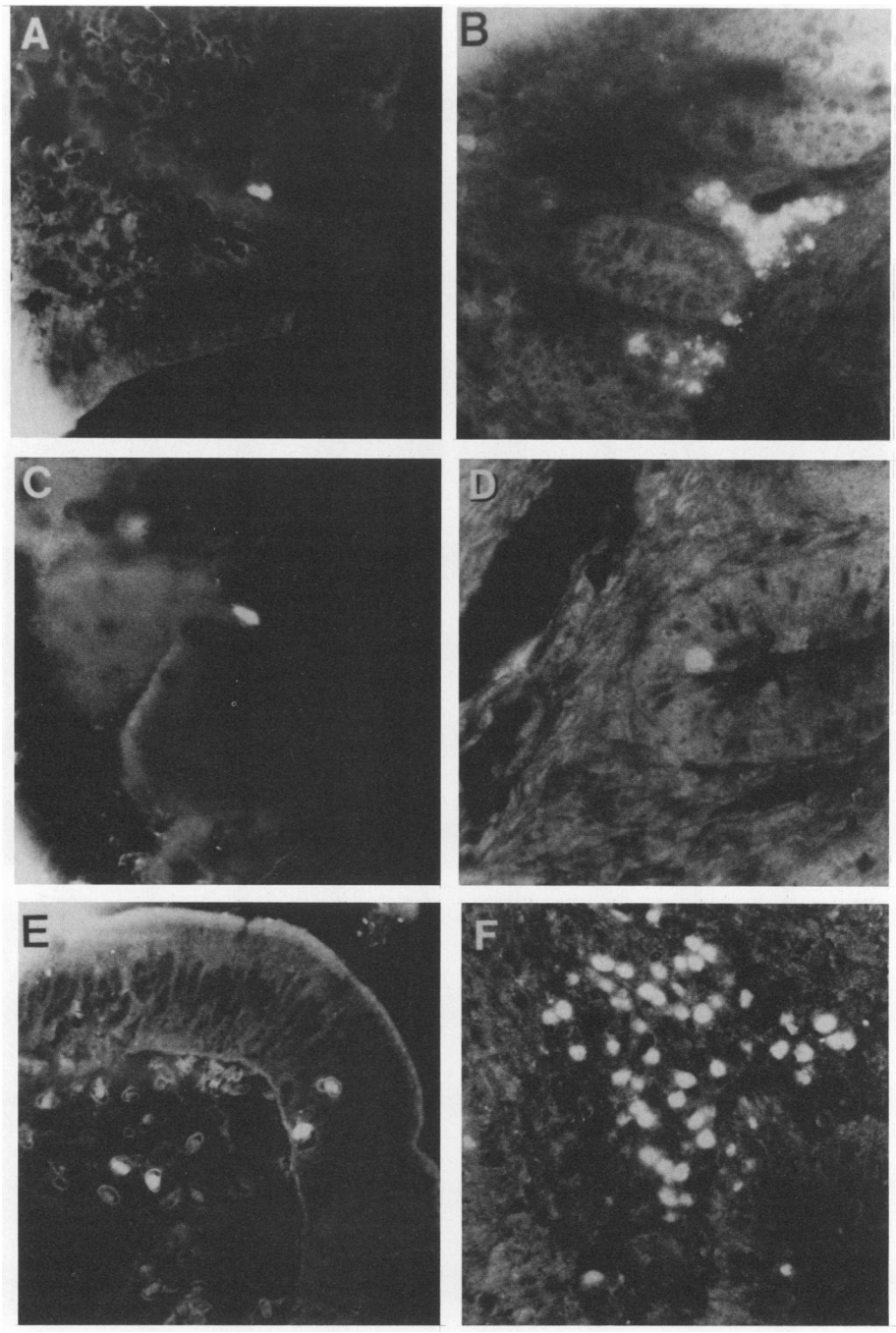

FIG. 3. Detection of sporozoites and merogonic stages by indirect immunofluorescence staining of intestinal tissue from chickens inoculated with nonirradiated or irradiated *E. tenella* oocysts. (A, C, and E) Intracellular sporozoites detected 24 h postinfection with nonirradiated (A), 20-kilorad-irradiated (C), or 30 kilorad-irradiated (E) oocysts. (B) Mature first-generation schizonts at 48 h postinfection arising from nonirradiated sporozoites. (D) Immature first-generation schizonts at 48 h postinfection arising from 20-kilorad-irradiated sporozoites. (F) Mature second-generation schizonts at 72 h postinfection arising from nonirradiated sporozoites.

lated with 20-kilorad-irradiated oocysts (Table 1). The number of merogonic stages increased with time in animals that received nonirradiated parasites, being too numerous to count at 96 h postinfection. In contrast, no asexual stages were observed beyond 48 h in chickens that received irradiated oocysts (Table 1). Although the morphologies of irradiated and nonirradiated sporozoites were indistinguishable (Fig. 3A, C, and E), immature schizonts arising from irradiated parasites had an abnormal, punctated appearance (Fig. 3D). No schizonts were observed in tissue of chickens infected with 30-kilorad-irradiated oocysts.

Assessment of in vitro development of irradiated and nonirradiated parasites. Invasions of PCK cells by irradiated and nonirradiated sporozoites were similar, as assessed by hematoxylin-eosin staining of the cultures 24 h postinfection (Fig. 4A to C). A noticeable difference in development of

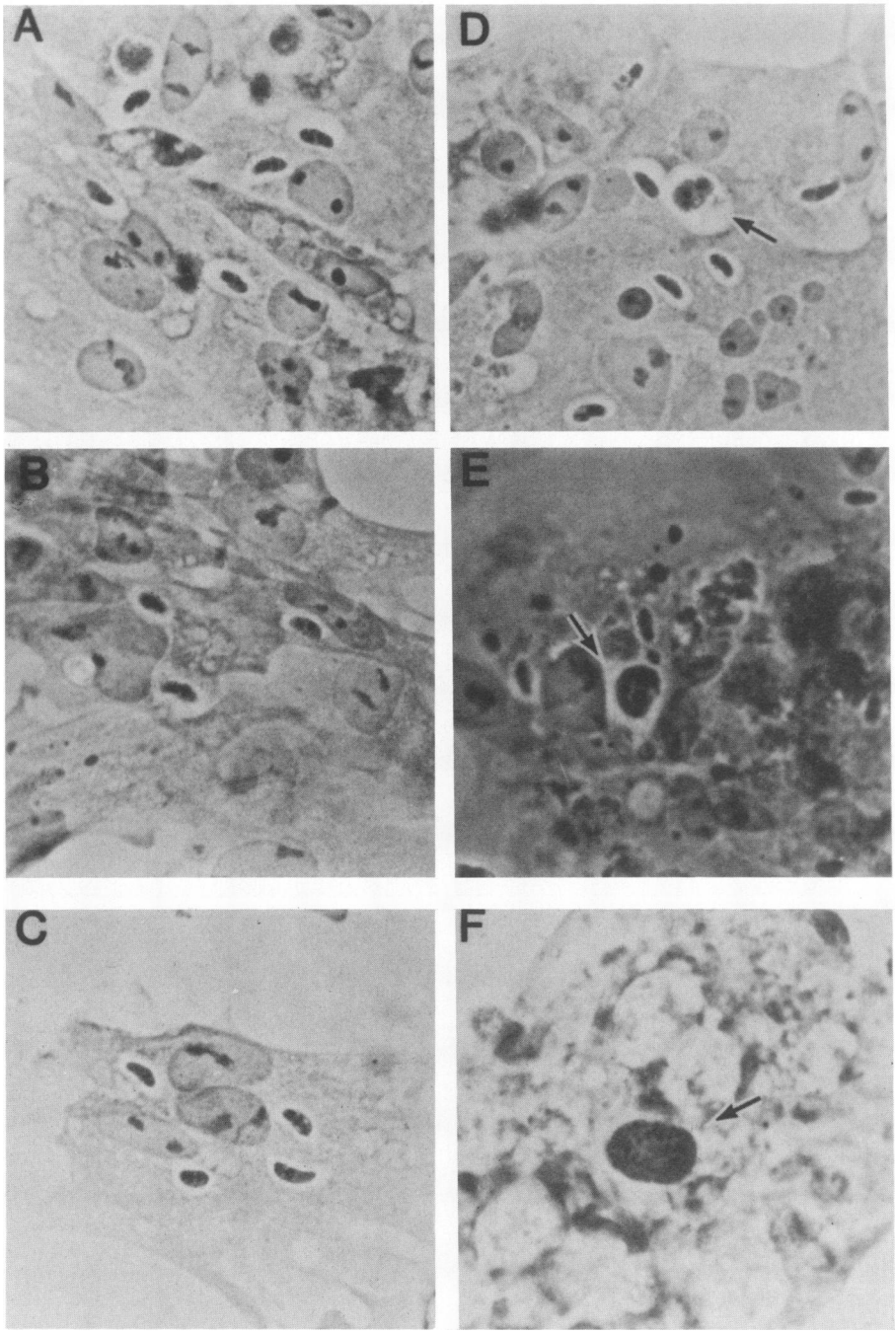

FIG. 4. Evaluation of in vitro sporozoite invasion and merogonic development in PCK cells infected with nonirradiated (A, E, and F), 20-kilorad-irradiated (B and D), or 30-kilorad-irradiated (C) *E. tenella* sporozoites by hematoxylin-eosin staining at 24 h (A to C) and 72 h (D to E) postinfection. Arrows indicate immature schizonts (D and E) and a mature schizont (F).

merogonic stages was observed at 48, 72, and 96 h between cultures inoculated with nonirradiated and irradiated sporozoites. Both immature and mature schizont stages were observed in cultures infected with the former (Fig. 4E and F). In contrast, 20-kilorad-exposed sporozoites exhibited minimal merogonic development, with only immature first-generation schizonts present (Fig. 4D). No schizont stages were found in cultures infected with 30-kilorad-irradiated sporozoites. Immunofluorescence staining with a sporozoite-specific MAb at 24 h (Fig. 5A and C) and 72 h (Fig. 5B and D) postinfection showed no detectable difference in the amount of "metabolic" antigen produced. PCK cells infected with 30-kilorad-irradiated sporozoites exhibited no detectable immunofluorescence (data not shown).

DISCUSSION

The present study provides conclusive evidence that protection against secondary *E. tenella* challenge does not require development of mature first-generation schizonts

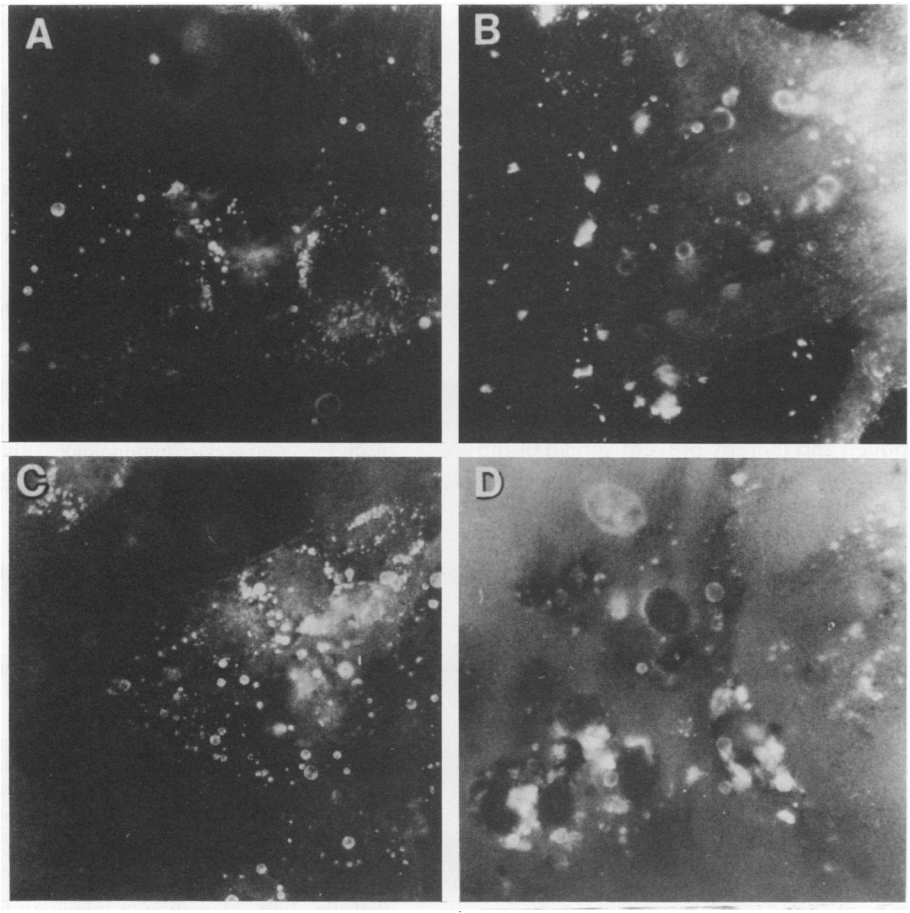

FIG. 5. Assessment of in vitro intracellular metabolism of nonirradiated (A and C) and 20-kilorad-irradiated (B and D) *E. tenella* asexual stages (sporozoites and merogonic stages) as detected by indirect immunofluorescence staining with a MAb that recognizes a low-M_r metabolic protein. Panels: A and B, 24 h postinfection; C and D, 72 h postinfection.

during the primary infection. Immunofluorescence staining of tissues from chickens infected with high doses of nonirradiated or 20-kilorad-irradiated oocysts showed profuse merogonic development in the former and minimal development in the latter. Both immunization schemes conferred significant protection against weight loss in 1- and 7-day-old chickens. Dissimilar to the results obtained with nonirradiated *Eimeria* parasites, resistance elicited by 20-kilorad-irradiated parasites was established in the absence of oocyst shedding. These findings are consistent with similar research on the upper intestinal coccidial species *E. acervulina* (9) and other apicomplexan parasites, such as *Plasmodium* sp. (18, 29).

The parasite antigen(s) that induces the protective response appears to be associated with sporozoite-infected host cells. However, sporozoite invasion of the intestinal epithelium alone is not sufficient to induce a protective response. This conclusion is based on the finding that parasites exposed to high doses of radiation (30 kilorads) were able to penetrate the host epithelial layer in numbers similar to those of nonirradiated and 20-kilorad-irradiated sporozoites but were incapable of eliciting resistance to challenge infection. It appears that intracellular sporozoite metabolism and/or limited meront development is required. As revealed by immunofluorescence staining with a MAb that recognizes a low-M_r metabolic antigen, nonirradiated or 20-kilorad-irradiated sporozoites, unlike parasites exposed to 30 kilorads of radiation, were active for up to 72 h postinfection. Under these culture conditions, nonirradiated parasites developed to the first- and second-generation merogonic stages. In contrast, 20-kilorad-irradiated parasites exhibited minimal development at 48 and 72 h and were not detected thereafter.

The immune mechanism induced by irradiated sporozoite-infected host cells and/or immature first-generation schizonts prevents one disease parameter (i.e., weight loss) but not the appearance of another (i.e., intestinal lesions). This is consistent with previous research utilizing drugs that inhibit schizont development (8). Recent attempts to achieve weight gain and lesion score protection against *E. tenella* challenge by immunization with a foreign host-specific coccidium, *E. adenoeides*, were successful as judged by the former parameter but not as judged by the latter (2, 3). As in the present work, no merogonic stages of *E. adenoeides* were detected in cross-sections of infected chicken gut epithelium, suggesting that intracellular asexual development had also been inhibited. Our data support the hypothesis that a toxic factor produced by inhibited parasites or the host itself in response to these parasites gives rise to intestinal lesions (8). It is intriguing that immunization with attenuated parasites (e.g., those exposed to 20 kilorads) does not prevent intestinal lesions and yet a patent infection with

nonirradiated parasites is effective against this parameter. It is possible that sporozoite- or immature-schizont-infected host cells induce two types of immune responses; one is involved in resistance to challenge by preventing intracellular merogonic development, and the other elicits tissue destruction by some unknown mechanism (e.g., delayed-type hypersensitivity). These findings are also consistent with past research which showed little correlation between weight gain protection and the absence of intestinal lesions in either medicated or vaccinated chickens (6; unpublished data).

The conclusion that sporozoite-infected host cells alone can elicit a protective response against *E. tenella* supports our hypothesis on a general mechanism for resistance against coccidial infections. During a challenge infection, the immune system may destroy infected cells that are surface expressing foreign antigen and thereby prevent spread of the parasite. Our hypothesis for *Eimeria* spp. must be modified to include immature schizonts, since these were detected both in vivo and in vitro in this study. Efforts to develop subunit coccidiosis vaccines may therefore need to concentrate on parasite genes that are expressed by sporozoite-infected cells. This may also require that expressed genes be complexed to the appropriate major histocompatibility complex antigens when presented to host immune system after immunization.

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