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A comparison of sporozoite transport after homologous and heterologous challenge in chickens immunized with the Guelph strain or the Florida strain of *Eimeria maxima*

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Abstract The two strains of *Eimeria maxima*, Guelph and Florida, used in this study were previously shown to only partially cross-protect immunologically with respect to lesion scores, weight gains and feed conversions after heterologous challenge. In this paper, we provide evidence that this partial lack of cross-protection is manifested at the level of sporozoite transport. In birds immunized and challenged with the homologous strain, sporozoites accumulated in the lamina propria and were blocked from further movement into the crypts by 72 h post-challenge, unlike the situation observed in naive birds. Fewer than 5% of sporozoites were found in the crypts by 72 h post-challenge. In immunized birds challenged with the heterologous strain, fewer sporozoites reached the crypts than in naive birds but at least four times as many sporozoites successfully migrated to the crypts, when compared with birds challenged with the homologous strain. The degree of cross-protection afforded by the heterologous strain as measured by sporozoite transport success was not equally reciprocal.

Introduction

Coccidiosis of domestic fowl is of major economic importance to the poultry industry. The disease is caused by intracellular protistan parasites of the genus *Eimeria* and is currently being controlled mainly through the continuous use of prophylactic medication. This has led to widespread drug resistance in the parasites and has renewed interest in the development of effective vaccines as an alternative means of controlling the disease. Both wild-type and attenuated oocysts of the various species are being used as vaccines today. Since there is no cross-protection between the seven species that infect chickens, these vaccines need to include all species (Shirley 1989; Shirley and Long 1990). The development of such vaccines has been further complicated by evidence of immunological variability between strains of the same species (Joyner 1969; Fitz-Coy 1992; Martin et al. 1997).

Lee (1993) demonstrated that although *E. maxima* was included in live vaccines, it did not always elicit sufficient immunity to subsequent challenge with heterologous strains. In a recent study, Martin et al. (1997) examined the extent of immunological cross-protection among five field strains of *E. maxima* isolated from various geographic regions in North America. Among these, the Florida and the Guelph strains only partially cross-protected against each other at the level of lesion score, weight gain and feed conversion. This is particularly important in the study of immunity to coccidiosis, as *E. maxima* has been shown to be highly immunogenic with near-complete protection against homologous challenge being afforded by previous infection (Lee and Fernando 1978).

The sporozoites of *E. maxima* enter villous epithelial cells of the chicken small intestine and are transported within mononuclear cells to the crypt epithelium, where further development of the parasite takes place (Fernando et al. 1987; Riley and Fernando 1988). Riley and Fernando (1988) also found that the pattern of transport of *E. maxima* sporozoites through the intestinal mucosa differed significantly between naive and immune chickens. In immune chickens, sporozoites within mononuclear cells tended to remain in the lamina propria for up to 72 h post-challenge (p.c.) rather than continue their migration to the crypt epithelium as was observed in naive chickens.

The focus of the current study was to determine whether the lack of complete cross-protection observed between the Florida (FL) and Guelph (GS) strains of *E. maxima* was also manifested at the level of sporozoite transport. The transport of *E. maxima* sporozoites through the intestinal mucosa was, therefore, examined

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over a period of 72 h p.c. in groups of naive and immunized chickens challenged with either the homologous or the heterologous strain of *E. maxima*.

Materials and methods

Domestic fowl

The Shaver strain of the White Leghorn chicken (male) was used in this study. Males were obtained as day-old chicks and raised in a coccidia-free isolation facility. In individual experiments, chickens were housed in disposable chicken cages in steam-cleaned rooms that had been disinfected with ammonia. One-week-old chickens were used in all experiments, except one in which 5-week-old chickens were used. They were given water and food ad libitum (unmedicated chick starter with 21% protein, containing no antibiotics and no anticoccidials).

Parasites

The GS and FL strains of *Eimeria maxima* were used in these studies. The GS strain was isolated in 1973 and has been maintained since in this laboratory at the University of Guelph, Guelph, Canada. The FL strain was isolated from litter samples obtained from commercial broiler houses in Florida in 1994 and 1995 (see Martin et al. 1997).

Oocysts used in this study were obtained from experimentally infected chickens. Procedures for the maintenance, isolation and sporulation of oocysts have been fully described (Long et al. 1976). Cultures used in these studies were less than 3 months old.

Homologous challenge of experimentally immunized chickens

Prior to examining the patterns of sporozoite transport in chickens, the ability of the two strains of *E. maxima* to afford complete protection against a homologous challenge infection was determined.

Two groups (A and B) of five 1-week-old chickens were used in this experiment. Chickens were each inoculated with 2×10^3 sporulated oocysts of either *E. maxima* GS (group A) or *E. maxima* FL (group B) respectively. Two weeks later, groups A and B were challenged with 50×10^3 sporulated oocysts of the homologous strain. Oocyst output (over 24 h) was monitored 5–8 days p.c., as indicated below.

This study was repeated using two groups of 5-week-old chickens. The inoculum dose was 5×10^3 sporulated oocysts and the challenge dose was 50×10^3 sporulated oocysts. Oocyst output was again monitored 5–8 days p.c.

The 24-h fecal samples were first examined for the presence of oocysts, using the technique of flotation in a saturated solution of sodium chloride. If fewer than 15 oocysts were found using salt flotation, then total 24-h fecal counts were not done, as the results were found to be unreliable. If 15 or more oocysts were found, the numbers of oocysts in the 24-h fecal collections were estimated, according to the method described by Long and Rowell (1958).

Sporozoite transport within the intestinal mucosa in immune and naive chickens

Sporozoite transport within the intestinal mucosa in naive chickens was compared with that in immune chickens challenged with either the homologous strain or the heterologous strain of *E. maxima*.

Four groups of 1-week-old chickens were used in this study (groups 1–3 had 24 chickens each and group 4 had three chickens). Group 1 was inoculated with *E. maxima* GS, group 2 with *E. maxima* FL and group 3 was not inoculated at this time. Each inoculated bird received 2×10^3 sporulated oocysts of the appropriate strain of *E. maxima*. Two weeks post-inoculation, the birds in each of groups 1, 2 and 3 were subdivided into two groups (A and B), each containing 12 birds. Birds in groups 1A (GS/GS) and 2A (FL/GS) were challenged with 10×10^6 oocysts of *E. maxima* GS each and birds in groups 1B (GS/FL) and 2B (FL/FL) were challenged with 10×10^6 oocysts of *E. maxima* FL each. Birds in groups 3A (0/GS) and 3B (0/FL) were inoculated with 10×10^6 oocysts of *E. maxima* GS and *E. maxima* FL each, respectively. Group 4 consisted of non-inoculated, non-challenged control chickens. Three chickens from each subgroup were killed at 7, 24, 48 and 72 h p.c. and their intestines were fixed in freshly prepared Carnoy's fixative (one part glacial acetic acid, six parts absolute ethanol, three parts chloroform) for 1.5 h. The tissue was transferred through 95% alcohol to 70% alcohol.

Three areas of the gut were routinely used for the detection of sporozoites: (1) duodenum just distal to the bile ducts, (2) jejunum midway between the duodenum and Meckel's diverticulum and (3) jejunum 5 cm above Meckel's diverticulum. Longitudinal segments of tissue from each of the three areas of gut were dehydrated through alcohol and embedded in paraffin at a temperature of less than 60 °C. Sections, 6–8 μm thick, were mounted on positively charged glass slides (Superfrost Plus, Fisher Scientific, Toronto, Canada) and dried at 37 °C. Sections were rehydrated, rinsed in water and an immunofluorescent staining technique was used to detect the sporozoites as described below.

Immunohistochemical detection of sporozoites

For tissues taken at 7 h and 24 h p.c., a mouse monoclonal antibody (mAb 1209) against refractile body protein (see Danforth and Augustine 1983) was used as the primary antibody. For tissues taken at 48 h and 72 h p.c., rabbit anti-sporozoite serum (RaSpz) prepared and used in an earlier study by Riley and Fernando (1988) was the primary antibody. The bound antibodies were detected using antibodies labeled with either goat-anti mouse fluorescein-isothiocyanate (GaM FITC) or goat anti-rabbit (GaR) FITC (Caltag Laboratories, San Francisco, Calif.).

Slides were viewed at 100×, 400× and 1,000× magnification on a Provis microscope (Olympus, New York) at a wavelength of 490–520 nm. Sporozoites within the intestinal mucosa were counted and recorded as described below.

Parasite counts

The numbers of parasites found in each of three compartments within the intestinal mucosa, i.e. the villous epithelium, lamina propria and crypt epithelium, were counted in 50 crypt-villous (CV) units (Riley and Fernando 1988) from each of the three areas of intestine described above. Only CV units that were sectioned longitudinally were used in this evaluation, so as to include the crypt and the entire length of the villus. The average number of parasites found in each of the three intestinal compartments (villous epithelium, lamina propria and crypt epithelium) at each of the four time periods was calculated for each chicken. For each subgroup, the average number of parasites within each mucosal compartment for each time period was determined and expressed as total parasites in each compartment per time period. The number of parasites per mucosal compartment was also expressed as a percentage of the total parasites detected per time period.

Statistical analysis

The effect of homologous or heterologous challenge in immunized birds on the distribution of sporozoites in each compartment of the mucosa over time was analyzed statistically using a split-plot analysis of variance. Counts of sporozoites in the villous epithelium, lamina propria and crypts were first calculated per 50 CV units. These numbers were then converted to common logarithms prior to analysis, in an attempt to stabilize any variance. The

statistical analysis was performed using the Mixed procedure of the SAS System for Windows (version 6.12; SAS Institute, Cary, N.C.).

A two-tailed *t*-test was used to compare the total parasites in the intestinal mucosa of two independent groups. Significance was determined at $P < 0.05$ for all groups.

Results

Homologous challenge of experimentally immunized chickens

Using a salt flotation technique, no oocysts were detected in the feces of 1-week-old chickens inoculated and challenged with *E. maxima* GS. A few oocysts (too few to count reliably using the 24 h fecal count technique) were detected in the feces of the 1-week-old birds inoculated and challenged with *E. maxima* FL. Five-week-old birds immunized and challenged with *E. maxima* FL or GS did not shed detectable oocysts.

Localization and pattern of transport of *E. maxima* sporozoites

Few sporozoites were found in the tissue sections taken from the duodenum. Sections taken from the region above Meckel's diverticulum contained more sporozoites than the duodenum. The area of the intestine that consistently contained the most sporozoites was the jejunum midway between the duodenum and Meckel's diverticulum.

The numbers of parasites found in each of the three intestinal compartments, the villous epithelium, lamina propria and crypt epithelium, at the four time periods were determined. In order to observe the trend(s) in parasite migration, the numbers of parasites were expressed as a percentage of total parasites detected per time period (Figs. 1 and 2).

At 7 h p.c., the majority of parasites were found in the villous epithelium in both naive birds and immune birds challenged with either the homologous or the heterologous strain of *E. maxima* (Figs. 1 and 2). In naive birds, the numbers of sporozoites in the epithelium decreased steadily, while the proportion in the lamina propria and crypt epithelium increased over the next 65 h (Figs. 1C and 2C). In the immunized groups that received either a homologous or heterologous challenge, the numbers of parasites in the villous epithelium also decreased in a pattern similar to the control groups. However, a large proportion of the sporozoites in both these groups remained in the lamina propria. In the groups with the homologous strain, 75–95% of sporozoites remained in the lamina propria, compared to between 60–80% in the heterologous-challenge groups (Figs. 1 and 2). There were noticeable differences in the sporozoite migration patterns between the two groups challenged with the heterologous strains of *E. maxima*. At 24 h p.c. in the homologous-challenge groups, few parasites were seen in the crypts but sporozoites were still present in large numbers in the lamina propria. In the groups challenged with the heterologous strains, a larger number of parasites were seen in the crypts than in those challenged with the homologous strains. In the GS/FL groups, up to 40% of sporozoites were observed in the crypt epithelium at 48 h p.c. In the FL/GS groups however, only about 20% of sporozoites were observed in the crypt epithelium at the same time period. Overall, it appeared that more parasites reached the crypt epithelium in the

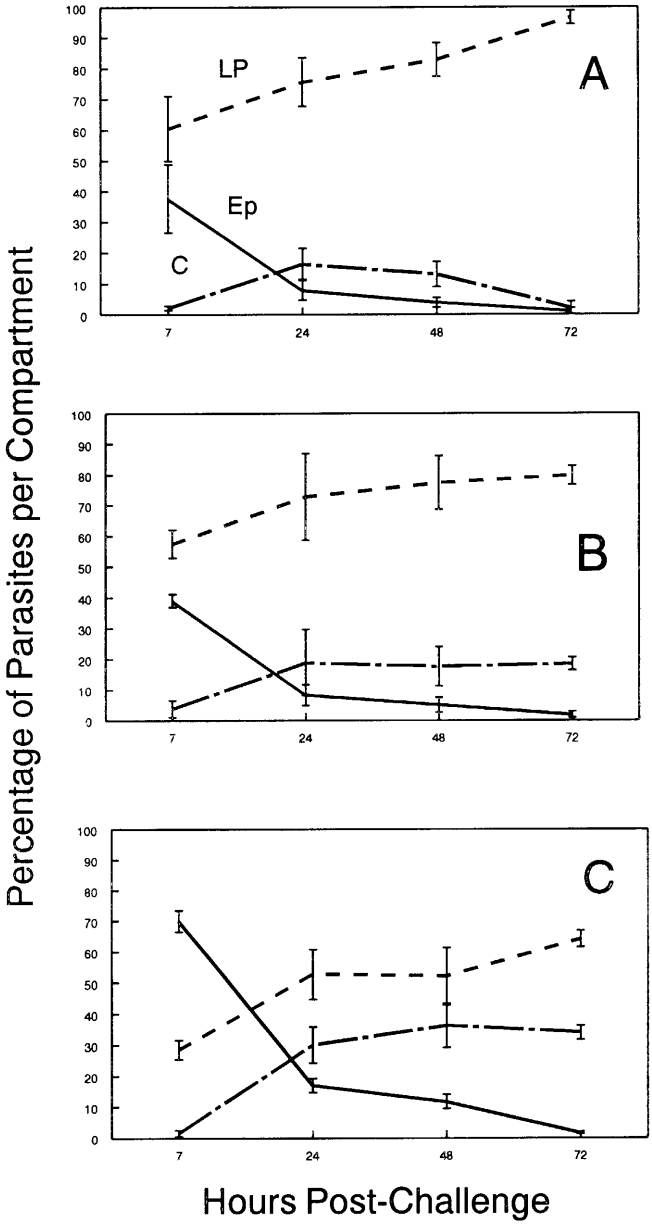

Fig. 1A–C Comparison of the distribution of *Eimeria maxima* in the intestines of chickens at 7, 24, 48 and 72 h post-challenge with the Guelph strain. Chickens were (**A**) immunized with the Guelph strain, (**B**) immunized with the Florida strain and (**C**) non-immunized controls. For each time period, the percentage of parasites per mucosal compartment is the mean percentage of total parasites in 50 crypt-villous units ± one standard deviation. *C* Crypt epithelium, *Ep* villous epithelium, *LP* lamina propria

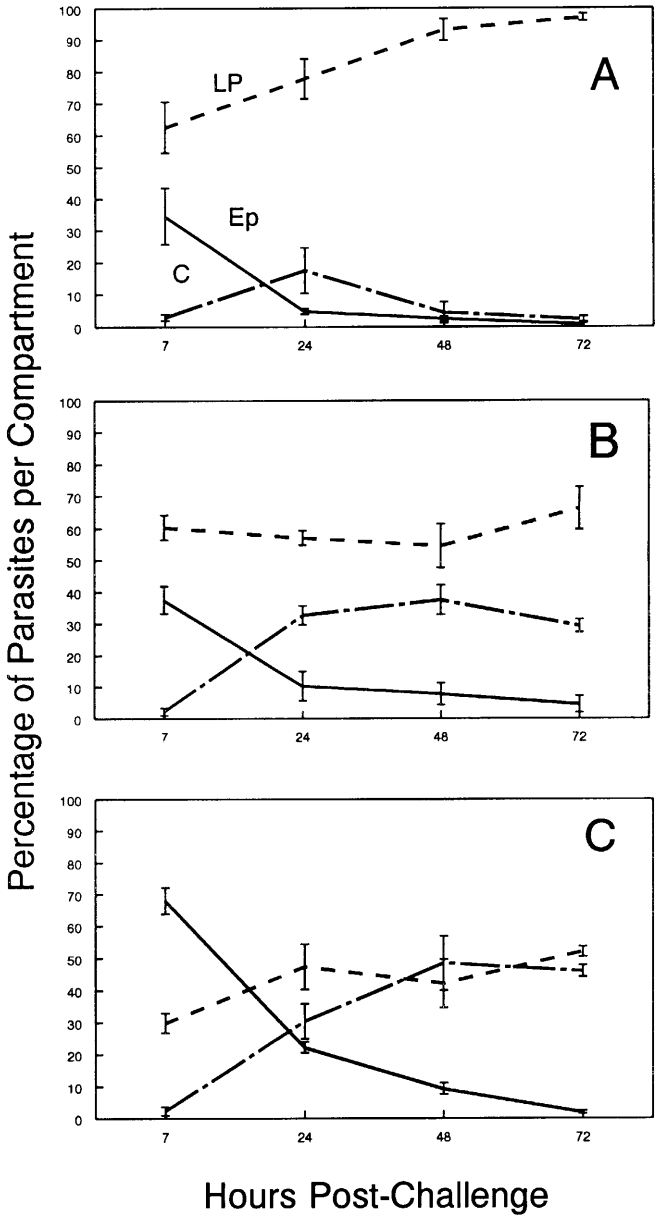

Fig. 2A–C Comparison of the distribution of *E. maxima* in the intestines of chickens at 7, 24, 48 and 72 h post-challenge with the Florida strain. Chickens were (**A**) immunized with the Florida strain, (**B**) immunized with the Guelph strain and (**C**) non-immunized controls. For each time period, the percentage of parasites per mucosal compartment is the mean percentage of total parasites in 50 crypt-villous units ± one standard deviation

chickens immunized with *E. maxima* GS and challenged with *E. maxima* FL than in those immunized with *E. maxima* FL and challenged with *E. maxima* GS (Figs. 1B and 2B).

Table 1 shows the results of a statistical analysis of the pattern of transport of sporozoites in control chickens and in immune chickens that received either a homologous or a heterologous challenge. No significant difference in the pattern of transport between the villous epithelium and the lamina propria was found, except between groups FL/FL and GS/FL. The pattern of

Table 1 Results of a statistical analysis of the pattern of transport of sporozoites in naive and immunized chickens challenged with either the homologous or the heterologous strain of *E. maxima*. The patterns of transport between the intestinal epithelium (*e*) and the lamina propria (*l*) and between the lamina propria and the crypt epithelium (*c*) were analyzed between: (1) homologous- and control-challenge groups, (2) heterologous- and control-challenge groups and (3) homologous- and heterologous-challenge groups. *DDF* Degrees of freedom for error statement, *NDF* degrees of freedom for contrast statement

Source	NDF	DDF	*F* value	$P_r > F$
0/FL vs FL/FL e→l	1	96	7.41	0.0077
0/FL vs FL/FL l→c	1	96	87.20	4.0×10^{-15}*
0/FL vs GS/FL e→l	1	96	2.52	0.1155
0/FL vs GS/FL l→c	1	96	1.99	0.01611
0/GS vs GS/GS e→l	1	96	1.45	0.2320
0/GS vs GS/GS l→c	1	96	67.72	9.0×10^{-13}*
0/GS vs FL/GS e→l	1	96	0.09	0.7592
0/GS vs FL/GS l→c	1	96	4.86	0.0298
FL/FL vs GS/FL e→l	1	96	18.58	4.0×10^{-5}*
FL/FL vs GS/FL l→c	1	96	62.82	4.0×10^{-12}*
GS/GS vs FL/GS e→l	1	96	2.28	0.1343
GS/GS vs FL/GS l→c	1	96	36.29	3.0×10^{-8}*

* Patterns of sporozoite transport found to be significantly different ($P < 0.00857$, $F > 7.203$)

transport of parasites between the lamina propria and the crypt epithelium was significantly different between immunized groups challenged with the homologous strain and their respective non-immunized-challenge groups (Table 1). The pattern of transport of sporozoites between the lamina propria and the crypt epithelium was also significantly different between the heterologous-challenge groups and their respective homologous-challenge groups. However, there was no significant difference in the pattern of sporozoite transport from the lamina propria to the crypt epithelium between the groups given a heterologous challenge and their respective control non-immunized groups (Table 1).

The total number of parasites present in the intestinal mucosa was compared for the GS strain and FL strain (Figs. 3 and 4 respectively). At 7 h p.c., there were significantly more sporozoites present in the mucosa of the 0/GS group than in the mucosa of the FL/GS group ($P < 0.05$). At 48 h and 72 h p.c., there were significantly more sporozoites present in the mucosa of the FL/FL group than in the mucosa of the GS/FL group. Significantly more sporozoites were present in the mucosa of the GS/GS group than in the mucosa of the 0/GS group at 48 h p.c. ($P < 0.05$). A significantly higher number of sporozoites was found in the mucosa of the FL/FL group than in the mucosa of the 0/FL group ($P < 0.05$).

Discussion

Immunological responses in chickens to infection with *Eimeria* spp have been well documented (see Wakelin and Rose 1990; Lillehoj and Lillehoj 2000). Parasite-

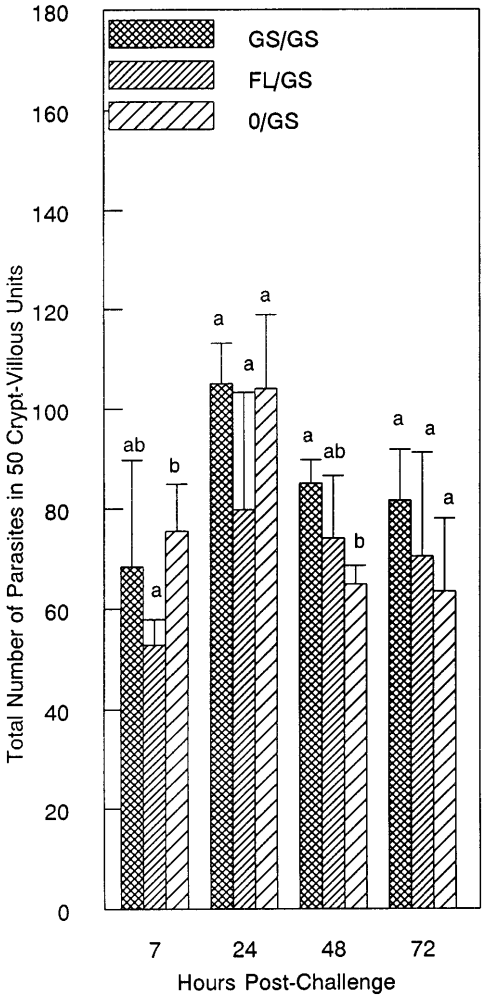

Fig. 3 Comparison of the total number of sporozoites of *E. maxima* in 50 crypt-villous units at 7, 24, 48 and 72 h post-challenge with the Guelph strain. For each time period, the total number of parasites in 50 crypt-villous units is the mean of the total numbers of parasites in 50 crypt-villous units in the three areas of the intestines examined in three chickens ± one standard deviation. Within a time period, means that are significantly different ($P < 0.05$) are denoted by different letters. *FL/GS* Chickens immunized with the Florida strain, *GS/GS* chickens immunized with the Guelph strain, *0/GS* non-immunized control chickens

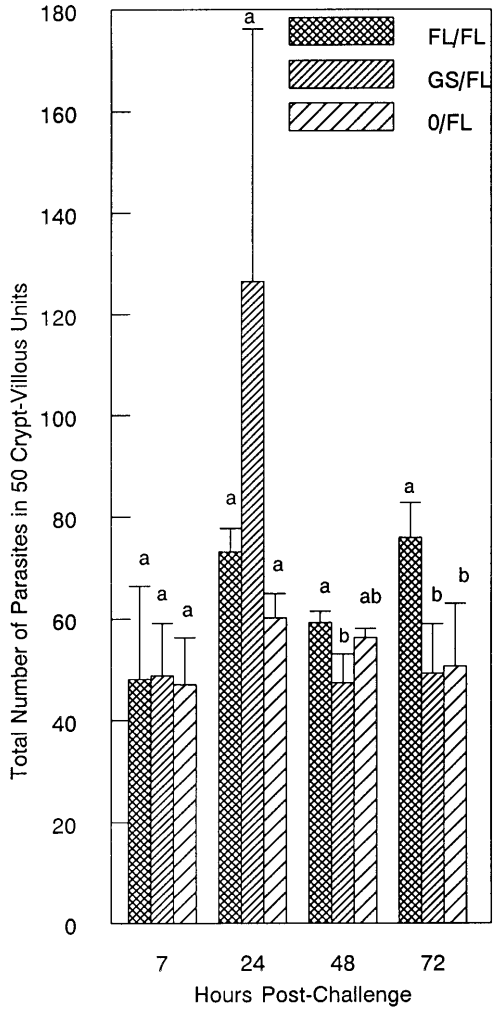

Fig. 4 Comparison of the total numbers of sporozoites of *E. maxima* in 50 crypt-villous units at 7, 24, 48 and 72 h post-challenge with the Florida strain. For each time period, the total number of parasites in 50 crypt-villous units is the mean of the total numbers of parasites in 50 crypt-villous units in the three areas of the intestines examined in three chickens ± one standard deviation. Within a time period, means that are significantly different ($P < 0.05$) are denoted by different letters. *FL/FL* Chickens immunized with the Florida strain, *GS/FL* chickens immunized with the Guelph strain, *0/FL* non-immunized control chickens

specific serum and biliary antibodies are detected 4–7 days after oral inoculation with oocysts of several eimerian species (Lillehoj and Ruff 1987), but the immune responses that control eimerian infections are T-cell dependent (Rose and Hesketh 1982; Lillehoj 1998). Rose and Hesketh (1982) in fact showed that lymphocytes taken from the spleen, lymph nodes, thoracic duct and peripheral blood of immune chickens were capable of transferring immunity to naive birds. Recent studies have concluded that it is the CD 8^+ cytokine T cells which are primarily responsible for protection against challenge infection with *Eimeria* spp. (Lillehoj 1998).

For *E. maxima* in particular, previous studies have shown that a single oocyst (Joyner and Norton 1976) or a single sporocyst (Lee and Fernando 1978) is capable of inducing immunological protection against a homologous challenge. The results reported here confirm that the GS and FL strains are able to induce protective immunity against a homologous challenge. A few oocysts were detected in the feces of the younger birds immunized and challenged with the FL strain of *E. maxima*. Martin et al. (1997) reported that *E. maxima* FL afforded 80–91% protection against homologous challenge (relative to unimmunized controls). The authors did not report the oocyst output of the challenged birds; and therefore it is not known whether the FL-immunized birds produced any oocysts. They also reported that *E. maxima* GS afforded 97–100% protection against homologous challenge (relative to unimmunized controls). These results may indicate a potential differ-

ence in the immunogenicity of the two strains of *E. maxima*. *E. maxima* GS affords essentially complete protection against homologous challenge and therefore no oocyst output would be expected. Since *E. maxima* FL affords only up to a 91% protection, there is a potential for the production of oocysts.

Several studies have provided evidence that protective immunity does target the early stages of sporozoite migration (Tyzzer et al. 1932; Horton-Smith et al. 1963; Rose et al. 1984). The development of immunity to *E. maxima* appears to be associated (in part) with a high proportion of the sporozoites remaining in the lamina propria and not continuing their migration to the intestinal crypts (Riley and Fernando 1988). In the current study, both immunized groups given a homologous challenge had high percentages (75–95%) of sporozoites in the lamina propria up to 72 h p.c.

The patterns of sporozoite transport in the groups challenged with the heterologous strain indicate that only partial protection is afforded by the initial immunizing strain of *E. maxima*. It appears however that initial immunization with the FL strain imparts a greater level of immunity against the GS strain than vice versa. This resulted in a greater number of sporozoites reaching the crypt epithelium in the GS/FL group than in the FL/GS group, supporting the findings of Martin et al. (1997; see Figs. 1B and 2B).

This study has shown that partial recognition occurs at the level of sporozoite transport between the two strains of *E. maxima*. Sporozoite counts however, showed a greater percentage of parasites reaching the crypt epithelium in the GS/FL group (40%) than in the FL/GS group (20%), indicating that *E maxima* FL afforded more protection to a challenge with *E. maxima* GS than vice versa. Oocyst production by the heterologous-challenge groups was not monitored in the current study, nor by Martin et al. (1997). If oocysts shed by GS-immunized birds challenged with *E. maxima* FL were collected and passaged repeatedly through GS-immunized birds, could this cause an immunological drift such that *E. maxima* GS no longer imparts any protection against the newly created *E. maxima* strain? These two strains of *E. maxima* could then be analyzed for any differences that might help us understand the basis for this lack of inter-strain cross-protection. The antigenic differences between FL and GS strains still remain unknown. Barta et al. (1998) have demonstrated that the sporozoite proteins of the two strains of *E. maxima* (GS and FL) are indistiguishable by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis. The authors suggested that either the strain variation may not be visible using this type of analysis or the antigenic variance is expressed by another stage of the life cycle.

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