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Some aspects of *Leucocytozoon caulleryi* reinfection in chickens*

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Abstract. The role of first- and second-generation schizonts in acquired immunity to *Leucocytozoon caulleryi* in chickens was studied. The chickens, which had recovered from a primary infection with various doses of sporozoites at 22–95 days of age, were challenged with sporozoites. First-generation merozoites were found in all of the challenged chickens, but no second-generation merozoites and gametocytes were seen in 30 of 32 chickens challenged with sporozoites. Almost all of the chickens that had recovered from a primary infection with sporozoites showed complete resistance to reinfection, and those that had recovered from a primary infection with first-generation merozoites showed resistance to reinfection with sporozoites. These results indicate that the second-generation schizont of *L. caulleryi* appears to be more immunogenic than the first-generation schizont and that some immune factors acquired by the chickens in the second generation of schizogony may inhibit the development of second-generation schizonts.

Leucocytozoon caulleryi is a pathogenic protozoan of chickens commonly found in several Asian countries (Mathis and Léger 1909; Fallis et al. 1974; Morii et al. 1981). It has been reported (Morii and Kitaoka 1970; Morii et al. 1986) that chickens that had recovered from a primary infection with *L. caulleryi* showed complete resistance to reinfection and that acquired immunity to *L. caulleryi* in chickens might be expressed against schizogony. *L. caulleryi* undergo the first and second generations of schizogony in various tissues and organs of chickens. Sporozoites invade the endothelial cells of such organs as the liver, spleen, and lung and develop into first-generation schizonts, which mature and release first-generation merozoites between the 5th and 7th day postinfection. These merozoites, in turn, invade the endothelial cells of various tissues and organs and become second-generation schizonts that release second-generation merozoites on day 14 postinfection. The second-generation merozoites invade erythrocytes and become gametocytes.

The immunogenic relationship between first- and second-generation schizonts in acquired immunity to *L. caulleryi* in chickens has not yet been well studied. The present study was undertaken to observe the roles of first- and second-generation schizonts in the acquisition of immunity to *L. caulleryi* in chickens.

Materials and methods

The Shizuoka strain of *L. caulleryi,* isolated in July 1977 from a naturally infected chicken in Shizuoka Prefecture, Japan, was used throughout the present experiments. Since its isolation this strain has been maintained by cyclic transmission in chickens, and the vector, *Culicoides arakawae,* were colonized at the authors' laboratory. Male white leghorn chickens, 22–95 days of age at primary infection, served as experimental birds. The procedures for rearing and feeding *C. arakawae* for infection with *L. caulleryi* and preparing sporozoites for inoculation into chickens were as described in previous reports (Morii and Kitaoka 1968a–c; Morii et al. 1984, 1986). Morii and Kitaoka (1970) have reported that chickens primarily inoculated with sporozoites of *L. caulleryi* at an age of <11 days were less resistant to reinfection than chickens inoculated

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at 14 days of age. To observe the occurrence of reinfection in chickens that had recovered from a primary infection with sporozoites at an age of >14 days, those that had recovered from a primary infection with doses varying from 5×10^1 to 1×10^3 sporozoites were subsequently challenged with doses varying from 1×10^4 to 1.5×10^5 sporozoites at various times after the disappearance of second-generation merozoites and gametocytes from their peripheral blood.

To examine the appearance and infectivity of first-generation merozoites in the peripheral blood of chickens subjected to sporozoite reinoculation after a primary infection with the latter, 30-day-old chickens were inoculated I.V. with 2 ml peripheral blood samples collected from each recovered chicken on days 5 and 6 after sporozoite reinoculation. The number of first-generation merozoites inoculated was estimated on the smears made from an aliquot of the peripheral blood samples; the smears were stained with Giemsa stain. Blood smears of chickens reinoculated with sporozoites or inoculated with blood samples from recovered chickens were prepared daily and stained with Giemsa for determination of the occurrence of reinfection or infection, respectively. The parasites were counted by the method previously described by Morii et al. (1986).

To observe the acquisition of immunity to *L. caulleryi* in chickens infected with first-generation merozoites, 10 chickens that had recovered from a primary infection with such merozoites were subsequently challenged with sporozoites within 10 days after the disappearance of parasites from their peripheral blood. The first-generation merozoites were obtained from the peripheral blood of chickens on the 5th day after sporozoite inoculation. Blood smears of the challenged chickens were prepared daily and stained with Giemsa stain to monitor the occurrence of reinfection. The occurrence of infection or reinfection was determined by the appearance of second-generation merozoites and gametocytes in the peripheral blood.

To detect second-generation schizonts in various tissues and organs of chickens subjected to sporozoite challenge after a primary infection with sporozoites or first-generation merozoites, the chickens were killed on day 13 after sporozoite inoculation and their tissues and organs were examined by the method previously described by Kitaoka et al. (1972).

Morii (1972) recognized specific soluble antigens (serum-soluble antigens) and their antibodies in the sera of chickens infected with *L. caulleryi*. In the present study, serum-soluble antigens and antibodies were detected using a microscopic slide modification of the Ouchterlony double-diffusion-in-gel technique (Ouchterlony 1953). To observe the presence of serum-soluble antigens and the change in the titer of antibodies in chickens reinoculated with sporozoites, 5 chickens that had recovered from a primary infection with a dose of 2×10^2 sporozoites at 57 days of age were challenged with a dose of 1.2×10^5 sporozoites at 120 days of age. Serum samples were collected from the challenged chickens 1 week before and at weekly intervals for 8 weeks after sporozoite reinoculation. The titer of antibodies in the challenged chickens was determined using the method previously described by Morii et al. (1986).

Results

The appearance of first-generation merozoites and the rate of reinfection in chickens subjected to sporozoite reinoculation after a primary infection with various doses of sporozoites are summarized in Table 1. First-generation merozoites were found in all of the challenged chickens on days 5 and 6 after sporozoite reinoculation, but no second-generation merozoites or gametocytes were seen in 30 of 32 chickens challenged with sporozoites. These first-generation merozoites showed infectivity to chickens. Second-generation merozoites appeared in the peripheral blood of chickens inoculated with first-generation merozoites 9 days after inoculation, reached a peak at 10 days, and disappeared after 15 days. Gametocytes appeared at 14 days after inoculation, reached a peak at 16 days, and disappeared after 19 days. The duration of the appearance of second-generation merozoites and gametocytes in the peripheral blood of chickens inoculated with first-generation merozoites was similar to that in chickens infected with sporozoites.

Two chickens challenged with sporozoites in groups 3 and 6 were reinfected. The prepatent and patent periods of the reinfection were similar to those of the primary infection, but the degree of parasitemia of the former was lower than that of the latter. Second-generation merozoites appeared in the peripheral blood of chickens on day 14 after sporozoite reinoculation, reached a peak at 16 days, and disappeared after 20 days. Gametocytes first appeared at 19 days after inoculation, reached a peak at 21 days, and disappeared after 24 days; they appeared in the peripheral blood of reinfected chickens on days 19–22 after sporozoite reinoculation and showed infectivity to *C. arakawae*. Sporozoites were found in the salivary

Table 1. Resistance to reinfection with *L. caulleryi* in chickens

Group	First sporozoite inoculation		Second sporozoite inoculation			
	Dose of sporozoites	Age of chickens (days)	Dose of sporozoites	Age of chickens (days)	First-generation merozoites	Second-generation merozoites and gametocytes
1	5×10^1	22	1×10^4	70	4/4[a]	0/4[a]
2	1×10^2	29	1.2×10^5	106	4/4	0/4
3	2×10^2	42	1×10^5	230	5/5	1/5
4	1×10^3	52	2×10^4	108	4/4	0/4
5	2×10^2	57	1.2×10^5	120	5/5	0/5
6	3×10^2	65	6×10^4	125	5/5	1/5
7	1×10^3	80	1.5×10^5	190	3/3	0/3
8	1×10^3	95	1.5×10^5	250	2/2	0/2

[a] Number of positive chickens/number of chickens inoculated

Table 2. Development of *L. caulleryi* second-generation schizonts in chickens subjected to sporozoite challenge after a primary infection with sporozoites

First sporozoite inoculation		Second sporozoite inoculation			
Dose of sporozoites	Age of chickens (days)	Dose of sporozoites	Age of chickens (days)	First-generation merozoites	Second-generation schizonts
2×10^2	42	1.5×10^5	150	6/6[a]	0/6[a]
1×10^4	150	–		3/3	3/3

[a] Number of positive chickens/number of chickens inoculated

glands of *C. arakawae* on day 3 after feeding. Almost all of the chickens that had recovered from a primary infection with sporozoites showed complete resistance to reinfection.

Table 2 shows the results of the detection of second-generation schizonts in chickens subjected to sporozoite challenge after a primary infection with sporozoites. First-generation merozoites were observed in the peripheral blood of challenged chickens on days 5 and 6 after sporozoite reinoculation, but no second-generation schizonts were detected. Numerous second-generation schizonts were found in chickens primarily inoculated with sporozoites.

The antibody response to *L. caulleryi* in sera from five chickens subjected to sporozoite challenge after a primary infection with sporozoites is shown in Fig. 1. The antibody titers maintained a low level before sporozoite reinoculation, then gradually began to increase, reaching a high level at 21 and 28 days after sporozoite reinoculation, after which they gradually decreased.

First-generation merozoites appeared in the peripheral blood of challenged chickens on days 5 and 6 after sporozoite reinoculation, but no second-generation merozoites or gametocytes were found. Serum-soluble antigens were not observed in the sera from challenged chickens.

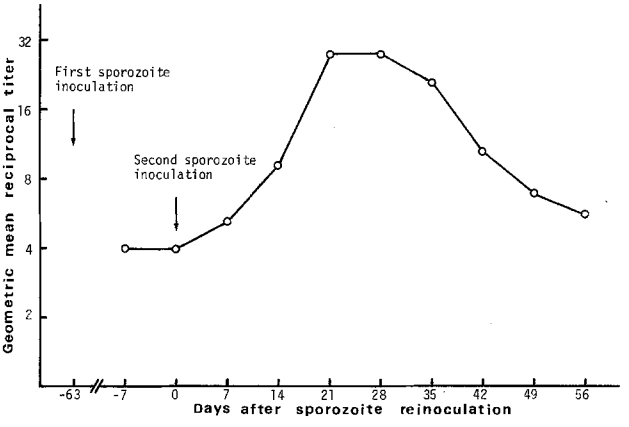

Fig. 1. Antibody response to *L. caulleryi* in five chickens challenged with sporozoites after a primary infection

The results of resistance to reinfection with sporozoites of chickens that had recovered from a primary infection with first-generation merozoites are summarized in Table 3. First-generation merozoites appeared in the peripheral blood on day 5 and 6 after sporozoite inoculation, but no second-generation merozoites, gametocytes, or sec-

Table 3. Resistance to reinfection with *L. caulleryi* sporozoites in chickens that recovered from a primary infection with first-generation merozoites

Primary infection with first-generation merozoites			Sporozoite challenge				
Dose of first-generation merozoites	Age of chickens (days)	Second-generation merozoites and gametocytes	Dose of sporozoites	Age of chickens (days)	First-generation merozoites	Second-generation schizonts[a]	Second-generation merozoites and gametocytes
3.2×10^3	35	5/5	1×10^5	60	5/5[b]	0/5[b]	
3.2×10^3	35	5/5	1×10^5	60	5/5		0/5[b]
–			1×10^3	60	5/5	5/5	
–			1×10^3	60	3/3		3/3

[a] The chickens were killed to detect second-generation schizonts on day 13 after sporozoite inoculation
[b] Number of positive chickens/number of chickens inoculated

ond-generation schizonts were found in challenged chickens. Various stages of the parasite were seen in chickens primarily inoculated with sporozoites.

Discussion

Some of the chickens primarily infected with sporozoites of *L. caulleryi* within 14 days of age were reinfected with sporozoites (Morii and Kitaoka 1970). In the present experiments, 2 chickens that had recovered from a primary infection with sporozoites at 42 and 65 days of age, respectively, were reinfected by the sporozoite challenge, but almost all of the chickens challenged with sporozoites after a primary infection at various ages showed complete resistance to reinfection. The results of the present study are similar to those of previous experiments (Morii and Kitaoka 1970; Morii et al. 1986).

First-generation merozoites appeared in all of the chickens; however, in almost all of the chickens challenged with sporozoites after a primary infection with sporozoites, no second-generation schizonts, second-generation merozoites, or gametocytes were found. These results indicate that sporozoites develop into first-generation schizonts, which release first-generation merozoites into the blood, but some immune factors acquired by the chickens in the course of the primary infection inhibit the development of second-generation schizonts.

Morii (1974) reported that serum-soluble antigens originated from second-generation schizonts. In the present experiments, serum-soluble antigens were used to examine the antibody titers in challenged chickens. The titers of antibody to *L. caulleryi* in sera from challenged chickens gradually began to increase after sporozoite reinoculation. These results indicate that the antigenicity of first-generation schizonts is similar to that of second-generation schizonts and that first-generation schizonts may stimulate the production of antibodies.

Although first-generation merozoites appeared in chickens subjected to sporozoite challenge after a primary infection with first-generation merozoites, no second-generation schizonts, second-generation merozoites, or gametocytes were found. From the results of the present experiments, it may be concluded that the second-generation schizont of *L. caulleryi* appears to be more immunogenic than the first-generation schizont and that acquired immunity to *L. caulleryi* in chickens may be expressed against second-generation schizogony.

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