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Immunogenicity of *Leucocytozoon caulleryi* sporozoites and their reactivity with specific immune sera

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Abstract The immunogenicity of *Leucocytozoon caulleryi* sporozoites for chickens and their reactivity in vitro with specific immune sera were studied. Almost all of the chickens that had been immunized with the sporozoite antigens survived the sporozoite challenge. The degree of parasitemia observed in the immunized chickens was significantly lower than that found in the nonimmunized chickens. Specific antibodies against sporozoites were tested by the circumsporozoite precipitation (CSP) reaction. Antibodies were demonstrated in the sera of chickens that had been immunized with the sporozoite antigens or chickens that had recovered from a primary infection with *L. caulleryi* sporozoites. When viable mature sporozoites were incubated in vitro with serum from immune chickens, agglutination and a long, thread-like precipitate at one end of the sporozoite could be seen within a few minutes under a phase-contrast microscope. The effects of specific immune serum on the infectivitiy of sporozoites were examined by the sporozoite neutralization activity (SNA) test. Sporozoites that had been incubated in vitro with serum from immune chickens lost their infectivity to chickens. The CSP reaction and the SNA test in *L. caulleryi* infection were stage- and species-specific.

Introduction

Since *Leucocytozoon caulleryi*, one of the pathogenic protozoa of chickens, was first observed by Mathis and Léger (1909) in the peripheral blood of domestic fowls in Tonkin in Southeast Asia, chicken leucocytozoonosis caused by infection with this parasite has been recognized in many Asian countries (Mathis and Léger 1909; Akiba 1970; Fallis et al. 1974; Morii et al. 1981, 1986). *L. caulleryi* infection starts when an infected *Culicoides* biting midge takes a blood meal from a chicken and simultaneously injects sporozoites into the bloodstream of the chicken (Akiba 1960; Morii and Kitaoka 1968c; Morii et al. 1984). Sporozoites transmitted to a chicken initiate the first generation of schizogony in the capillary endothelial cells in the spleen, lung, liver, and bursa of Fabricius (Morii et al. 1989; Morii and Fukuda 1992).

It has been reported that chickens that have recovered from a primary infection with *L. caulleryi* sporozoites are completely resistant to reinfection (Morii and Kitaoka 1970; Morii et al. 1986, 1989) and that the acquired immunity to this protozoan in chickens is strongly expressed against the second generation stages of schizogony (Morii et al. 1989, 1990).

The immunogenicity and characteristics of sporozoites of malaria parasites have been studied by many investigators, and the results of these studies have been reviewed by Nussenzweig and Nussenzweig (1989). However, the immunogenicity of *L. caulleryi* sporozoites for chickens has not yet been well studied. Therefore, the present study was undertaken to examine the immunogenicity of *L. caulleryi* sporozoites for chickens and their reactivity in vitro with specific immune sera from chickens.

Materials and methods

The Shizuoka strain of *Leucocytozoon caulleryi*, which has been maintained by cyclic transmission in chickens, and the vector *Culicoides arakawae*, colonized at the authors' laboratory (Morii and Kitaoka 1968a), were used throughout the present experiments. Male White Leghorn chickens aged 30–60 days served as experimental birds. The procedures for the rearing and feeding of *C. arakawae* for infection with *L. caulleryi* and for the preparation of sporozoites for inoculation into chickens were the same as those described in previous reports (Morii and Kitaoka 1968a–c; Morii et al. 1986).

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Female *C. arakawae* harboring sporozoites in their salivary glands on day 3 or 4 after infective blood meals were anesthetized slightly with chloroform and then ground in phosphate-buffered physiological saline (PBS, pH 7.2) in a loosely fitting glass microhomogenizer to obtain the sporozoites. The resulting homogenate was filtered through a sheet of 500-mesh stainless-steel gauze and poured into a test tube. The sporozoite suspension was centrifuged at 3,000 *g* for 15 min at 4° C. After centrifugagation the supernatant was discarded and the sediment, resuspended in PBS. This procedure of centrifugation and resuspension was repeated three times. The sporozoite concentration in the suspension was determined by means of a hemocytometer. Sporozoites suspended in PBS were homogenized thoroughly in a Teflon tissue grinder. The resulting suspension was mixed with 0.4% (v/v) formalin and then supplemented with an equal volume of PBS containing 1.54 mg $Al(OH)_3$ (as aluminum hydroxide gel) per milliliter as an adjuvant. The suspension was used as a source of sporozoite antigens.

Four separate experiments were conducted using a total of 1×10^8, 2×10^8, or 3×10^8 sporozoites as the immunizing agent and a dose of 1×10^4, 2×10^4, or 3×10^4 sporozoites as the challenging organism. The mean number of sporozoites per milliliter of antigen suspension was 5×10^7, 1×10^8, or 1.5×10^8. Each chicken was immunized with two intramuscular injections of 1 ml of the suspension of sporozoite antigens given at 2-week intervals. All chickens used in the immunization experiments were 30 days old at the time of the first immunization. Nonimmunized control chickens received injections of PBS containing 1.54 mg $Al(OH)_3$/ml on the same schedule. The chickens were challenged by intravenous inoculation of 1×10^4, 2×10^4, or 3×10^4 sporozoites on day 7 after the last immunization.

The mortality and the degree of parasitemia were affected by an increase in the number of sporozoites inoculated (Morii and Kitaoka 1969). To observe the degree of parasitemia in surviving chickens, a low dose of 1×10^4 sporozoites was used in experiment II. Blood smears from chickens challenged with sporozoites were prepared daily and stained with Giemsa stain for determination of the degree of parasitemia. The numbers of parasites and erythrocytes were counted by the methods previously described by Morii et al. (1986).

To examine the presence of antibodies against sporozoites in chickens immunized with the sporozoite antigens, serum samples were collected from all chickens just before sporozoite challenge. The presence of humoral antibodies to sporozoites was examined by means of the circumsporozoite precipitation (CSP) reaction described by Vanderberg et al. (1969) in rodent malaria. A 0.01-ml drop of the immune serum and a 0.01-ml drop of a suspension of viable sporozoites isolated from the salivary glands of *C. arakawae* on day 3 or 4 after infective blood meals in Medium 199 (Morgan et al. 1950) were placed close together on a microscope slide and then mixed. The sporozoite suspension had a concentration of 5×10^5 sporozoites/ml. The cover slip (24×24 mm) was placed on top of the suspension and the preparation was then sealed with nail polish. These preparations were incubated at 37° C for 60 min. Each slide preparation was examined under a phase-contrast microscope.

L. caulleryi sporozoites isolated from the salivary glands of *C. arakawae* beginning 3 days after the infective blood meals showed a high degree of infectivity to chickens (Morii et al. 1984). It has been suggested that the surface component of sporozoites that is recognized by anti-sporozoite antibodies and the infectivity of sporozoites are closely related as judged from the results obtained with *Plasmodium* sporozoites (Nussenzweig and Nussenzweig 1989). To examine the relationship between the CSP reactivity and the infectivity of immature or mature *L. caulleryi* sporozoites, sporozoites isolated from various sites in *C. arakawae* at different times after infective blood meals were used in the present experiments. The procedures used for the isolation of sporozoites from different sources within *C. arakawae* and for their inoculation into chickens were the same as those described in a previous report (Morii et al. 1984). Immune serum from chickens immunized with the sporozoite antigens was used for the CSP reaction.

The effect of immune serum on the infectivity of sporozoites was examined by the sporozoite neutralization activity (SNA) test, which was designated by Nussenzweig et al. (1969). Viable sporozoites suspended in Medium 199 were incubated in vitro at 37° C for 15 min with an equal volume of undiluted immune serum. Following this step, sporozoite suspensions were injected intravenously into normal chickens. Control chickens received sporozoites of the same batch that had been incubated with normal serum under the same conditions. Blood smears of the chickens that had been inoculated with sporozoites were prepared daily after inoculation and stained with Giemsa stain to monitor the status of infection.

To examine the stage specificity of the CSP reaction and the SNA test in *L. caulleryi* infection, antisera against the antigens of second-generation schizonts, second-generation merozoites, and gametocytes were obtained from chickens that had been immunized with the respective antigens. The antigens used for immunization were prepared by the technique described in a previous report (Morii 1972).

To observe the species specificity of these reactions, serum samples were also collected from chickens that had recovered from an artificial or a natural infection with sporozoites of *L. caulleryi*, *L. sabrazesi*, or *Plasmodium juxtanucleare*. The Nara strain of *P. juxtanucleare*, which had been maintained by cyclic transmission in chickens and colonized *Culex pipiens pallens*, was used in the present study. The CSP reaction of *P. juxtanucleare* sporozoites with various immune sera was examined.

Results

Immunogenicity of sporozoites for chickens

The immunizing effects of the sporozoite antigens against *Leucocytozoon caulleryi* infection in chickens are summarized in Table 1. Almost all of the immunized chickens survived the sporozoite challenge. All nonimmunized chickens (experiments I, III, and IV) died on day 13 after sporozoite challenge and showed typical macroscopic changes of leucocytozoonosis in their internal organs at autopsy. The degree and the duration of parasitemia in chickens challenged with sporozoites in experiment II are shown in Fig. 1. Two nonimmunized chickens died on day 14 after sporozoite challenge. The geometric means of counts of merozoites and gametocy-

Table 1 Immunizing effect of sporozoite antigens against *Leucocytozoon caulleryi* sporozoite infection in chickens

Experiment number	Immunizing dose of sporozoites	Sporozoite challenge		
		Dose of challenge sporozoites	Rate of infection[a]	Mortality[b]
I	1×10^8	2×10^4	5/5	1/5
	None	2×10^4	5/5	5/5
II	1×10^8	1×10^4	8/8	0/8
	None	1×10^4	8/8	2/8
III	2×10^8	2×10^4	10/10	0/10
	None	2×10^4	10/10	10/10
IV	3×10^8	3×10^4	8/8	0/8
	None	3×10^4	8/8	8/8

[a] Number of chickens infected/number of chickens challenged
[b] Number of chickens that died/number of chickens challenged

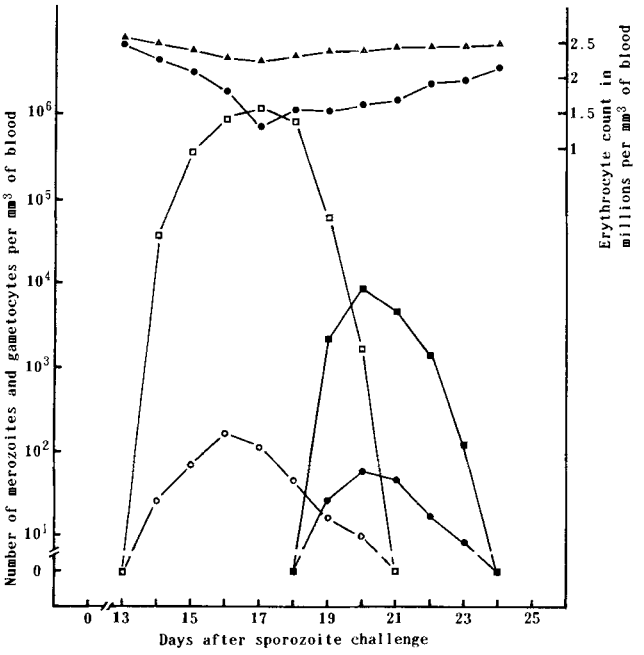

Fig. 1 Mean levels of parasitemia found in immunized chickens (*white circles* merozoites, *black circles* gametocytes) and nonimmunized control chickens (*white squares* merozoites, *black squares* gametocytes) following challenge with *Leucocytozoon caulleryi* sporozoites. *Right*: Mean erythrocyte counts obtained in immunized chickens (*triangles*) and nonimmunized control chickens (*black circles*)

tes and the erythrocyte counts determined in each group are shown. The prepatent period and the duration of parasitemia determined in immunized chickens after sporozoite challenge were essentially the same as those found in nonimmunized chickens, but the degree of parasitemia observed in the former was significantly lower than that found in the latter. Severe anemia was observed in the intermediate phase of parasitemia in non-immunized chickens.

CSP reaction

When viable mature sporozoites were placed in sera from chickens immunized with the sporozoite antigens of *L. caulleryi*, sporozoite agglutination and a long, thread-like precipitate could be observed at one end of the sporozoite by a phase-contrast microscope (Fig. 2). Sporozoite agglutination and the CSP reaction occurred within a few minutes, but a 60-min incubation at 37° C was uniformly carried out for determination of the reaction. No such reaction was observed when sera from nonimmunized control chickens were used (Fig. 3).

Table 2 shows the summarized results of the CSP reaction of *L. caulleryi* sporozoites. All chickens immunized with the sporozoite antigens showed the production of CSP antibodies on the day before sporozoite challenge. The CSP reaction was also observed in serum from chickens that had recovered from a primary infection with *L. caulleryi* sporozoites. *L. caulleryi* sporozoites showed no CSP reactivity in sera from chickens that had recovered from a natural infection with *L. sabrazesi* or an artificial infection with *Plasmodium juxtanucleare* sporozoites. *P. juxtanucleare* sporozoites reacted only with serum from chickens that had recovered from infection with the homologous sporozoites. No CSP reaction was observed in sera from chickens immunized with the antigens of each developmental stage of *L. caulleryi* except for anti-sporozoite serum.

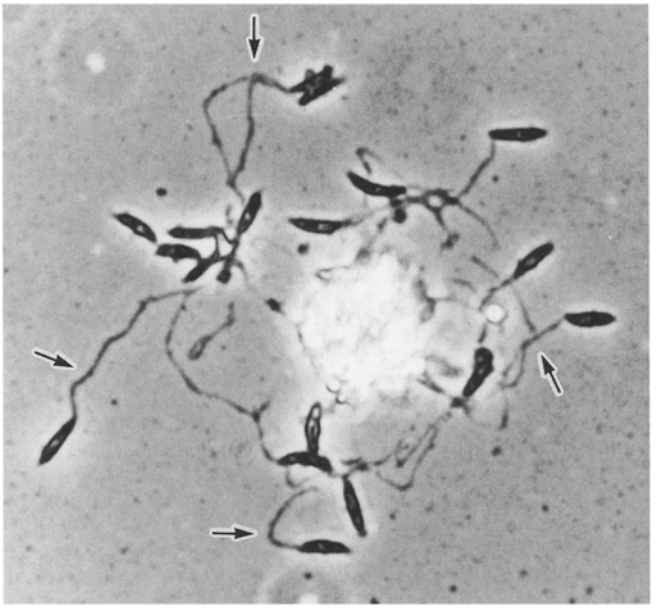

Fig. 2 Positive CSP reaction of *L. caulleryi* sporozoites as observed under a phase-contrast microscope. Sporozoite agglutination and a long thread-like precipitate that was formed at one end of the sporozoite (*arrows*) are visible

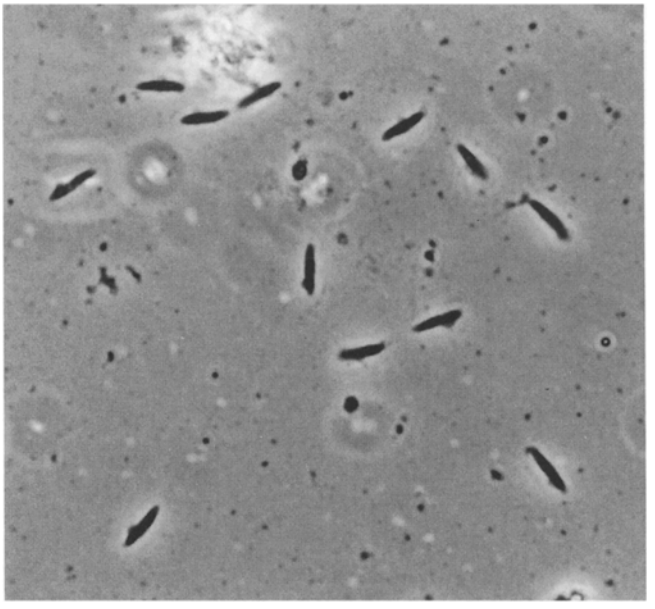

Fig. 3 Negative CSP reaction of *L. caulleryi* sporozoites as observed under a phase-contrast microscope. No circumsporozoite precipitate has been formed

Table 2 CSP reaction of *L. caulleryi* sporozoites with various immune sera from chickens (+ Positive reaction, – negative reaction)

Immune serum	CSP reaction
Anti-*L. caulleryi* sporozoite serum	+
Anti-*L. caulleryi* 2nd-generation schizont serum	–
Anti-*L. caulleryi* 2nd-generation merozoite serum	–
Anti-*L. caulleryi* gametocyte serum	–
Serum from chickens that recovered from a primary infection with a dose of 5×10^2 sporozoites of *L. caulleryi*	+
Serum from chickens that recovered from a natural infection with *L. caulleryi*	+
Serum from chickens that recovered from a natural infection with *L. sabrazesi*	–
Serum from chickens that recovered from a primary infection with a dose of 5×10^3 sporozoites of *Plasmodium juxtanucleare*	–
Normal serum	–

Table 3 CSP reactivity and infectivity of *L. caulleryi* sporozoites from different sources within *Culicoides arakawae* at different times after infective blood meals

Days after infective meal	Source of sporozoites	CSP reactivity	Rate of infection[a]
	Midguts	–	0/5
2	Abdominal hemocoel	–	0/5
	Thoracic hemocoel	–	0/5
	Salivary glands	–	0/5
3	Salivary glands	+	5/5
4	Salivary glands	+	5/5
5	Salivary glands	+	5/5
10	Salivary glands	+	5/5

[a] Number of chickens infected/number of chickens inoculated with a dose of 1×10^3 sporozoites

Table 4 *L. caulleryi* SNA of various immune sera from chickens

Immune serum	Rate of infection[a]
Anti-*L. caulleryi* sporozoite serum	0/5
Anti-*L. caulleryi* 2nd-generation schizont serum	5/5
Anit-*L. caulleryi* 2nd-generation merozoite serum	5/5
Anti-*L. caulleryi* gametocyte serum	5/5
Serum from chickens that recovered from a primary infection with a dose of 5×10^2 sporozoites of *L. caulleryi*	0/5
Serum from chickens that recovered from a natural infection with *L. caulleryi*	0/5
Serum from chickens that recovered from a natural infection with *L. sabrazesi*	5/5
Serum from chickens that recovered from a primary infection with a dose of 5×10^3 sporozoites of *P. juxtanucleare*	5/5
Normal serum	5/5

[a] Number of chickens infected/number of chickens inoculated with a dose of 1×10^3 sporozoites that had been incubated in vitro with each serum sample

Table 3 shows the results recorded for the CSP reactivity and the infectivity of *L. caulleryi* sporozoites isolated from various sites of *Culicoides arakawae* after infective blood meals. Mature sporozoites isolated from the salivary glands of biting midges at 3, 4, 5, and 10 days after infective blood meals showed CSP reactivity and infectivity to chickens. Sporozoites obtained from the midguts, the abdominal and thoracic hemocoel, and the salivary glands of biting midges on day 2 after infective blood meals did not show CSP reactivity or infectivity to any of the chickens inoculated.

SNA test

The results obtained regarding the effects of various immune sera on the infectivity of *L. caulleryi* sporozoites are summarized in Table 4. Sporozoites preincubated with anti-*L. caulleryi* sporozoite serum or with serum from chickens that had recovered from infection with *L. caulleryi* sporozoites lost their infectivity to chickens. The other serum samples tested did not show any effect on the infectivity of sporozoites.

Discussion

Leucocytozoon caulleryi sporozoites invade the capillary endothelial cells of several organs of chickens and develop into first-generation schizonts (Morii et al. 1989; Morii and Fukuda 1992). The degree of parasitemia and the mortality in chickens infected with *L. caulleryi* rise with an increase in the number of sporozoites inoculated (Morii and Kitaoka 1969).

In the present study, almost all of the chickens that had been immunized with the sporozoite antigens survived the sporozoite challenge. The results presented herein suggest that immunization with the sporozoite antigens protects chickens against *L. caulleryi* sporozoite infection. This protection may be attributed to partial suppression of the development of *L. caulleryi* sporozoites in immunized chickens after sporozoite challenge. Although further research is needed to determine the immunogenic components of sporozoites and the immunological mechanisms against *L. caulleryi* sporozoite infection in chickens, the sporozoite antigens of *L. caulleryi* may be useful as a preventive immunological measure against *L. caulleryi* infection in chickens.

All chickens that had been immunized with the sporozoite antigens or chickens that had recovered from a primary infection with *L. caulleryi* sporozoites produced circulating antibodies against sporozoites. These results indicate that sporozoites of *L. caulleryi* have antigenicity to chickens and that the CSP reaction is available for the detection of antibodies against *L. caulleryi* sporozoites.

Agglutination and the CSP reaction of sporozoites of malaria parasites in the presence of immune sera have been recognized (Mulligan et al. 1940; Vanderberg et al.

1969). These reactions are stage- and species-specific. In the present experiments, chickens that had been immunized with the sporozoite antigens of *L. caulleryi* or had recovered from a primary infection with *L. caulleryi* sporozoites produced agglutination, CSP, and SNA antibodies. The morphology of the CSP reaction of *L. caulleryi* sporozoites was similar to that described by Vanderberg et al. (1969) in *P. berghei* infection. These reactions of *L. caulleryi* sporozoites may occur by the same mechanisms as do those of malaria parasites.

In the present experiments, mature sporozoites isolated from the salivary glands of biting midges between the 3rd and 10th day after infective blood meals showed CSP reactivity and infectivity to chickens, but immature sporozoites isolated from various sites in biting midges on day 2 after feeding did not show these reactions. These results indicate that the cell-surface membrane of immature *L. caulleryi* sporozoites lacks certain substances that play an important role in their infectivity in vivo and in their CSP reactivity.

Mature sporozoites that had been incubated with anti-sporozoite serum or serum from chickens that had recovered from infection with *L. caulleryi* sporozoites lost their infectivity to chickens. The CSP reaction and the SNA test in *L. caulleryi* infection in chickens were found to be stage- and species-specific.

Further studies are needed to clarify the relationship among the CSP reactivity, infectivity, and immunogenicity of immature or mature *L. caulleryi* sporozoites and their surface components.

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