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Immunization of Chickens with Schizont and Serum-soluble Antigens of *Leucocytozoon caulleryi* Against Leucocytozoonosis

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Abstract

Three-week-old chickens were immunized with schizont antigen or serum-soluble antigen of *Leucocytozoon caulleryi* to observe the protective effect against chicken leucocytozoonosis. The chickens thus treated were challenged with live sporozoites 2 weeks after the last immunization. Changes in clinical signs, parasitemia and antibody response were observed. In chickens immunized with schizont antigen, the reduction in the severity of sickness was noticeable although similar reduction was not noticeable in chickens immunized with serum-soluble antigen. Antibody responses were different between the two groups of chickens. Results indicated that schizont antigen and serum-soluble antigen of *L. caulleryi* were rather different and the immunization of chickens with schizont antigen had a somewhat protective effect against chicken leucocytozoonosis.

Key words: *Leucocytozoon caulleryi*, chicken, protozoa, immunization, schizont antigen, serum-soluble antigen

Introduction

Leucocytozoon caulleryi, a causative agent of chicken leucocytozoonosis, was first described by Mathis and Leger (1909), and in Japan by Akiba *et al.* (1958). Leucocytozoonosis in chickens has been observed in various Asian countries and affects the productivity of the poultry industry by causing a reduction in egg production, weight loss and sometimes death.

Chicken leucocytozoonosis has been controlled by the use of drugs such as pyrimethamine (Akiba *et al.*, 1963) and some sulfa drugs (Akiba *et al.*, 1964). These compounds will continue to be an important factor in the prevention of chicken leucocytozoonosis for the foreseeable future, but there are some problems arising from this type of treatment. In the field, *L. caulleryi* have been repeatedly exposed to the above drugs, and as a result, some *L. caulleryi* isolates are showing a decrease in drug sensitivity (Akiba, 1970). This problem has been offset somewhat by the introduction of new compounds, but few new compounds are being cleared for use. Moreover, in Japan, using any drug on laying hens is prohibited by law because the drug remains in the eggs and may be harmful to humans when eaten. Should this trend continue, the poultry industry will have to find other means of effective control. One alternative approach that has been suggested for many years is the use of immunity to control *L. caulleryi* infection. It has been well documented that chickens develop a good protective immune response to leucocytozoonosis after infection with the live parasite (Morii and Kitaoka, 1970). Unfortunately, the chickens must become infected to develop immunity in such a case. Until recently, protection against leucocytozoonosis could not be obtained without using live *L. caulleryi*. This study attempts the immunization of chickens with nonviable schizont antigen and serum-soluble antigen of *L. caulleryi* to reduce in the severity of sickness.

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Materials and Methods

Parasites. The strain of *L.caulleryi.* used in the present experiment was derived from a naturally infected chicken in Gifu Prefecture, in July, 1982. It has been maintained at the authors' laboratory by cyclic transmission in specific-pathogen-free (SPF) chickens, the final host of this parasite, and in colonized *Culicoides arakawae,* the vector of the same. The procedures for rearing, breeding and feeding of *C. arakawae* for infection with *L.caulleryi* and the preparation of sporozoite suspension for inoculation of chickens were the same as that described previously (Isobe *et al.*, 1984).

Chickens. The PDL-1 strain of SPF chickens was used. They were maintained in the authors' laboratory in the same manner as that described by Furuta *et al.* (1980). All chickens had constant access to feed and water.

Antigens.

Serum-soluble antigen (*S.A.*): Six 39- to 78-day-old chickens were inoculated with 6.5×10^2 to 2.7×10^4 sporozoites intravenously. Thirteen days after inoculation, they were killed by exanguination. The sera were collected from them, pooled and clarified by centrifugation at 8,000 g, for 20 min at 4°C. The resulting supernatant was precipitated with 50% (v/v) saturated ammonium sulphate solution. The precipitates were dialyzed against phosphate buffered saline (PBS, pH 7.2) for 24 hr with two buffer changes. The precipitates were concentrated and assayed for antigenic activity. Antigenic activity was measured by the agar gel precipitation (AGP) test using the antiserum as described below. For the production of antiserum for *L.caulleryi,* five 56-day-old chickens were inoculated with 6×10^3 sporozoites intravenously. The birds were bled 28 days after inoculation. Sera were pooled and used as a reference antisera. The protein concentration of antigen was estimated by absorption at 280 nm in a spectrophotometer (Hitachi, 101). Antigen titer was 1:128 and the protein concentration was 20 mg/ml.

Schizont antigen: Twenty-six 66- to 89-day-old chickens were inoculated with 6×10^1 to 1×10^5 sporozoites intravenously. Thirteen to 15 days after inoculation, all chickens were dead or killed by exanguination. The spleens, kidneys and bursas of Fabricius were harvested from them and preserved at −80°C until use. The other antigen preparation methods were the same as those described previously (Isobe and Suzuki, 1986). The measurement of antigenic activity and protein concentration was the same as above. The antigen titer was 1:8 and the protein concentration was 43 mg/ml.

Immunization.

Antigens were injected into 21-day-old chickens intramuscularly with adjuvant (group 1) or PBS (group 2). As an adjuvant, Freund's complete adjuvant (Iatron Laboratories) was used. The volume of the injection was 1.0 ml; i.e., 0.5 ml of antigen and 0.5 ml of adjuvant or PBS. One ml of PBS was injected into non-immunized (group 4) and non-challenged controls (group 5) intramuscularly. One half ml of adjuvant mixed with 0.5 ml of PBS was injected into another group of non-immunized controls (group 3). For the second immunization, antigens were injected into immunized chickens (groups 1 and 2) intravenously 3 weeks after the first immunization. The volume of injection was 0.5 ml. Chickens in the groups of non-immunized (groups 3 and 4) and non-challenged controls (group 5) were sham-inoculated with 0.5 ml of PBS intravenously. Two weeks after the second immunization, chickens were challenged with sporozoites intravenously: 1×10^3 sporozoites were inoculated into chickens immunized with schizont antigen and 3.5×10^3 sporozoites were inoculated into chickens immunized with S. A. Two to 4 weeks after the challenge, blood smears were prepared everyday from all chickens, fixed in methanol, stained with Giemsa, and observed light microscopically. Clinical signs were also observed everyday in the same period. Sera were collected from all chickens at 5, 2 weeks and just before challenge, 2 and 4 weeks after challenge. Antibody titers were measured by AGP test using S. A. (Isobe and Akiba, 1982) and the enzyme-linked immunosorbent assay (ELISA)

with schizont antigen (Isobe and Suzuki, 1986).

Results

In all groups immunized with S. A., typical infections were observed after challenge. Following challenge, all chickens inoculated with the adjuvant (group 3) died due to the disease. Among chickens inoculated with S. A. in presence of the adjuvant (group 1), S. A. with PBS (group 2) and sham-inoculated with PBS (group 4), there was no remarkable difference in infection rate, mortality, parasitemia and clinical signs (Table 1).

On the other hand, in the immunization test using schizont antigen, the chickens immunized with schizont antigen and PBS (group 2) became infected after challenge. But the severity of the infection was slighter than that of the control ones (group 4) – among the chickens of group 2, no chicken died, the appearance of typical parasitemia was 57% and the clinical signs such as anemia and discharge of green feces were less noticeable than those of group 4 (Table 2). Between the chickens

Table 1 Responses of chickens immunized with serum-soluble antigen of *Leucocytozoon caulleryi* after challenge

Group	Number of chickens	1st immun.*	2nd immun.†	Challenge‡	Mortality (%)	Infection rate (%)	Detection rate of typical parasitemia§(%)	Clinical signs‖
1	10	S. A.#+Adj¶	S.A.	+	40	100	100	++
2	10	S.A.+PBS**	S.A.	+	40	100	100	++
3	10	Adj+PBS	PBS	+	100	100	ND††	ND
4	10	PBS	PBS	+	50	100	100	++
5	10	PBS	PBS	−	0	0	0	−

*: First immunization, intramuscularly.
†: Second immunization, intravenously.
‡: Challenged with 3.5×10^3 sporozoites of *L. caulleryi*.
§: Detection of merozoites from 14 to 18 days after infection and gametocytes from 18 to 23 or 24 days after infection in blood smears.
‖: Anemia, emaciation and discharge of green feces, ++: heavy, −: none.
#: serum-soluble antigen
¶: adjuvant
**: phosphate buffered saline
††: not done

Table 2 Responses of chickens immunized with schizont antigen of *Leucocytozoon caulleryi* after challenge

Group	Number of chickens	1st immun.*	2nd immun.†	Challenge‡	Mortality (%)	Infection rate (%)	Detection rate of typical parasitemia§(%)	Clinical signs‖
1	9	Sch#+Adj¶	Sch	+	22	100	100	++
2	7	Sch+PBS**	Sch	+	0	100	57	+
3	10	Adj+PBS	PBS	+	20	100	100	++
4	10	PBS	PBS	+	30	100	100	++
5	10	PBS	PBS	−	0	0	0	−

*: First immunization, intramuscularly.
†: Second immunization, intravenously.
‡: Challenged with 1×10^3 sporozoites of *L. caulleryi*.
§, ‖, #, ¶, **: The same as described in Table 1, ++: heavy, +: light, −: none.

immunized with schizont antigen emulsified with the adjuvant (group 1) and the chickens inoculated with the adjuvant (group 3), there was no difference in mortality, parasitemia and clinical signs after challenge (Table 2). In non-challenged controls (group 5), no changes were noticed in either experiment (Tables 1 and 2).

In the serological test, antibody responses after immunization with S. A. were detected by the AGP test, but not by ELISA (Fig. 1). In the chickens of group 1 immunized with S. A., the geometric mean (GM) AGP antibodies rose to 9.2 after the first immunization, but the titer decreased slightly to 8.0 after the second immunization. In the chickens of group 2 immunized with S. A., antibodies were not detected after the first immunization and the GM AGP antibodies first rose to 3.0 after the second immunization. Two weeks after challenge, the GM AGP antibody titers of both groups 1 and 2 had decreased to 1.2 and 1.1, respectively (Fig. 1a). In contrast to these results, antibody responses after immunization with schizont antigen were detected by ELISA, but not detected by the AGP test (Fig. 2). In the chickens of group 1 immunized with schizont antigen, ELISA values rose to 0.37 after the first immunization, and reached 1.22 after the second immunization. In the chickens of group 2 immunized with schizont antigen, antibodies were not detected after the first immunization and ELISA values first rose to 1.41 after the second immunization. Two weeks after challenge, ELISA values of both groups 1 and 2 had decreased to 0.21 and 0.78, respectively (Fig. 2b). In both experiments, however, high antibody responses were detected by both the AGP test and ELISA in all challenged chickens 4 weeks after challenge. They reached peak levels in each group, ranging from 1.7 to 2.0 in ELISA value and 9.7 to 50.9 in GM AGP titer (Figs. 1 and 2).

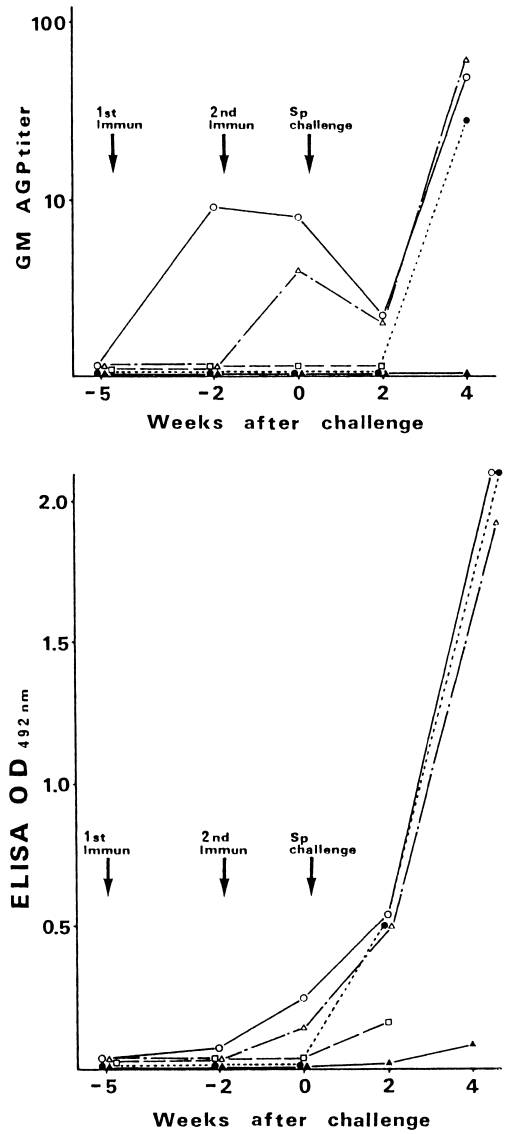

Fig. 1 Antibody responses in chickens immunized with serum-soluble antigen of *L. caulleryi*. (a) Measured by agar gel precipitation test. (b) Measured by enzyme-linked immunosorbent assay. ○: Immunized with serum-soluble antigen with the adjuvant, △: Immunized with serum-soluble antigen + PBS, □: Inoculated with the adjuvant + PBS (non-immunized control), ●: Sham-inoculated with PBS (non-immunized control), ▲: Non-challenged control, GM AGP titer: Geometric mean agar gel precipitation titer, Immun: Immunization, Sp: Sporozoite, ELISA OD 492 nm: Optical density measured at 492 nm in enzyme-linked immunosorbent assay.

Discussion

In both experiments, after the first immunization, the chickens immunized with antigen

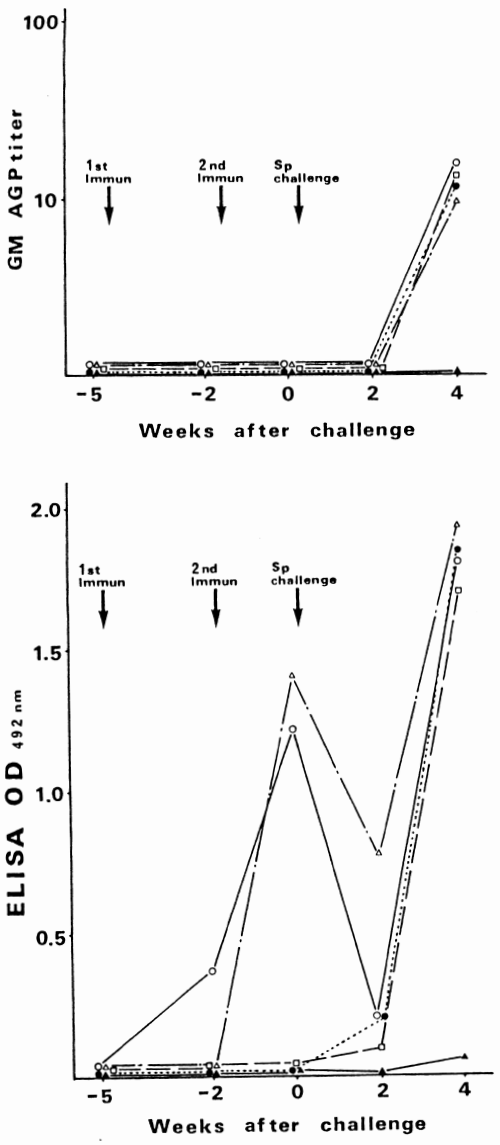

Fig. 2 Antibody responses in chickens immunized with schizont antigen of *L. caulleryi*. (a) Measured by agar gel precipitation test. (b) Measured by enzyme-linked immunosorbent assay. ○: Immunized with schizont antigen with the adjuvant, △: Immunized with schizont antigen + PBS, □: Inoculated with the adjuvant + PBS (non-immunized control), ●: Sham-inoculated with PBS (non-immunized control), ▲: Non-challenged control, GM AGP titer, Immun, Sp, ELISA OD $_{492\ nm}$: the same as described in Fig. 1.

in the presence of the adjuvant (group 1) showed a rise in antibody titers although the chickens immunized with antigen in the absence of the adjuvant (group 2) showed no rise in antibody titer (Figs. 1a and 2b). From this result, the effect of adjuvant was noted. However, in chickens immunized with schizont antigen in the presence of the adjuvant, the protective effect was not demonstrated although the reduction in the severity of sickness was observed in chickens immunized with schizont antigen without the adjuvant (Table 2). The reason for the lack of effect of the adjuvant is not clear. It is necessary to investigate this more in detail.

After the second immunization, in chickens immunized with S. A. in presence of the adjuvant (group 1), GM AGP antibody titer decreased although the chickens immunized with antigen without the adjuvant (group 2) showed a rise in GM AGP titer (Fig. 1). Similarly, after the second immunization, in chickens immunized with schizont antigen in presence of the adjuvant (group 1), ELISA values were lower than those of chickens immunized with schizont antigen alone (group 2) (Fig. 2b). From these results, it is supposed that the antibodies which appeared after the first immunization were neutralized with the antigen of the second immunization. In the same way, two weeks after challenge, in chickens of groups 1 and 2, the antibody titers suddenly decreased in both experiments using S. A. and schizont antigen. This phenomenon also appears to depend on the neutralization of antibodies with the antigen appearing after challenge infection.

The antibody responses after immunization with S. A. were detected by the AGP test, but poorly detected by ELISA. However, the antibody responses after immunization with schizont antigen were detected by ELISA, but not detected by the AGP test (Figs. 1 and 2). The schizont antigen has been used in ELISA (Isobe and Suzuki, 1986) and S. A. has been used in the AGP test (Isobe and Akiba, 1982). These results suggest that S. A. is slightly different from schizont antigen seroimmunologically in spite of Morii's suggestion (1974). In

chickens immunized with schizont antigen alone, the reduction in the severity of sickness was observable, whereas in chickens immunized with S. A., no such effect was observed. It might depend on the seroimmunological difference between S. A. and schizont antigen.

In both immunization tests using S. A. and schizont antigen, antibody responses were detected by both the AGP test and ELISA in all challenged chickens 4 weeks after challenges. It might depend on the appearance of both antibodies for S. A. and schizont antigen after the challenge infection.

This time, S. A. and schizont antigen were used for vaccination against chicken leucocytozoonosis and the protective effect of the vaccines was investigated by examining clinical signs, parasitemia and antibody responses. A reduction in the severity of sickness was noticed when chickens were immunized with schizont antigen without adjuvant. From this, the possibility of vaccinating chickens against leucocytozoonosis with schizont antigen was suggested.

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