Characterization of Monoclonal Antibodies against the Second-Generation Schizonts of *Leucocytozoon caulleryi*

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ABSTRACT. Monoclonal antibodies (MAbs), R1 and M5, were established against the second-generation schizont of *Leucocytozoon caulleryi* (L. caulleryi). Both antibodies reacted to membrane and internal structure proteins of the second-generation schizont by immunofluorescence microscopy. Molecular weight of the second-generation schizont (2GS) antigen was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. At least 40 protein bands were detected in 2GS antigen by SDS-PAGE under reduced condition and ranged from 10 to 270 kDa. MAb R1 reacted to polypeptides of 150-268 kDa in 2GS antigen, whereas MAb M5 did with that of 66 kDa. Injection with a protein of 2GS antigen fractionated by affinity chromatography using MAbs R1 and M5 protected chickens against challenge with sporozoites of *L. caulleryi*. These results suggest that MAbs, R1 and M5, recognize 2GS antigen of *L. caulleryi*.

KEY WORDS: *Leucocytozoon caulleryi*, monoclonal antibody, second-generation schizont antigen.

Chickens that recovered from the primary infection with *Leucocytozoon caulleryi* (L. caulleryi) become solidly immune to reinfection, and thus vaccination has been considered as an effective method to prevent the infection. Proteins demonstrating antigenic and immunogenic properties against *L. caulleryi* have been reported to reside in the second-generation schizonts (2GS) [3, 14, 15]. Similar proteins designated as the serum-soluble antigen (SSA) have likewise been noted from the second-generation schizonts [6, 16]. In the present study, two different monoclonal antibodies (MAbs), designated as MAb R1 and MAb M5, were established in mice injected with 2GS antigen. Some properties of the MAbs were analyzed.

Three-week-old specific-pathogen-free chickens (Line M, Nisseiken Co., Ltd.) and free of both the antigen and antibody against *L. caulleryi* were used in the preparation of 2GS antigen. The Shizuoka strain [11–13] of *L. caulleryi* used was kindly supplied by Dr. T. Morii in 1989 and has been maintained by cyclic transmission in chickens and its vector, *Culicoides arakawae* (C. arakawae), which likewise have been reared in our laboratory.

Experimental exposure of *C. arakawae* to *L. caulleryi*, and the preparation of sporozoites for inoculation into chickens and collection of the second-generation schizonts from the lungs, kidneys, thymus and Bursa of Fabricius of chickens followed those earlier described by Morii [9]. Thirteen days after infection of chickens with *L. caulleryi*, the second-generation schizonts were collected and suspended in phosphate-buffered saline (PBS, pH 7.0) at a concentration of 1:1. The suspension was homogenized, filtered, sonicated for 5 min, and centrifuged at 14,000 x g for 15 min, and the resulting supernatant was used as a 2GS antigen in this study. Concentration of protein in 2GS antigen was determined using BCA Kit (Bio-Rad Laboratories, U. S. A.).

Six-week-old mice (BALB/c) were intraperitoneally immunized with 2GS antigen (120 µg of protein/0.1 ml/mouse) mixed with an equal volume of Freund’s complete adjuvant (FCA). Forty-nine days after the first injection, mice were injected intraperitoneally with 2GS antigen (240 µg/0.1 ml/mouse) without adjuvant. Mouse spleen cells were harvested and fused with P3X63Ag8U1 myeloma cells using the standard procedure [8]. Generated hybridomas that elicited antibodies against 2GS antigen were selected by enzyme-linked immunosorbent assay (ELISA). MAb was purified with a commercial affinity chromatography column, Con A-Sepharose (Pharmacia Biotech. AB, Uppsala, Sweden) or MAb Trap G (Pharmacia Biotech. AB, Uppsala, Sweden).

Isotype of MAb was determined by using Mouse Typer kit (Bio-Rad Laboratories, U. S. A.). Reactivity of MAbs to the second-generation schizonts of *L. caulleryi* was examined by using indirect-immunofluorescence microscopy [2]. Two different MAbs that reacted to 2GS antigen were established by ELISA. Both MAbs reacted to membrane and internal structure proteins of the second-generation schizonts of *L. caulleryi* (Fig. 1). Reactivity of MAb R1 to membrane protein of the second-generation schizonts was stronger than that of MAb M5. Isotype of MAb R1 was determined as IgG1, whereas MAb M5 was noted as IgM.

Molecular weight of 2GS antigen was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously [5, 7]. The 2GS antigen separated by SDS-PAGE was transferred electro-photically onto nitrocellulose membrane and incubated with MAb, then mixed with the optimum concentration of horseradish peroxidase-conjugated goat anti-mouse IgG (Bethesda Research Laboratory, U. S. A.) [17]. At least 40...
protein bands were detected in 2GS antigen by SDS-PAGE (Fig. 2, Lane a). Molecular weights of the bands ranged from 10 to 270 kDa, but most of them were under 100 kDa. By Western blotting, MAb R1 recognized seven polypeptides of 150, 165, 175, 185, 235, 256 and 268 kDa in the 2GS antigen (Fig. 2, Lane b), whereas MAb M5 reacted only to the 66 kDa polypeptide (Fig. 2, Lane c). Although we can not explain on the reactivity of MAb R1 to several polypeptides, previous studies reported similar observation in Plasmodium falciparum proteins reactive to specific MAbs [1].

The 2GS antigen adjusted to a protein concentration of 1.2 mg/ml was fractionated by affinity column chromatography using MAb R1 or MAb M5. Each fractionated antigen was collected and concentrated by ultrafiltration using MINICON B15 (Amicon, U. S. A.). Protein concentration of the antigen fractionated with MAb R1 was 5 µg of protein/ml, and that of the antigen fractionated with MAb M5 was 10 µg/ml. Immunogens were prepared with a mixture of each antigen emulsified with Freund’s completes adjuvant (FCA). Protein concentration of each fractionated antigen in immunogen was adjusted to 3 µg/0.8 ml. Chickens 23 days of age were each injected intramuscularly with 0.8 ml of immunogen and were injected with a booster dose of 0.8 ml at 37 days of age. The control received PBS mixed with FCA. On day 14 after the last injection of the immunogen or PBS + FCA, chickens in both experimental and control groups were challenged by intravenous inoculation of 2 × 10^4 sporozoites/0.15 ml.

Clinical signs, such as anemia, emaciation, and discharge of green feces, were observed everyday for 19 days after challenge. Sera were collected from all the chickens on days 0 and 13 after challenge. Each serum sample was 100 times diluted with PBS, after then 2-folds serial dilution was made, and the diluted samples were measured by ELISA [4] using 2GS antigen (1.0 µg/well). Antibody titers were denoted as a reciprocal of the highest serum-dilution showing positive ELISA value (≥ O.D. value of negative serum + × 3 standard deviation value). SSA titers of the sera at 13 days post-challenge were checked by agar gel precipitation (AGP) test by Morii [10]. Blood samples of all the chickens were collected at 19 days after parasite challenge and were examined for the parasites [12].
After sporozoite challenge, all the control chickens showed anemia, emaciation, and/or discharge of green feces during the observation period. SSA titers remained on 64 at 13 days post-challenge (Table 1) and great numbers of gametocytes (data not shown) were observed in all the control chickens at 19 days post-challenge, these findings demonstrating the development of leucocytozoonosis in the control group.

Post-challenge, the immunized chickens appeared clinically normal and showed significantly high anti-2GS antibody titers (Table 1). Neither SSA antigen nor parasites were detected in 75% of the immunized chickens, although one chicken showed a few gametocytes at 19 days post-challenge (data not shown).

In the present study, we have produced MAb R1 and MAb M5 that reacted to membrane and internal structure proteins of the second-generation schizont of *L. caulleryi*. Chickens produced anti-2GS antibodies by injecting a 2GS antigen fractionated by affinity chromatography using MAb R1 and M5. In addition, the immunized chickens developed relatively high antibody titers at challenge, and were protected from the establishment of infection and disease. Humoral antibodies against 2GS antigen produced in the immunized chickens, as shown in the present study, collaborate with each other and reinforce the actions of the 2GS antigen to play an important role in the control of chicken leucocytozoonosis [6, 14, 15].

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Table 1. Protective activity of the second-generation schizont antigen against infection with sporozoites of *L. caulleryi*

<table>
<thead>
<tr>
<th>Imunizationb)</th>
<th>Chicken No.</th>
<th>2GS antibody titersa) at challengeb)</th>
<th>Serum-soluble antigen titersa) at 13 days post-challenge</th>
<th>No. of chickens showing clinical signsa) /No. of chickens tested</th>
<th>Parasitemiaa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2GS antigenb)</td>
<td>1</td>
<td>12,800</td>
<td>&lt;1</td>
<td>0/4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1,600</td>
<td>&lt;1</td>
<td>0/4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6,400</td>
<td>&lt;1</td>
<td>0/4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>800</td>
<td>&lt;1</td>
<td>0/4</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>&lt;100</td>
<td>8</td>
<td>4/4</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>&lt;100</td>
<td>64</td>
<td>4/4</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>&lt;100</td>
<td>8</td>
<td>4/4</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>&lt;100</td>
<td>64</td>
<td>4/4</td>
<td>+</td>
</tr>
</tbody>
</table>

a) Immunization time: 23 and 37 days of age.
b) Challenge Dose (Shizuoka strain=\(2 \times 10^4\) sporozoites/0.15 ml/chicken). Challenge: 51 days of age.
c) For 19 days post-challenge.
d) Observed at 19 days post-challenge.
e) A mixture (1:1) of MAb R1- and MAb M5-fractionated antigens was used as immunogen (3 µg/0.8 ml/chicken).

REFERENCES