FULL PAPER  Immunology

Field Efficacy of Recombinant R7 Vaccine against Chicken Leucocytozoonosis

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ABSTRACT. Effectiveness of vaccine that used recombinant R7 protein (rR7) as antigen that is derived from second-generation schizont (2GS) of Leucocytozoon caulleryi was verified under a field condition against chicken leucocytozoonosis. Chickens reared in a poultry farm where the chickens are attacked by leucocytozoonosis in every year were inoculated with oil-adjuvanted rR7 vaccine (O-rR7), and the immunized chickens were found to have production of antibodies against 2GS at a high level by one shot. Leucocytozoonosis was observed at post-injection. During the epidemic period of leucocytozoonosis, the unique clinical signs of the disease such as discharge of green feces and anemia, and also parasitemia were observed, however, compared to chickens in control group, those in O-rR7 vaccinated group had significantly slight symptoms (P<0.05). In addition to this, immunized chickens had better result of egg production than the unvaccinated chickens did, and the maximum difference of egg production rate, 22%, was observed at the peak of the disease. In conclusion, it is verified that O-rR7 vaccine has efficacy against leucocytozoonosis under field condition, and this vaccine can be put into practical use.

KEY WORDS: field trial, Leucocytozoon caulleryi, leucocytozoonosis, recombinant R7 vaccine.

Leucocytozoon caulleryi, a pathogenic protozoan parasite of chickens, is common in many of the Asian countries including Japan [1, 2, 9, 14, 15]. Chicken leucocytozoonosis is an important disease for poultry industry, causing reduction of egg production, weight loss, and sometimes death. Drugs have been used for control of the disease, but there have been problems with decreased drug sensitivity [2] and restriction to use drug for layer birds. Therefore, development of vaccine against chicken leucocytozoonosis has been deeply desired.

Chickens that have recovered from a primary infection with L. caulleryi sporozoites acquired resistivity to reinfection, and it has been suggested that immunity to L. caulleryi in chickens is induced by parasitic stage of second-generation shizonts (2GS) [5, 16]. Gotandaa el at. established monoclonal antibody that recognize immunogenic antigen of 2GS [3]. Recently, the gene encoding an immunogenetical protein of 2GS, named gene R7, has been cloned and vaccine with recombinant R7 protein (rR7) have protective effects strongly against sporozoite of L. caulleryi challenge [8]. The present study was to evaluate the effects of the vaccine used rR7 against L. caulleryi on clinical and parasitic parameters under field condition in a typical Japan poultry.

MATERIALS AND METHOD

The examination poultry farm: The experiment was performed at a typical poultry farm that holds about 18,000 chickens in Gunma prefecture. There are no poultry farms within a radius of 3 km from the experimental farm, and this farm is surrounded by paddy fields where Curicoides arakawai lives [1, 2, 10]. The outbreak of chicken leucocytozoonosis is observed in this farm every summer.

Chickens: Two thousands commercial layers (white leghorn) in a same flock that have been done vaccinations against marek’s disease, infectious bronchitis, newcastle disease, fowl pox, infectious bursal disease, infectious coryza, infectious laryngotracheitis and egg drop syndrome 1976 were employed for the experiment. The chickens were separated into two groups: one group (1,000 chickens) was vaccinated, and the other group was unvaccinated (as control). These chickens were reared in cages as two birds per cage, close to the rice fields, in which the outbreaks of leucocytozoonosis have been excessively serious.

Injection of oil-adjuvanted rR7 vaccine into chickens: Oil-adjuvanted rR7 (O-rR7) vaccine that was prepared in the exactly same way with one made in previous experiment [8]. The vaccine was injected into leg muscle of the chickens with the amount of 0.25 ml/dose (10,000 antigen unit/dose as antigen titer) [8], at 91 days of age. This vaccination was done on June 30th, 1998, about one and a half months before the mid-August, in which the outbreak of leucocytozoonosis is observed every year in this farm.

Detection of antibodies against 2GS antigen: For the purpose of observing duration of antibodies after vaccination, serum samples of 60 same chickens in vaccinated group were obtained at pre-vaccination, 30 and 91 days after vaccination, which gave us total of 180 samples. Similarly, serum samples of 60 same chickens in control group were obtained to confirm L. caulleryi infection. Enzyme-linked immunosorbent assay (ELISA) was used to detect serum anti-2GS antibody titers [4, 8]. Samples from vaccinated group that showed positive response at the 1,600 or higher anti-2GS antibody titers were considered as positive to protective antibody [8]. On the other hand, samples from control group that showed positive response at the 100 or higher
anti-2GS antibody titers were considered as positive to infectious antibody. Finally, the rate of chickens that had positive responses was determined for both vaccinated and control groups.

**Observation of symptom of leucocytozoonosis:** All tested chickens were observed their clinical signs, number of chickens which died by chicken leucocytozoonosis during the period the disease was observed. Clinical signs, such as discharge of green feces and anemia [2], were observed once a week. For judgement on anemia, we considered an individual whose comb obviously changes into white compared to normal one to be infected. A symptom of reduced egg production caused by *L. caulleryi* infection, was also investigated once a week.

**Observation of parasitemia caused by infection of *L. caulleryi***: Blood samples were collected three times, once within approximately 10 days, from total of 65 chickens in both groups that were suspected to be attacked by *L. caulleryi*, or were reared close to infected ones, while the clinical signs were observed. Agar gel precipitation test [11–13] was used to detect serum-soluble antigen (SSA) and antibody induced by *L. caulleryi* infection, and second-generation merozoites and gametocytes were observed in sample blood smears under light microscope [2, 10]. These smears were prepared in this way; samples were smeared on micro slide glass, fixed in methanol, stained with Giemsa solution.

**RESULTS**

**Duration of anti-2GS antibody titers after vaccination:** Table 1 shows the results of the anti-2GS antibody titers at pre-vaccination, 30 and 91 days post-vaccination. O-rR7 vaccinated chickens had titer peak at 30 days of post-injection, and after that, the titer declined gradually. Geometric mean of the anti-2GS antibody titers of 60 chickens at the peak was 14,703, which indicates significant rise of the anti-2GS antibody titers. In vaccinated group, the percentage of positives to protective antibody was 100%, 86.7% at 30 days, and 91 days of post-inoculation, respectively, which were high percentage. On the other hand, the anti-2GS antibodies became positive at 91 days after the experiment started in control group, and the percentage of positive to antibody induced by infection of *L. caulleryi* was 100%.

**Appearance of clinical signs and comparison of status of egg production:** Clinical signs of leucocytozoonosis in tested groups were seen from mid-August (48 days after O-rR7 vaccination) to the mid of September. Table 2 shows the number of individuals that showed clinical signs of leucocytozoonosis during the observation period. Compare O-rR7 vaccinated group with the control group, the number of chickens that had discharge of green feces were 38 (3.8%) in the vaccinated group and 172 (17.2%) in the control group. Also, 73 (7.3%) individuals in the vaccinated group and 232 (23.2%) ones in the control group showed symptom of anemia. For each clinical sign, the number of chickens that had

<table>
<thead>
<tr>
<th>Table 1. Antibody response of chickens in the vaccinated and control groups against second-generation shizont (2GS) of <em>Leucocytozoon caulleryi</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No. of Anti-2GS antibody titers</strong></td>
</tr>
<tr>
<td><strong>Vaccinated</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Control</strong></td>
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</table>

* a) Serum samples of 60 same chickens in each group were obtained (total of 180 samples/group).
* b) Geometric mean of anti-2GS titer measured by ELISA.
* c) Days after vaccination.
* d) Scores show that standard deviation of n when antibody titer was shown in $2^n \times 100$.
* e) Ratio of the chicken which showed anti-2GS antibody titers more than 1,600 as positive to protective antibody.
* f) Ratio of the chicken which showed anti-2GS antibody titers more than 100 as positive to infectious antibody.

**Table 2. Protective effects of vaccine evaluated by prevention of appearance of clinical signs against *L. caulleryi* infection in the field**

<table>
<thead>
<tr>
<th>Days after vaccination</th>
<th>Days after onset of infection</th>
<th>No. of chickens showed clinical signs of leucocytozoonosis</th>
<th>Anemia</th>
<th>Discharge of green feces</th>
<th>death</th>
<th>Vaccinated</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>(onset day)</td>
<td>0</td>
<td>40</td>
<td>6</td>
<td>77</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>51</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>55</td>
<td>7</td>
<td>3</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>62</td>
<td>14</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>8</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td>72</td>
<td>24</td>
<td>60</td>
<td>157</td>
<td>27</td>
<td>85</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>76</td>
<td>28</td>
<td>3</td>
<td>10</td>
<td>0</td>
<td>1</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>83</td>
<td>35</td>
<td>3</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>73</td>
<td>(onset day)</td>
<td>232</td>
<td>38</td>
<td>172</td>
<td>6</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

* a) About all individuals of each group, clinical observation was carried out.
* b) Total number of chickens which showed each clinical signs.
* c) Statistically significant value compared to the control group: * (P<0.05).
the symptoms of the disease in the vaccinated group, was significantly less than that in the control group ($P < 0.05$).

Additionally, O-rR7 inoculated chickens had slighter symptoms of leucocytozoonosis than the non-vaccinated ones did. Concerning Hen-day egg production rate in both groups during the observation period, vaccinated group had more egg production than the control group (Fig. 1). Especially, vaccinated group had significantly higher value of egg production rate at from 55 days to 62 days after vaccina-

tion that overlaps the outbreak of the disease ($P < 0.05$).

**Comparison of parasitemia by L. caulleryi infection**: Table 3 shows the comparison of parasitemia by *L. caulleryi* infection. Using serum samples that were obtained during the observation period, agar gel precipitation test that detect only antibodies that were induced by *L. caulleryi* infection was performed. Fifty-one out of 65 chickens (78.5%) in vaccinated group, and 60 out of 65 chickens (92.3%) in control group had positive responses, and this proved serologically that the both groups had been infected by the parasite during the observation time. SSA antigen that proves the presence of 2GS directly was detected only 5 out of 65 (7.7%) in the vaccinated group and 9 out of 65 (13.8%) in the control group. In the observation of parasite in the same chickens whose blood were taken for the serological examination with smeared blood, 18 out of 65 (27.7%) in the vaccinated group and 39 out of 65 (60.0%) in the control group had *L. caulleryi* in their blood.

**DISCUSSION**

In the previous studies for immunological prevention against leucocytozoonosis, vaccines that used inactivated 2GS of *L. caulleryi* [5, 17], or vaccine that is made with live sporozoites [16, 18], and etc. have been tried [20]. However, besides problems in safety, it is quite difficult to produce those vaccines; these facts have prevented to put those vaccines to practical use. Recombinant-R7 antigen, which was used in this study as vaccine antigen, is a protein that was come from 2GS made by gene recombinant technology, and can be produce in large quantities in *E. coli* [8]. That is, this rR7 vaccine has possibility to be manufactured. As the results stated above, it suggests O-rR7 vaccine can prevent leucocytozoonosis that efficacy of protection against *L. caulleryi* infection in field condition was confirmed in immunized chickens that were inoculated O-rR7 vaccine and this vaccine can be put into practical use.

In this study, as the index that evaluates efficacy of prevention of leucocytozoonosis, unique clinical signs such as anemia, discharge of green feces, and egg production rate were studied. All these clinical signs were mild in the vac-

### Table 3. Protective effects of vaccine against *L. caulleryi* infection evaluated by prevention of parasitemia in the field

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of chickens</th>
<th>AGP antibody</th>
<th>Index of parasitemia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SSA$^a$</td>
<td>2GM or Gt$^a$</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>65</td>
<td>51 (78.5%)$^b$</td>
<td>5 (7.7%) 18$^{*b}$ (27.7%)</td>
</tr>
<tr>
<td>Control</td>
<td>65</td>
<td>60 (92.3%)$^b$</td>
<td>9 (13.8%) 39 (60.0%)</td>
</tr>
</tbody>
</table>

a) Detection of antibody induced by *L. caulleryi* infection by agar gel precipitation test.

b) Serum-soluble antigen (SSA) detected by agar gel precipitation test.

c) Second-generation merozoites (2GM) or gametocytes (Gt) detected in blood.

d) Statistically significant value compared to the control group: $^* (P < 0.05)$.

e) Positive ratio of each indicator.

**Fig. 1.** Progress of Hen-day egg production rate in both groups during the epidemic period of leucocytozoonosis. Statistically significant values compared to the control group: $^* (P < 0.05)$. 

1. $\times$ 0.05.
cinated group during the observation period. Especially, it is considered to be significantly important that inoculation of rR7 vaccine prevented the reduction of egg production that is one of the symptoms of leucocytozoonosis and is the biggest economical problem to poultry farms. Nakamura et al. [19] reported the mechanism of reduction of egg production by L. caulleryi infection, which results from granulomatous and lymphocytic inflammation, edema, and pressure atrophy that are caused by 2GS’s parasite on ovary and ovic-duct. From this fact, it is suggested that rR7 vaccination prevented lesions in ovary and ovicduct by inducing antibodies that acted on and destroyed 2GS, and as a result, reduction of egg production caused by L. caulleryi infection was prevented or lessened.

In vaccinated chickens, protective efficacy against parasitemia was verified during the observation period. Specifically, the number of second generation merozoites and gametocytes were significantly low in blood vaccinated chickens. This is considered to result from it that antibodies induced by rR7 vaccine acted on 2GS and blocked its life cycle to a parasitic stage in blood cells. This is supposed to have made the symptoms of anemia slight. Meanwhile, the rate of detected SSA, which is serum-soluble antigen that came from 2GS, was low in both groups. This is considered as because it is difficult to detect SSA after 14 days of infection, when the clinical signs were seen strongly, since SSA can be detected only from about 9 to 13 days after L. caulleryi infection [13].

In chickens immunized with O-rR7 vaccine, anti-2GS antibodies were produced at high level at 30 days of post-injection. However, the level of antibody production was lower than that of laboratory examination [8]. Probably this is because of not only the possibility such as an environmental factor, which is to rear chickens in field, difference between the kinds of tested chickens, and injection by automatic syringe on the assumption of use in the field, but also the result of consumption of antibody by L. caulleryi infection. In this study, leucocytozoonosis started to be observed at 48 days of post-injection. During the epidemic period, at 62 days of post-injection when the egg productivity between vaccinated and control group showed the greatest difference, the geometric means of antibody titers of each group were 4,850 and 3,940 for vaccinated and control respectively (data not shown). And at 91 days of post-vaccination and control respectively, the anti-2GS antibody titer with ELISA. This suggests that the antibodies induced by rR7 vaccine are used in order to destroy 2GS, and as a result, reduct.

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REFERENCES


