Immune Response and In Vivo Distribution of the Virus in Chickens Inoculated with the Cell-Associated Vaccine of Attenuated Infectious Laryngotracheitis (ILT) Virus

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ABSTRACT. Chickens inoculated with the cell-associated (CA) vaccine acquired higher protective immunity to ILT. In chickens vaccinated with CA or cell-free (CF) vaccine, respectively, virus-neutralizing and IgG- and IgM-ELISA antibodies were detected in the serum, but no antibody was detected in the tracheal washes of the vaccinated chickens. More apparent antibody response was seen in chickens vaccinated with the CA vaccine than with the CF vaccine. The antibody titers did not correlate closely with the protection against challenge with ILT virus. After subcutaneous injection of either CA or CF vaccine, ILT virus was isolated from the liver, spleen, thymus, lungs and other organs of the chickens from 1 to 6 days after injection, and there was no correlation of the isolation rate for the CA-vaccinated and the CF-vaccinated chickens.—KEY WORDS: cell-associated vaccine, chicken, immunity, infectious laryngotracheitis.


Our previous report [12, 13] dealt with the immunological properties of the cell-associated ILT vaccine (CA vaccine), that can afford a satisfactory immunity to 1-, 20-, and 73-day-old chickens by subcutaneous or intramuscular injection. With the cell-free vaccine (CF vaccine) prepared from the culture fluid of chicken embryo fibroblast cells infected with the CE strain of ILT virus, chickens can be inoculated at 14 days of age or later and through the ocular or intranasal route as in natural infections. Since the age of the recipient chickens and the route of the CA-vaccination are different from those of the CF-vaccination, it is expected that the in vivo distribution of the virus and the antibody response are also different. Post-vaccination immune response [1, 2, 7, 15] and in vivo distribution of the virus [6, 11] have been reported, indicating the importance of cell-mediated immunity to the immune mechanism [3, 4].

In this report, we will show the results of the studies on the antibody response and virus distribution in the chicken after the CA or CF-vaccination.

MATERIALS AND METHODS

Chickens and eggs: Specific-pathogen-free (SPF) white Leghorn chickens supplied by Aso Branch, the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan) were used. Embryonating eggs laid by these chickens were used.

Vaccination and challenge: The CA vaccine was prepared from primary chicken embryo fibroblasts (CEF) cells infected with an attenuated CE strain of ILT virus, as reported previously [12], and the CF vaccine was prepared from the culture fluid of CEF cells infected with the CE strain. The infective titers of the CA and CF vaccines were $10^{5.1}$ and $10^{5.0}$ median tissue culture infective doses (TCID$_{50}$)/chicken, respectively. One-day-old SPF chicks were inoculated subcutaneously on the neck with either vaccine and the serum and the tracheal washes were collected periodically. The tracheal washes were collected by the methods of York et al. [15].

Vaccinated and unvaccinated control chickens were challenged by the intratracheal route with 200 median chicken infective dose (CID$_{50}$) of virulent ILT strain NS-175. All chickens were observed daily for clinical signs for 10 days post challenge. Chickens showing clinical signs of lacrimation, rales, coughing, or gasping were considered to have reactions caused by the challenge.

Virus-neutralization (VN) test and ELISA: VN-antibody titer was measured as previously described [12].

Egg-grown strain NS-175 of ILT virus was purified by sucrose density (10-40% W/V) gradient centrifugation (90 min at 10,000 x g) and used as an ELISA antigen. Commercially available (Cappel) horseradish peroxidase (HRP)-labeled anti-chicken IgG rabbit serum (HRP-labeled anti-chicken IgG) was used. HRP-labeled anti-chicken IgM and anti-chicken IgA were prepared by labeling goat anti-chicken IgM and IgA (ICN Immuno Biologicals) with HRP (Toyobo, Japan) by the method of Nakane and Kawarai [8] to use in the experiments described below.

The ELISA procedure was adapted from that described by Meulemans and Halen [7]. The ELISA antigen was diluted with 0.05 M carbonate buffer (pH 9.6) to obtain a final protein concentration of 10 µg/well. The optical density (OD) of well at 492 and 630 nm was measured with a micro-ELISA reader (EAR400AT, SLT- Labinstrument). The ELISA antibody titer of each specimen was expressed in the OD value for each well. The cut-off value of antibody negative serum was when the absorbance reading exceed 2 standard deviations (S.D.) for the unvaccinated chickens: 0.16 for anti-IgG and 0.13 for anti-IgM.

Distribution of virus in the chicken: One-day-old SPF chickens were injected subcutaneously on the neck with
the CA vaccine (10^5.1 TCID_50/chicken) or CF vaccine (10^5.0 TCID_50/chicken). At 1, 3, 6, 8, 11, 13 and 15 days PV, four chickens were killed and the liver, spleen, kidneys, lungs, trachea, bursa of Fabricius, ceca, small intestines, pancreas, heart, thymus, brain, sciatic nerve, tissue from the injection site, and blood were collected aseptically. A ten percent suspension of each tissue was centrifuged for 10 min at 1,200 × g, and each supernatant was used to inoculate the monolayers of chicken kidney (CK) cell cultures in test tubes. After incubation for 7 days at 37°C, the cells were observed microscopically and the appearance of CPE was considered to indicate positive virus isolation. When no CPE was observed, the culture supernatant was subcultured for two generations in CK cells to confirm negative virus isolation.

RESULTS

Protective potency of vaccines: The results are shown in Table 1. With respect to the CA vaccine, the protection rate was 85% at 14 days PV and a protection rate higher than 60% was maintained until 70 days PV. However, in chickens vaccinated with the CF vaccine, the maximal protection rate reached 70% at 14 days PV and decreased rapidly thereafter.

VN titers: Serum VN titers in four chickens injected with the CA or CF vaccine at one day of age are shown in Fig. 1. In the CA-vaccinated group, antibody (≥ 0.8 log) was detected in one of four chickens at 9 days PV. The VN titer of four chickens turned positive at 11 days PV and rose to 1.63 at 21 days PV. On the other hand, in the CF-vaccinated group, some chickens developed antibody at 13 days PV, but the mean VN titer was 0.82. There was significant difference between the CA-vaccinated and CF-vaccinated groups in the VN titer at 9, 10 and 15 to 21 days PV. No VN antibody was detected in the tracheal washes of any chicken (data not shown).

ELISA titers: The IgG- and IgM-ELISA titers of four chickens in each group are shown in Fig. 1. The IgG antibody of the CA-vaccinated group was detected at 5 days PV and thereafter increased gradually until 21 days PV. In the CF-vaccinated group, however, IgG antibody was detected at 7 days PV, but the titers were low and had decreased by 15 days PV. IgM antibody was detected from 3 to 18 days PV in the CA-vaccinated group and from 3 to 15 days PV in the CF-vaccinated group. The titers were always higher in the former group. IgA antibody was not detected on any sampling day. There were significant differences between the CA-vaccinated and CF-vaccinated groups in the IgG-ELISA titers from 7 to 21 days PV, and in the IgM-ELISA titers from 5 to 11 days PV.

The IgG-, IgM- and IgA-ELISA titers of the tracheal washes of the CA-vaccinated and CF-vaccinated groups are shown in Fig. 2. On any days of sampling, no antibody was detected in the tracheal washes.

Relation between VN and ELISA antibody titers: The correlation between serum VN titers and IgG-ELISA

![Graph showing virus neutralizing antibody, ELISA antibody(IgG), and ELISA antibody(IgM) levels over time.](image)

Fig. 1. Virus neutralizing titer, ELISA (IgG, M) titer of serum in chickens vaccinated with the CA or CF vaccine. Each point represents the mean ± standard deviation (n=5).

Table 1. Protective potency of the cell-associated vaccine and cell-free vaccine against a virulent infectious laryngotracheitis (ILT) virus

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Route</th>
<th>14^a</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-associated (CA)</td>
<td>Subcutaneous</td>
<td>17/20^b</td>
<td>14/20</td>
<td>17/20</td>
<td>14/20</td>
<td>14/20</td>
</tr>
<tr>
<td>Cell-free (CF)</td>
<td>Subcutaneous</td>
<td>13/20</td>
<td>7/20</td>
<td>4/20</td>
<td>1/20</td>
<td>0/20</td>
</tr>
<tr>
<td>Unvaccinated control</td>
<td>—</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

a) Days after vaccination.
b) Number of chickens protected / Number of chickens tested.
titers was examined in the CA-vaccinated chickens. As shown in Fig. 3, they closely correlated, with a correlation coefficient of 0.86, indicating that the IgG-ELISA titer is reflected by the VN titer. No correlation was observed, however, between the serum VN titers and IgM ELISA titers (data not shown).

Relation between antibody titers and protection against challenge: The correlation between the antibody levels at challenge and the protection rate against challenge was examined in the CA-vaccinated group. The correlations between the protection rate and serum VN titers and IgG-ELISA or IgM-ELISA titers are shown in Fig. 4. When the VN titers were below 0.4, the protection rate was 70%. The protection rate increased as the VN titer increased: when the VN titer was higher than 2.0, the protection rate was 100%. As was the case with the VN titers, the IgG-ELISA titers correlated approximately with the protection rate. A similar tendency was observed with the IgM-ELISA titer, but not so clearly as with the IgG-ELISA antibody titers.

Figure 5 shows the relation between the IgG- and IgM-ELISA titers and the time of appearance of the protective effect. The IgM antibody appeared at 3 days PV, when the protective effect appeared. The IgM antibody titer reached its maximum at 7 days PV, when the protective rate became higher than 70% and it decreased thereafter. The IgG antibody titer also showed a similar pattern as the protective rates, starting to increase from 5 days PV and reaching a plateau in 9 days PV.

Distribution of the virus: Recoveries of the virus from the CA- and CF-vaccinated chickens are shown in Table 2. In the CA-vaccinated groups, the virus was isolated from
Table 2. Distribution of ILT virus after CA or CF vaccination

<table>
<thead>
<tr>
<th>Vaccination</th>
<th>Specimen</th>
<th>1</th>
<th>3</th>
<th>6</th>
<th>8</th>
<th>11</th>
<th>13</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA vaccine</td>
<td>Liver</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Lungs</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Thymus</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Injection site</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CF vaccine</td>
<td>Liver</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>Lungs</td>
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<tr>
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<tr>
<td></td>
<td>Injection site</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a) Virus was not isolated from the other tissues (kidney, trachea, bursa of Fabricius, ceca, small intestines, pancreas, heart, proventriculus, brain and sciatic nerve).
b) Days after vaccination.
c) Number of chickens virus-isolation positive/four chickens tested.

the liver, spleen, lungs, thymus, kidneys, and tissues of the injection site up to 6th day PV. In the CF-vaccinated group, the virus was isolated from almost the same specimens as in the CA-vaccinated group. There was no meaningful difference between the CA-vaccinated and CF-vaccinated groups in the virus distribution.

**DISCUSSION**

The protective potency of the CA vaccine was superior to that of the CF vaccine. The present study was aimed at the elucidation of this difference. However, from the present results, the reason why the CA vaccine exert higher immunogenicity than the CF vaccine remains to be elucidated.

In ILT of chickens, since no passive immunization with the antibody is successful and the immunity is acquired in chickens from which the bursa of fabricius has been dissected to exclude the B-cell function [3], many reports deny the participation of the antibody in the immunity. However, since detection of the antibody in the washes of the tracheal mucous membrane has been reported [15], it is still possible that the local secretory antibody participates in the protection.

An previous report by us [12] and the present results clearly demonstrated that young chickens inoculated with the CA vaccine acquired more sufficient immunity than with the CF vaccine. In the present study, we attempted to find if the humoral antibody is involved in the immunity by analyzing the antibody in the serum and the tracheal washes after vaccination. Although no humoral antibody was detected in the tracheal washes, no clear correlation was demonstrated between protection against challenge and serum VN, IgG- and IgM-ELISA antibody. Similar results have been reported with other ILT vaccines [5]. From these findings, those vaccines and the CA vaccines seem to act the same as far as humoral antibody is concerned. We are planning to clarify the immune mechanism by examining the blast transformation with myogen of lymphocytes derived from the vaccinated chickens and the immune response of bursectomized and thymectomized chickens.

In chickens inoculated intranasally or ocularly with the conventional vaccine, the virus was recovered from the respiratory organs [11], while subcutaneous injection of the CA or CF vaccine resulted in proliferation of the virus in the liver, spleen, and thymus, where natural ILT infection does not occur. Little has been reported on the site of viral distribution after subcutaneous injection of ILT virus [9]. It seems important to examine the relation between these organs supporting the proliferation of the virus and the local immunity in the trachea to explain the high immunogenicity of the CA vaccine.
REFERENCES


