The Role of Cell-Mediated Immunity in Chickens Inoculated with the Cell-Associated Vaccine of Attenuated Infectious Laryngotracheitis Virus

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ABSTRACT. The cell-mediated immune responses of the chickens inoculated with the cell-associated (CA) ILT vaccine were studied. Lymphocyte blastogenic response was tested by MTT assay with spleen, thymus, bursa of Fabricius and peripheral blood lymphocytes of the vaccinated chickens. The blastogenic response of peripheral blood and spleen lymphocytes was enhanced by a T-cell mitogen. The CA vaccine induced an immunity satisfactorily in bursectomized chickens. To study the role of immune cells in the vaccinated chickens, the bursectomized chickens were inoculated intravenously with the splenic, thymic, bursal and peripheral blood lymphocytes of the vaccinated chickens. Protective effects were shown in the chickens received the splenic cells and peripheral blood lymphocytes, indicating that these play an important role in eliciting the protective effects of the CA vaccine. From these results, it was found that the immune mechanism with CA vaccine against ILT involves mainly cell-mediated immunity.—KEY WORDS: blastogenic response, bursectomy, cell-associated live ILT vaccine, chicken, infectious laryngotracheitis.

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Infectious laryngotracheitis (ILT) is an acute respiratory disease of chickens caused by ILT virus belonging to the herpesvirus group, and it is one of the most important infectious disease of chickens [8, 12, 13, 23]. The live virus vaccines have been used for the prevention of ILT, but the cell-free (CF) vaccines would give only insufficient immunity when inoculated into chickens of 14-day-old or younger [3].

In addition, the possibility exists that chickens suffer damage by the reaction of vaccine, because they are inoculated through such natural routes of infection as eye-dropping, in drinking water and aerosol spray [2, 4, 10].

We have studied the safety and efficacy of the cell-associated (CA) vaccine prepared by the cells infected with ILT virus, and confirmed that CA vaccine was highly effective by administering subcutaneously or intramuscularly into 1-day-old chickens and that the defects of CF vaccines can be avoided [21, 22].

In the previous communication, we have reported that there was not clear correlation between protection against challenge and the humoral immunity in chickens inoculated with CA vaccine [11], but the role of cell-mediated immunity to CA vaccine has not been studied yet.

Many workers have reported that the immunity to cell-free ILT virus depends on a cell-mediated immune response [5, 19].

In the present study, for the purpose of investigating the role of cell-mediated immunity and humoral immunity to CA vaccine, blastogenic response of the lymphocyte originating from the vaccinated chickens by MTT assay [1, 18, 20], the immune response in bursectomized or thymectomized chickens inoculated with CA vaccine and the adoptive transfer of resistance with immune cells derived from vaccinated chickens were scrutinized.

MATERIALS AND METHODS

Chickens and eggs: Inbred specific-pathogen-free (SPF) white Leghorns 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 chick embryo fibroblast (CEF) cells infected with attenuated CE strain of ILT virus [15] as reported previously [21]. The infective titer of the CA vaccine was $10^{4.7}$ median tissue culture infective dose ($TCID_{50}$) per chicken. Chickens were inoculated subcutaneously on the neck with CA vaccine.

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

Virus-neutralization (VN) test: VN-antibody titer was measured by a decreasing-virus constant-serum assay previously described [21].

Collection of lymphocytes: One-day-old chickens were inoculated with the CA vaccine. Four vaccinated and 4 unvaccinated control chickens were exsanguinated and autopsied at 0, 1, 4, 7, 10, 14, 21 and 28 days post-vaccination (PV), and the peripheral blood, thymus, spleen, and bursa of Fabricius were collected aseptically. Afterwards, the lymphocytes were separated from them by the density centrifugation over Ficoll-Paque (Pharmacia, U.S.A. × 400 g, 1 hr). Lymphocytes (10^6 cells/m*l* were suspended in RPMI-1640 medium containing 5% fetal calf serum, and incubated in each well of a 96-well microplate at 37°C under the presence of 5% CO_2 gas for

4 days.

Lymphocyte blastogenic response: Optimum conditions required for application of MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay to the lymphocyte blastogenic response in this study were determined according to the method of Takamatsu and Murata [20]. To a cultural fluid of the lymphocytes in the microplate, an MTT aqueous solution (5 mg/m*l*) was added to each well in 100 μ*l*/well, and the microplates were incubated at room temperature for 5 min. The supernatant was discarded and dimethyl sulfoxide (DMSO) was added to each well in 100 μ*l*/well. Then, the formasan formed was extracted with DMSO and its absorbance was read at 550 nm (test wavelength) and 620 nm (reference wavelength) with spectrophotometer.

Phytohemagglutinin-P (PHA-P, 20 μg/m*l*), Concanavalin A (Con A, 20 μg/m*l*), and lipopolysaccharide (LPS, 10 μg/m*l*) were used as mitogen in the blastogenic response. The ratio of the absorbance (stimulating index: SI) with a mitogen to that without mitogen was calculated.

Experiment with bursectomized chickens: Three-day-old embryonated eggs were dipped in a 2% testosterone propionate (TP) ethanol solution for a few seconds and then allowed to hatch. After hatching, cyclophosphamide (Cytoxan: CY) aqueous solution (2.5 mg/0.1 m*l*/day) was injected through the intraperitoneal route into the chickens for 4 days [9, 17]. At the age of 21-day, they were inoculated with the CA vaccine and challenged at 2 weeks PV. At challenge and 2 weeks PC, blood samples were taken to titrate for the neutralizing antibody. The chickens were autopsied at the end of the test period for examination for remnant of the bursa of Fabricius.

Experiment with thymectomized chickens: Thymus was removed surgically from SPF chickens at the age of 0 day. Some of the surgically thymectomized chickens were injected intraperitoneally with anti-thymocyte serum (Anti-T) prepared by the method of Forget *et al.* [7], having the cytopathic activities higher than × 256 on the thymocytes, continually for 4 days from 0-day to 3-day old. These chickens were inoculated with the CA vaccine at the age of 21 days and challenged at 2 weeks PV. Blood samples were taken at challenge and 2 weeks PC to perform the VN test.

Adoptive cell-transfer method: Lymphocytes (spleen, thymus, bursa of Fabricius and peripheral blood) from chickens inoculated with CA vaccine at 1-day old were taken at 10 days PV and injected intravenously into the 21-day-old bursectomized chickens as described by Fahey *et al.* [6]. These chickens and unvaccinated control chickens were challenged at 2 weeks after the cell-transfer. Blood samples were taken from the chickens at challenge and in 2 weeks PC to perform the VN test.

Statistical analysis: Differences in the SI value between the vaccinated and unvaccinated groups were analyzed by the Student's *t* test. A level of $P<0.05$ was considered significant.

RESULTS

The blastogenic response of lymphocytes obtained from the vaccinated chickens: Results of the blastogenic response are shown in Figs. 1 and 2. In PHA-P added group, the SI value from peripheral blood and spleen lymphocytes became higher ($P<0.05$) in the vaccinated than in unvaccinated group after 4 days PV. In Con-A added group, the SI value from peripheral blood and spleen lymphocytes became higher ($P<0.05$) in the vaccinated than in unvaccinated group after 7 days PV. While the values were kept on nearly the same levels as those of the group with LPS added. There were no differences between the vaccinated and the unvaccinated group in the SI value from thymic and bursal lymphocytes (data were not shown).

Immune responses of the bursectomized chickens: The CA vaccine was inoculated at serial 10-fold dilutions to compare the minimum effective doses of the CA vaccine in bursectomized and in unbursectomized control chickens. As shown in Table 1, the protection rate was 100% in the TP-CY-treated group inoculated with $10^{4.7}$ $TCID_{50}$/chicken, 80% in the group inoculated with $10^{3.7}$ $TCID_{50}$/chicken, and 30% in the group inoculated with $10^{2.7}$ $TCID_{50}$/chicken and the rate were 100%, 80% and 50% in the untreated group, respectively. Thus, no significant difference in the effective dose of the vaccine was found between the groups with and without the TP-CY treatment. The neutralizing antibody titers measured at chal-

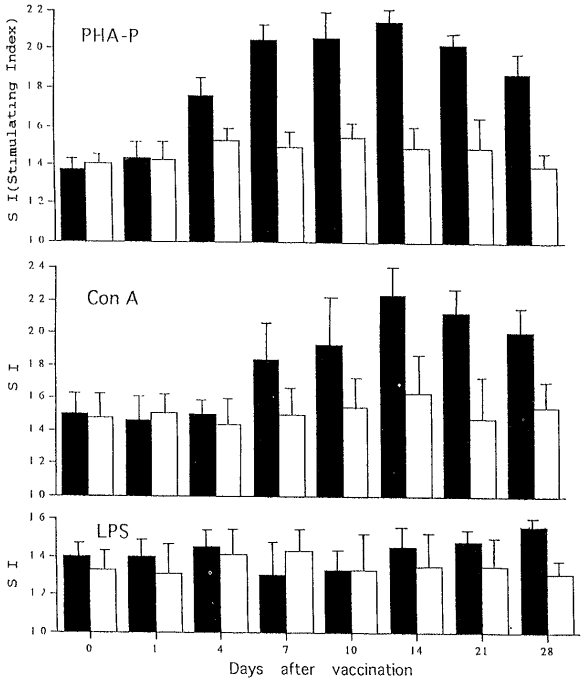

Fig. 1. Blastogenic response of chicken peripheral blood lymphocyte to PHA-P, Con A and LPS measured by MTT assay. ■; with vaccination □; without vaccination
Data represents mean ± S.D. of 4 samples.

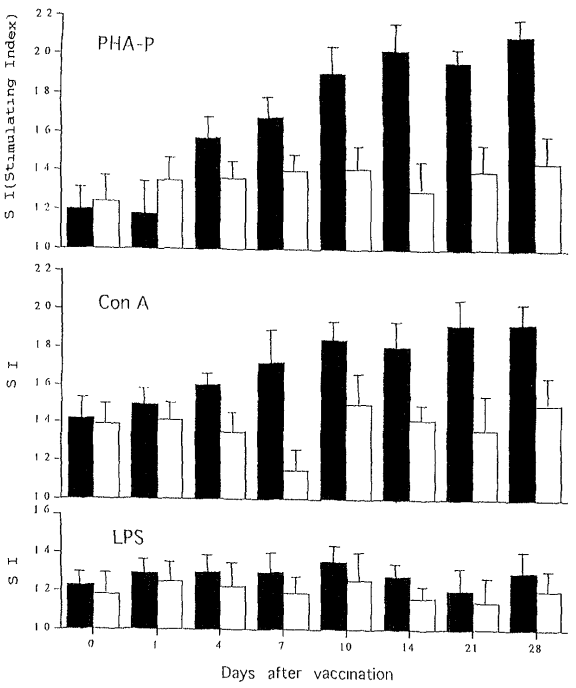

Fig. 2. Blastogenic response of spleen lymphocyte to PHA-P, Con A and LPS measured by MTT assay. ■; with vaccination □; without vaccination. Data represent means ± S.D. of 4 samples.

lenge and 2 weeks PC are also shown in Table 1. All the TP-CY-treated chickens showed negative neutralizing antibody (<0.8) at or after challenge in any of the vaccinated group. The untreated-vaccinated chickens possessed neutralizing antibody titers of 0.8 to 1.4 at the challenge and higher titers after the challenge. The untreated-unvaccinated control chickens showed high neutralizing antibody titer after the challenge. The above results show that even such chickens in which the bursa of Fabricius became extinct by the TP-CY treatment and was lacking in the humoral antibody-producing ability are bestowed protection to a similar extent to in normal chickens by inoculation with CA vaccine. The TP-CY-treated chickens were autopsied at the end of the test to check for remnants of the bursa of Fabricius. Even a trace of bursa of Fabricius was not found in any chicken, no marked changes were detected in any other organs.

Immune response of the thymectomized chickens: The antibody responses to vaccination of the chickens thymectomized by different methods, surgical removal of the thymus (TX) and/or injection of Anti-T, were compared. As shown in Table 2, the protection rates were 70% for the group received Anti-T only, 40% for the group received thymectomy only, and 20% for the group received both. All these rates were lower than the rate for the untreated-vaccinated group, but in the treated (TX+Anti-T) group, some chickens survived challenge without developing symptoms. The difference was statistically significant ($P<0.05$) in the protection rate between treated- and untreated-vaccinated groups. The vaccinated chickens treated with TX and TX, anti-T or anti-T showed mostly positive neutralizing antibody (≧0.8), which increased after the challenge. No marked difference in the antibody response was seen among the groups, showing that the humoral immune response was kept normal in the thymectomized chickens.

Adoptive cell-transfer assay: Table 3 shows the results of adoptive cell-transfer assay. In any of the groups, the neutralizing antibody was negative (data not shown). In the challenge test, among the groups having received the cells of the vaccinated chickens, the splenic cell-transferred group showed 70% protection and the peripheral blood-transfered group showed 44% protection. The other groups were all developed symptoms, except that group received the splenic cells of unvaccinated chickens showed 10% protection.

DISCUSSION

The lymphocyte function of the chickens inoculated

Table 1. The effect of bursectomy (TP-CY) on the protective efficacy and antibody response to the CA vaccine

Treatment[a]	Vaccine[b]	Protection rate[c]	Virus neutralizing titer for ILT[d]	
			At challenge	At 2 weeks post-challenge
TP-CY	$10^{4.7}$	10/10(100%)	0.16±0.22	0.16±0.21
TP-CY	$10^{3.7}$	8/10(80%)	0.08±0.14	0.10±0.11
TP-CY	$10^{2.7}$	3/10(30%)	0.11±0.15	0.12±0.17
Untreated	$10^{4.7}$	10/10(100%)	1.02±0.20	2.28±0.47
Untreated	$10^{3.7}$	8/10(80%)	NT[e]	NT
Untreated	$10^{2.7}$	5/10(50%)	NT	NT
TP-CY	—	0/10(0%)	0.06±0.10	0.18±0.20
Untreated	—	0/10(0%)	0.10±0.14	2.24±0.49

a) Dipping eggs in Testosterone propionate (TP) and injection with cyclophosphamide (CY).
b) $TCID_{50}$/chicken.
c) No. protected/No. tested.
d) Virus neutralizing titer for ILT: Mean value±S.D.
e) Not tested.

Table 2. The effect of thymectomy on the protective efficacy and antibody response to the CA vaccine

Treatment[a)]	Vaccinated[b)]	Protection rate[c)]	Virus neutralizing titer for ILT	
			At challenge	At 2 weeks post-challenge
TX-Anti T	+	2/10(20%)	1.34±0.21[d)]	2.56±0.31
TX	+	4/10(40%)	1.22±0.11	2.52±0.33
Anti T	+	7/10(70%)	1.14±0.25	2.38±0.27
Untreated	+	10/10(100%)	1.32±0.36	2.40±0.33
Untreated	Nil	0/10(0%)	0.10±0.17	2.20±0.31

a) TX: Surgical thymectomy at 1-day-old.
Anti T: Injection intraperitoneally with anti-thymocyte serum continually for 4 days.
b) CA vaccine: $10^{4.7}$ $TCID_{50}$/chicken.
c) No. protected/No. tested.
d) Mean value±S.D.

Table 3. Relative efficacy of immune cells in conferring protection

Donor chickens	Source of donor cells[b)]	Number of transfered cells/chicken	Protection rate[c)]
Vaccinated[a)]	Spleen	1.3×10^7	7/10(70%)
	PBL	1.0×10^7	4/ 9(44%)
	Thymus	1.7×10^6	0/ 9(0%)
	BF	4.0×10^6	0/10(0%)
Unvaccinated	Spleen	1.0×10^7	1/10(10%)
	PBL	5.0×10^6	0/ 7(0%)
	Thymus	4.6×10^6	0/ 9(0%)
	BF	1.0×10^6	0/10(0%)

a) $10^{4.7}$ $TCID_{50}$/chicken
b) PBL: Peripheral blood lymphocyte BF: Bursa of Fabricius
c) No. protected/No. tested.

with the CA vaccine was examined by MTT assay, and it was found that blastogenic response of peripheral blood and spleen lymphocytes was enhanced by a T-cell mitogen. In addition, it was successful to transfer adoptively protection to ILT with peripheral blood and spleen lymphocytes. These data support that cellular response is important in the immunity against ILT. Fahey *et al.* concluded that the immunity with CF vaccine to ILT virus depends on a cell-mediated immune response [6]. From results of present study, it was confirmed that the immune mechanism of CA vaccine also involves mainly cell-mediated immunity. The CA vaccine can give the protective effects to the same extent to the chickens deprived of ability of antibody production as to normal chickens. The cell-mediated immunity is generally considered to be the principal mechanism of protection against herpesvirus infection [14, 16]. It was found that this was also the case in this vaccine.

It was proved that there were some remnants of the thymus remaining in some of the treated chickens. This fact shows that the treatments performed in the present study failed to remove completely the thymus. More complete removal of the thymus functions seems necessary. Even so, the results demonstrated that the chickens undergone a thymectomy treatment showed much lower protection than the untreated, vaccinated chickens, probably due to the lowered thymus function.

Fahey *et al.* reported that the ILT resistivity was able to be transferred by spleen cells or peripheral blood leukocytes [6]. In the present study, we failed to transfer the protective effects by the transfer of the thymocytes or the bursal cells indicating that these immune organs are not direct sources of immune effector cells in chickens inoculated with CA vaccine. The CY-treated chickens received splenic cells from unvaccinated chickens showed 10% protection against challenge with ILT virus. From these results, the direct involvement of nonspecific mechanisms of protection to ILT such as natural killer cells can not completely ruled out. It is necessary to characterize the effector cells in splenic and peripheral blood lymphocytes to confer the protection.

As mentioned above, it was confirmed that the cell-mediated immune mechanisms were involved in immunity induced with CA vaccine to ILT. It was found that this was also the case in CF vaccine. However, the protective potency of the CA vaccine was superior to that of the CF vaccine. The cause by which the CA vaccine exert higher immunogenicity than the CF vaccine remains to be elucidated.

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