Protection of Mice against Aujeszky’s Disease Virus Infection by Intranasal Vaccination with Inactivated Virus

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(Received 8 October 1993/Accepted 14 February 1994)

ABSTRACT. Intranasal vaccination of mice with inactivated Aujeszky’s disease virus (ADV) induced IgA and IgG antibody responses to the virus in the secretion of the respiratory tract, resulting in complete protection of the animals against intranasal challenge with virulent ADV. The immune response was enhanced by the use of the cholera toxin B subunit (CTB) as an adjuvant. On the other hand, subcutaneous vaccination of mice with inactivated ADV, even together with CTB, scarcely stimulated secretory antibody responses, resulting in only partial protection. The present results suggest that development of a vaccination procedure to stimulate the mucosal immune response should improve the protective effects of the inactivated herpesvirus vaccines, and thereby make it possible to control the infections by prohibiting virus replication at the site where primary infection takes place, as well as inhibiting subsequent latency and reactivation of the virus. — KEY WORDS: Aujeszky’s disease virus, cholera toxin B subunit, intranasal vaccination, mouse.


Aujeszky’s disease is caused by an alpha-herpesvirus, Aujeszky’s disease virus (ADV), and inflicts a major economic loss in pig industries worldwide. It is well known that latent infection with ADV easily occurs in its natural reservoir, pigs, like other alpha-herpesvirus infections [4, 19]. As a result of environmental or physical stress, latent ADV may be reactivated and shed, resulting in the spread of ADV infection to other animals [6].

Vaccination of pigs against ADV infection with modified-live virus or inactivated virus induces neutralizing antibodies and sensitized lymphocytes in blood and lymphoid organs, but confers only partial protection [11]. Studies on inactivated ADV vaccines with cattle [1] and dogs [16] also showed that parenteral vaccination did not confer satisfactory protection on the animals against the challenge with the virus.

It has been established that local immunity plays an important role to protect animals from mucosal infections, independent of systemic immunity. Since the mucosal surface is the site of primary infection by a large number of viruses, protection against those viruses on mucosa is a prerequisite for prevention of the following generalized infections. McKenzie and Halsey [12] showed that the cholera toxin B subunit (CTB) stimulated the mucosal immune response. Intranasal administration of influenza virus split vaccine together with CTB induced a protective immune response in mice [20]. Intranasal vaccination of cattle with the envelope glycoprotein, gl, of bovine herpes virus 1 together with CTB gave protective immunity [7].

As is the case for other secondary host species for ADV, the mouse is a ‘terminal’ host, unable to survive acute virus replication [3], and thus mouse virulence has been used as a marker for characterization of the ADV strains [2, 17]. Therefore, the mouse is a good model animal to evaluate protective effects of vaccines against ADV infection. In the present study, we examined whether or not intranasal administration of inactivated ADV induces a local immune response in mice to give a protective effect against primary infection with the challenge virus.

MATERIALS AND METHODS

Virus and cell culture: ADV strain YS-81 and CPK cells were kindly provided by Dr. M. Shimizu of the National Institute of Animal Health (Tsukuba, Japan). CPK cells were cultured in Eagle’s minimal essential medium (EMEM) supplemented with 10% calf serum. ADV was inoculated to CPK cell monolayers at a multiplicity of 0.1 PFU/cell. After adsorption for 1 hr, EMEM was added and the cultures were incubated at 37°C for 2 days. From the culture fluids harvested, the virus was purified by differential centrifugation and sedimentation through a 10–15% sucrose gradient [9] and inactivated with 0.1% formalin.

Mice: BALB/c female mice were purchased (Shizuoka Laboratory Animal Center, Shizuoka, Japan) and used when they were 7 or 8 weeks old. The mice were fasted for 12 hr before vaccination and virus challenge.

Vaccination and protection test of mice: Mice were anaesthetized by an intraperitoneal injection with sodium pentobarbital (0.5–0.75 mg) and then vaccinated by intranasal administration or subcutaneous injection with the required dose of inactivated ADV alone or together with the cholera toxin B subunit (CTB) (Sigma Chemical Co., St. Louis, MO, U.S.A.) in 20 μl of PBS. Two weeks later, the second vaccination was done, followed by the third vaccination a week after that. One week after the third vaccination, mice were challenged intranasally with 10 or 100×LD50 (105.7 PFU) of live virus in 5 μl of PBS.
under anesthesia.

Nasal and trachea-lung washes were collected as described by Niederd et al. [15]. Briefly, a silk suture was tied below the larynx after surgical exposure of the trachea. Using a syringe, half a milliliter of PBS was slowly injected into the trachea above or below the suture for nasal and trachea-lung washes, respectively. The nasal wash was collected from the nares in a tube and trachea-lung wash was pulled back into the syringe.

**Immunological assays:** Anti-virus antibodies were measured by enzyme-linked immunosorbent assays (ELISA) [8]. Disrupted viral antigen was prepared from purified ADV. Rabbit anti-mouse IgA and goat anti-mouse IgG antibodies conjugated to horseradish peroxidase were purchased (Zymed Laboratories, Inc., San Francisco, CA, U.S.A. and Bio-Rad Laboratories, Richmond, CA, U.S.A., respectively).

Western blotting was performed as described by Towbin et al. [21]. ADV proteins of the purified virus were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose sheets in 25 mM Tris-192 mM glycine-20% methanol buffer. The sheets were incubated with Block Ace (Snow Brand Milk Co., Ltd., Tomakomai, Japan) and then soaked into sample dilutions. After incubation with anti-mouse IgA or IgG antibodies conjugated to peroxidase, the sheets were soaked in 10 mM Tris-HCl buffer containing 4-chloro-1-naphthol (0.5 mg/ml), H2O2 (0.015%), and 50 mM NaCl (pH 7.5).

**Results**

**Immune responses of mice vaccinated intranasally or subcutaneously with inactivated ADV:** Attempts were made to establish a test system for evaluation of the protective effect of intranasal vaccination of mice with inactivated ADV. Two-dose vaccination with inactivated ADV (20 μg protein) intranasally or subcutaneously together with or without CTB did not confer protection against the challenge with 100×LD50 of virulent virus on mice. Since survival days of intranasally vaccinated mice after the challenge were longer than those of control mice (data not shown), we then examined animals with a three-dose vaccination formula.

Mice were given three doses of intranasal or subcutaneous vaccination with inactivated ADV (40 μg of protein) alone or together with CTB (5 μg). Five mice from each group were sacrificed 6 days after the last vaccination to collect the nasal washes, trachea-lung washes and sera. Antibody titers in these samples are shown in Table 1. Both IgA and IgG antibodies specific to ADV were demonstrated in the nasal and trachea-lung washes of the mice intranasally vaccinated with inactivated ADV. In the sera of these mice, low levels of anti-ADV IgA antibodies were detected, although IgG antibodies were demonstrated at high titers. Antibody titers of the nasal and trachea-lung washes and the sera of the mice vaccinated intranasally with inactivated ADV together with CTB were significantly higher than those of the mice vaccinated with ADV alone.

In contrast, in the nasal washes of the mice vaccinated subcutaneously, IgA and IgG antibodies were scarcely detected. Anti-ADV IgA antibody titers of the trachea-lung washes and the sera of these mice were very low or below the level of detection, while IgG antibodies were clearly demonstrated. No significant difference of antibody titers was found between the two groups of mice vaccinated subcutaneously with ADV alone and together with CTB.

**Protection of mice vaccinated with inactivated ADV against virus challenge:** To evaluate the protective effect of vaccination with inactivated ADV, five or ten mice of each group were challenged by intranasal inoculation with virulent ADV (10×LD50) 7 days after the last vaccination. Survival rates of these mice after the challenge are also shown in Table 1. All of the mice vaccinated intranasally with inactivated ADV, regardless of the use of CTB

<table>
<thead>
<tr>
<th>Route</th>
<th>Vaccine</th>
<th>Antibody titer</th>
<th>Survival rate</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Nasal wash</td>
<td>Trachea-lung wash</td>
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<td></td>
<td></td>
<td>IgG</td>
<td>IgA</td>
</tr>
<tr>
<td>IN</td>
<td>V</td>
<td>3.0±1.1</td>
<td>1.8±0.4</td>
</tr>
<tr>
<td>V+CTB</td>
<td></td>
<td>4.8±0.4</td>
<td>3.0±0.6</td>
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<tr>
<td>SC</td>
<td>V</td>
<td>0.4±0.5</td>
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<tr>
<td>V+CTB</td>
<td></td>
<td>0.4±0.5</td>
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<td>CONTROL</td>
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</table>

a) Samples for antibody titrations were collected from 5 mice from each group 7 days after the last vaccination and the remaining mice (10 or 5) were challenged intranasally with a lethal dose of live ADV (10×LD50).

b) IN: intranasal; SC: subcutaneous.

c) V: inactivated ADV; CTB: cholera toxin B subunit.

d) Antibody titer was determined by ELISA and expressed as log2 of the reciprocal of the end point sample dilution (mean±SD). —: Each of individual titers of the 5 mice was less than 1.0.
survived the challenge with a lethal dose of live ADV, while 90% of control mice died. Of each 5 mice that were vaccinated subcutaneously with inactivated ADV alone or ADV together with CTB, two (40%) and one (20%) survived, respectively.

Forty-two days after the challenge with 10×LD₉₀ of virus, surviving mice were again challenged with a higher dose of live virus (100×LD₉₀). Control mice and subcutaneously vaccinated mice died within 4 days of challenge. On the other hand, 30% and 90% of the mice intranasally vaccinated with inactivated ADV alone and ADV together with CTB, respectively, survived for 14 days (Fig.1). All the surviving mice were sacrificed 14 days after the second challenge and the nasal washes and the sera were examined for their antibody titers. Antibody levels in these samples were similar to those collected before the first challenge, indicating that virus replication did not occur after the second challenge.

Viral proteins which antibodies in the nasal washes recognized were determined by western blot analysis. Both IgA and IgG antibodies in the nasal washes of mice vaccinated intranasally mainly bound to 155K protein of ADV (Fig.2).

Protective effect in mice with different doses of vaccine and challenge virus: Since the survival rates of mice were different according as the dose of the challenge virus in the above protection test, we examined whether or not the protective effect was affected by the dose of the vaccine and challenge virus.

Mice vaccinated intranasally with different doses (10, 20 or 40 μg) of inactivated ADV three times were challenged with 10×LD₉₀ or 100×LD₉₀ of virus. These mice showed dose-dependent defensive responses (Table 2). Against the challenge with 10×LD₉₀ of virus, survival rates of mice vaccinated with 10, 20, and 40 μg of inactivated virus were 80, 100, and 100%, respectively. In contrast, neither mice vaccinated with 20 nor 40 μg of inactivated virus showed complete protection against the challenge with 100×LD₉₀ of virus (20 and 40% survival rates, respectively). None of the mice vaccinated with 10 μg of inactivated virus survived the challenge with 100×LD₉₀ of virus.

Table 2. Survival rates of mice vaccinated intranasally with inactivated ADV after virus challenge

<table>
<thead>
<tr>
<th>Challenge dose (μg)</th>
<th>Vaccine dose (μg/mouse)</th>
<th>40</th>
<th>20</th>
<th>10</th>
<th>0</th>
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</thead>
<tbody>
<tr>
<td>10×LD₉₀</td>
<td>100×(S/5)</td>
<td>100×(S/5)</td>
<td>80×(S/5)</td>
<td>0×(S/5)</td>
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</tr>
<tr>
<td>100×LD₉₀</td>
<td>40×(S/5)</td>
<td>20×(S)</td>
<td>0×(S/5)</td>
<td>0×(S/5)</td>
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a) Mice were intranasally vaccinated three times with each dose of inactivated ADV without using CTB.
b) Mice were challenged intranasally with 10 or 100×LD₉₀ of live virus after the last vaccination.

DISCUSSION

It is known that serum antibodies and cellular immunities induced by parenteral vaccination suppress the severity of the disease caused by ADV, but do not prevent primary infection which may lead to latent infection. Protection from primary infection with ADV could be achieved by induction of secretory antibodies which neutralize virus infectivity on the mucosal surface. In the present study, intranasal administration of inactivated ADV into mice induced ADV-specific antibody responses in their secretions of the respiratory tract where primary infection takes place, resulting in the protection of these
mice from ADV infection. In contrast, subcutaneous vaccination with inactivated ADV stimulated only a limited secretory antibody response and did not give complete protection.

Dominant antibodies in the nasal washes of the mice intranasally vaccinated with inactivated ADV were specific to 155K protein of ADV in the present study. By SDS-PAGE of structural proteins of strain Phylaxia, molecular weight of gII complex was estimated as 155K under nonreducing condition [10]. On the other hand, Nakamura et al. [13] showed that gII complex of strain Indiana S migrated as molecular weight of 230K protein under nonreducing condition. Such discrepancy of the molecular weight of gII protein may be due to different complex formation of molecular mass of the proteins [22]. It was shown that monoclonal antibodies to envelope glycoprotein gII neutralized the virus infectivity and inhibited its adsorption to the cells [5]. The gII protein is essential for virus penetration including fusion function [18]. Intraperitoneal immunization with purified gII glycoprotein together with adjuvant gave a protective effect in mice and pigs [14]. The present results, therefore, suggest that the local secretory antibodies specific to gII glycoprotein interfered with either attachment of the virus to the receptors on the mucosal epithelial cells or fusion of the viral envelope with the cellular membrane, resulting in complete protection of mice from viral invasion into the host. Intranasal vaccination of pigs with the gII glycoprotein of ADV may induce protective antibodies in the nasal secretion.

The protective effect was dependent on vaccine and challenge virus doses (Table 2). Mice vaccinated intranasally with inactivated ADV alone showed only partial protection against the challenge with 100×LD50 of live virus. On the other hand, intranasal administration of CTB as an adjuvant enhanced both systemic and local immune responses, and 90% of mice vaccinated together with CTB were protected from the challenge of 100×LD50 of virus (Fig. 1). To induce a sufficient immune response, use of a suitable mucosal adjuvant may be required for intranasal vaccination with the inactivated virus.

The present study is one of the promising attempts to control herpesvirus infection by vaccination. Secretory antibodies that act as a barrier to primary infection by viruses on mucosal epithelial cells could be induced by intranasal vaccination of animals with the relevant inactivated virus. The vaccination strategy for Aujeszky’s disease should be designed to stimulated local immune responses that are essential for protection against primary infection by the virus on the mucosal surface. Consequently, this would make it possible to prevent latent infections, and hence control the spread of ADV throughout a population.

REFERENCES


