Experimental Study on Mucosal Vaccination of Animals against Viral Infections

（ウイルス感染症に対する粘膜ワクチンの研究）

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Preface

Mucosal surfaces of the animals serve as portals of entry for viral, bacterial, or parasitic agents and hence most of infectious diseases of intestinal, respiratory, or genital tracts are contracted through a large surface area of mucosal membranes. Therefore, these tissues are furnished with the mucosal immune system which is, with properties and functions, independent of the systemic immune system. While the systemic immune system is required to actively eliminate absorbed antigens, mucosal immune system is primarily involved with keeping antigens out or preventing the interactions of potentially harmful agents with the epithelium.

Mucosal immunity is typified by secretory immunoglobulin A (SIgA), which is the predominant immunoglobulin class in exocrine secretions. SIgA which consists of two IgA molecules linked by J chain can be transported across the epithelial cells by polymeric immunoglobulin receptor, also called secretory component present on the basolateral membrane of the epithelial cells (Bienenstock and Befus, 1980; Brandtzaeg and Krajci, 1992; Underdown, 1992). Possible protective functions of SIgA are immune exclusion comprising inhibition of attachment and penetration of infectious agents, antibody-dependent T cell-mediated cytotoxicity, and stimulation of immune response through facilitation of antigen uptake and lymphocyte stimulation by anti-idiotypic antibody activity (Holmgren *et al.*, 1981). Recently, Mazanec *et al.* (1992; 1994) demonstrated that IgA inhibited replication of microbial pathogens intracellularly. IgA antibodies are actively transported through the epithelial cells by the polymeric

immunoglobulin receptor and may be able to bind to newly synthesized viral proteins in the cells.

A large number of studies have been demonstrated that the level of protection against diseases of the respiratory or intestinal tract correlate better with the level of local antibodies in corresponding external secretions than in the serum (McGhee and Mestecky, 1990; Mestecky and Jackson, 1994). Intramuscular or subcutaneous injection with vaccine stimulates systemic immunity but does not confer successful protection on the animals. Alternative routes of immunization are recently used to stimulate mucosal immune responses. Many studies convincingly demonstrated that precursors of mucosal B and T cells originate predominantly from organized lymphoepithelial tissues distributed along the mucosal tissues such as intestinal or respiratory tracts and that the stimulation of these inductive sites results in a generation of mucosal immune response (Mestecky, 1987; McGhee and Mestecky, 1990). In contrast to systemic injection of antigens, mucosal immunization is frequently capable of inducing both mucosal and systemic immunity. There is, thus, currently a great interest in the mucosal vaccination especially against mucosally or sexually transmitted viral diseases, such as influenza, herpes, or immunodeficiency virus infections. Against these infections, systemically immunized animals were not protected when challenge virus was introduced by mucosal routes (Israel *et al.*, 1988; Mestecky and Jackson, 1994). Therefore, induction of protective immunity at the mucosal surfaces, the most frequent portals of entry of the viruses, is being considered with increasing emphasis in the design of novel vaccines (McGhee and Mestecky, 1990; Husband, 1993).

Antigens are usually poorly immunogenic when administered by mucosal routes. The use of an adjuvant which is able to stimulate mucosal immune response against unrelated protein antigens has been one of the promising attempts designed to overcome this problem. Cholera toxin (CT) and its B subunit (CTB) were found to be potent mucosal adjuvants which stimulate both mucosal and systemic immune responses when administered in combination with unrelated proteins (McKenzie and Halsey, 1984). CT is composed of a pentameric B subunit that binds to GM_1 ganglioside, and a toxic A subunit that activates intracellular adenylate cyclase (Holmgren, 1981). CT or CTB stimulates the transepithelial flux of antigen into the nasal mucosa of rabbits (Gizurarson *et al.*, 1991; 1992). CT adjuvant promotes antigen priming of T cells and B cell isotype differentiation *in vitro* (Lycke and Strober, 1989; Lycke *et al.*, 1990; Hörnquist and Lycke, 1993). Oral immunization using CT adjuvant stimulates both antigen-specific memory T and B cells (Vajudy and Lycke, 1993). Therefore, CT and CTB have been used to enhance immune responses against Sendai virus (Nedrud *et al.*, 1987; Liang *et al.*, 1988), influenza virus (Tamura *et al.*, 1988), bovine herpesvirus 1 (Israel *et al.*, 1992), and human respiratory syncytial virus (Oien *et al.*, 1994). In this study, CTB was used as a mucosal adjuvant for intranasal immunization of chickens and mice against Newcastle disease and Aujeszky's disease, respectively.

Newcastle disease (ND) has been one of the major problems in the poultry industry worldwide (Alexander, 1988). The causative agent, Newcastle disease virus (NDV) is an enveloped RNA virus, belonging to the family *Paramyxoviridae*. Vaccinations of chickens with live-attenuated or

inactivated NDV have been examined (Meulemans, 1988). As the protective effects of the vaccination were closely correlated with the amount of serum neutralizing or hemagglutination-inhibition (HI) antibodies, efforts toward the ND vaccines have been designed to induce systemic antibody response. It was shown that vaccination of chickens with a live-attenuated NDV was capable of generating serum HI antibody, and also secretory antibody responses when introduced by the respiratory routes (Ewert *et al.*, 1979; Russel and Koch, 1993; Russell and Ezeifeka, 1995). However, attenuated NDVs used as live vaccines have potential of reversion to virulent strains with passage from bird to bird (Thornton, 1988). On the other hand, parenteral vaccination with inactivated virus generally induces serum neutralizing antibodies, and little local immune response. Although previous reports by others showed that vaccination with inactivated NDV by the mucosal routes induced local immune response (Yoshida *et al.*, 1971; Zakay-Rones *et al.*, 1971), protective effects and local immunity given by vaccination with inactivated virus by the respiratory routes have not been examined.

Aujeszky's disease virus (ADV), also known as pseudorabies virus, is a member of the *Alphaherpesvirinae* and an important pathogen of swine. In its natural host, pigs, latent infection easily occurs, like other alpha-herpesviruses (Sabo and Rajcani, 1976; Gutekunst, 1979). Latent ADV may be reactivated by environmental or physical stress, resulting in the spread of ADV infection to other animals (Howarth, 1969). ADV invade through the mucosal membrane of the respiratory tracts, which is thought to be the most common route of entry of the virus in natural infections. After

initial replication in the nasal and pharyngeal mucosa, the virus spreads towards the central nervous system by means of cranial nerves such as the olfactory and trigeminal nerves, resulting in meningoencephalitis (McFerran and Dow, 1965; Sabo and Rajcani, 1969; Wittmann *et al.*, 1980; Babic *et al.*, 1994). Therefore, infected animals, except pigs, can not survive acute virus infection even in the presence of systemic immune responses (Crandell, 1985). Accordingly, parenteral vaccination of animals has been failed to provide satisfactory protection against primary infection with the virus at the mucosal tissues (Pensaert *et al.*, 1980; Bironi *et al.*, 1982; Crandell, 1985; Martin *et al.*, 1986).

Since both NDV and ADV initially replicate in the epithelial cells lining the respiratory tracts, a local immune response against these viruses at the site of entry is expected to protect the animals from generalized infection which is subsequent to the primary infection, and hence the following latent infection. In the present study, local and systemic antibody responses induced by the intranasal vaccination of animals with inactivated virus or envelope glycoprotein were investigated. Protective effects of these vaccination against intranasal challenge with lethal doses of the relevant viruses were also evaluated. In comparison with parenteral vaccination, importance and advantages of mucosal vaccination of animals against viral infections are discussed.

Part 1

Protective Immune Response of Chickens against Newcastle Disease, Induced by the Intranasal Vaccination with Inactivated Virus

Introduction

Different routes of vaccination into chickens with live-attenuated NDV have been examined. Eidson and Kleven (1976) showed that intratracheal or aerosol administration was more effective than subcutaneous injection with an attenuated virus vaccine. Intranasal and intratracheal inoculation with live ND vaccine induced local antibody response in their saliva (Ewert *et al.*, 1979). However, attenuated NDVs used as live vaccines have potential of reversion to virulent strains with passage from bird to bird (Thornton, 1988). On the other hand, parenteral vaccination with inactivated virus generally induces serum neutralizing antibodies, and little local immune response. Zakay-Rones *et al.* (1971) demonstrated that local immune response was induced by intranasal vaccination of chickens with inactivated NDV. Yoshida *et al.* (1971) showed that intratracheal and intranasal administration of inactivated NDV elicited less antibody response in the mucosal secretion than with live vaccine. On the other hand, aerosol administration of inactivated ND vaccine was effective to confer the protection on chickens (Van Eck, 1990). Yet, protective effects of

intranasal vaccination of chickens and the classes of the local antibodies produced remained to be examined.

In the present study, it was examined whether local and systemic antibody responses were induced by intranasal vaccination with inactivated NDV alone or together with CTB in chickens. Virus replication at the site of inoculation with the virus and protective effects of intranasal vaccination on the birds from the challenge with a lethal dose of virulent NDV were also analyzed. In this part, correlation between induction of local and/or systemic antibody responses and inhibition of local and/or systemic infection is discussed.

Materials and Methods

Virus

NDV strains Miyadera and Sato from the repository of our laboratory were used for inactivated vaccine and challenge viruses, respectively. Strain Sato is the standard challenge virus for evaluation of ND vaccines in Japan. These viruses were propagated in the allantoic cavity of 10- to 11-day-old embryonated fowl eggs at 35℃ for 48 hours, and then purified from the allantoic fluids according to Kida and Yanagawa (1981). For the vaccine preparation, the purified virus was inactivated by 0.1% formalin at 4℃ for a week. Virus inactivation was confirmed by inoculation into 10 eggs with the vaccine.

Animals

White Leghorn chickens were hatched and raised in isolation in our laboratory, and then used at 6-8 weeks of age.

Vaccination and challenge

Chickens were vaccinated by intranasal administration or subcutaneous injection with 100 μg of inactivated NDV alone or together with 20 μg of CTB (Sigma Chemical Co.) in 0.1 ml of phosphate-buffered saline (PBS). After two weeks, the second vaccination was carried out, followed by the third vaccination a week later. One week after the last vaccination, chickens were challenged intranasally with 10^2 50% lethal doses (LD_{50})($5X10^{4.0}$ Plaque forming units)(PFU) of strain Sato in 0.2 ml of PBS per bird. All the surviving chickens were sacrificed 14 days post challenge. To examine the effect of a single vaccination, the nasal washes and the sera were obtained two weeks after the first vaccination and remaining chickens in this group were challenged on the same day.

Plaque assay for virus isolation

Chickens were sacrificed for virus isolation from the sinus and blood, 1 and 3 days post challenge. The tissue samples were ground with sterile sea sand to give 10-20% suspension in broth containing antibiotics. Blood samples were treated with heparin sodium (Wako Pure Chemicals). Serial dilutions of the samples were inoculated on Madin Darby bovine kidney (MDBK) cell monolayers. After 1 hour adsorption, the inoculum was removed and the cells were overlaid with Eagle's minimal essential medium

(EMEM) containing 1% Bacto-Agar (Difco). After incubation at 35℃ for 2 days in the 5% CO_2 atmosphere, cells were overlaid again with EMEM containing 1% Bacto-Agar and 0.005% neutral red, followed by plaque count.

Nasal wash and serum samples

Seven days after the final vaccination, five chickens of each group were sacrificed to obtain their nasal washes and sera. Serum samples were heat-inactivated at 56℃ for 30 min. The nasal washes were collected by washing the nasal cavity with 4 ml of PBS containing 0.1 % bovine serum albumin, after their heads were separated from the body with the submaxilla.

Antibody assays

Neutralization test was done by plaque reduction assay using MDBK cells according to Kida *et al.* (1982). HI test of the serum samples was done as reported by Sever (1962). Anti-NDV IgA, IgM, and IgG antibodies were measured by enzyme-linked immunosorbent assay (ELISA) (Kida *et al.*, 1982). Disrupted viral antigen was prepared from the purified virus, strain Miyadera. Goat anti-chicken IgA, IgM, and IgG antibodies conjugated to horseradish peroxidase were purchased (Bethyl Laboratories).

Results

Antibody responses of vaccinated chickens to NDV

Single or three-dose vaccination formulae were examined for antibody responses and protective effects of intranasal inoculation and subcutaneous injection with inactivated NDV. Serum HI antibody titers of chickens vaccinated intranasally or subcutaneously are shown in Table 1. Single intranasal vaccination did not induce any detectable antibody response in the sera. On the other hand, a single subcutaneous vaccination induced serum HI antibody response at titers of 1:4-1:128, which were similar to those of chickens intranasally vaccinated three times together with CTB (1:8-1:128). Titers of the chickens subcutaneously vaccinated three times were much higher than those of intranasally vaccinated birds. Hence, the following six groups were prepared to examine the local and systemic antibody responses, and protective effects (Table 2); chickens vaccinated intranasally three times with the inactivated virus alone (group 1) or together with CTB (group 2), chickens vaccinated subcutaneously three times with the virus alone (group 3) or together with CTB (group 4), chickens vaccinated subcutaneously once with the virus alone (group 5) and non-vaccinated chickens (group 6).

The nasal washes and the sera from five chickens of each group were pooled and neutralizing activity of these samples was examined (Table 2). The nasal wash and the serum samples of the birds vaccinated intranasally three times together with CTB neutralized infectivity of strain Sato at a titer of 1:15 and 1:160, respectively. In contrast, no neutralizing activity was

Table 1. Serum HI antibody response of chickens vaccinated intranasally or subcutaneously

Route[a]	Vaccine[b]	No. of times vaccinated	Serum HI titer[c]
IN	V	1	<2
	V+CTB	1	<2
	V	3	3.6±1.8
	V+CTB	3	4.8±1.5
SC	V	1	5.0±1.5
	V	3	8.4±1.0
	V+CTB	3	8.6±0.5
Control		0	<2

a) IN: Intranasal, SC: Subcutaneous.

b) V: Inactivated NDV, CTB: Cholera toxin B subunit.

c) Titer was expressed as $\log_2$ of the reciprocal of the highest antibody dilution inhibiting 4 HAU of virus (mean±SD of 5 birds).

Table 2. Antibody responses and protection of chickens vaccinated intranasally or subcutaneously with inactivated NDV

Group	Route[a]	Vaccine[b]	No. of times vaccinated	Neutralization titer[c]		No. of survivors/ no. challenged
				Nasal wash	Serum	
1	IN	V	3	<2	80	4/12
2	IN	V+CTB	3	15	160	12/14
3	SC	V	3	<2	3900	6/6
4	SC	V+CTB	3	<2	3600	6/6
5	SC	V	1	<2	160	4/10
6		Control	0	<2	<2	0/10

a) IN: Intranasal, SC: Subcutaneous.

b) V: Inactivated NDV, CTB: Cholera toxin B subunit.

c) Neutralization titers of nasal washes and sera are expressed as the reciprocals of the highest antibody dilution in agar overlay which caused 50% reduction of 100 PFU of virus.

detected in the nasal washes of subcutaneously vaccinated birds (groups 3 and 4), while neutralization titers of the sera were significantly higher than those of the birds vaccinated intranasally together with CTB. In the serum samples of the birds of group 5, neutralizing activity was detected at titers of 1:160, but not in the nasal wash.

Antibody classes in these samples were determined by ELISA (Fig. 1). IgA and IgM antibodies were detected in the nasal washes from birds vaccinated intranasally together with CTB (group 2). IgM antibodies were detectable also in the samples from birds vaccinated without CTB (group 1). On the other hand, only IgG antibodies were detected both in the nasal washes and sera from birds vaccinated subcutaneously three times, with or without CTB (groups 3 or 4). IgG antibodies were also detected in the nasal washes and the sera of birds vaccinated intranasally (groups 1 and 2). The antibody response was enhanced by the use of CTB, as was the case with IgA and IgM antibodies in the nasal washes. The levels of IgG antibodies in the sera of birds vaccinated subcutaneously (groups 3 and 4) were significantly higher than those of birds vaccinated intranasally together with CTB (group 2), while no difference was found in the nasal washes among those three groups. In the sera of the birds of group 5, IgG antibodies were detected at similar levels to those of intranasally vaccinated birds (group 2), but not in the nasal washes.

Protective effect of intranasal vaccination with inactivated NDV in chickens

Six to fourteen chickens of each group were challenged intranasally with 100 LD_{50} of virulent strain Sato to evaluate the protective effect of the

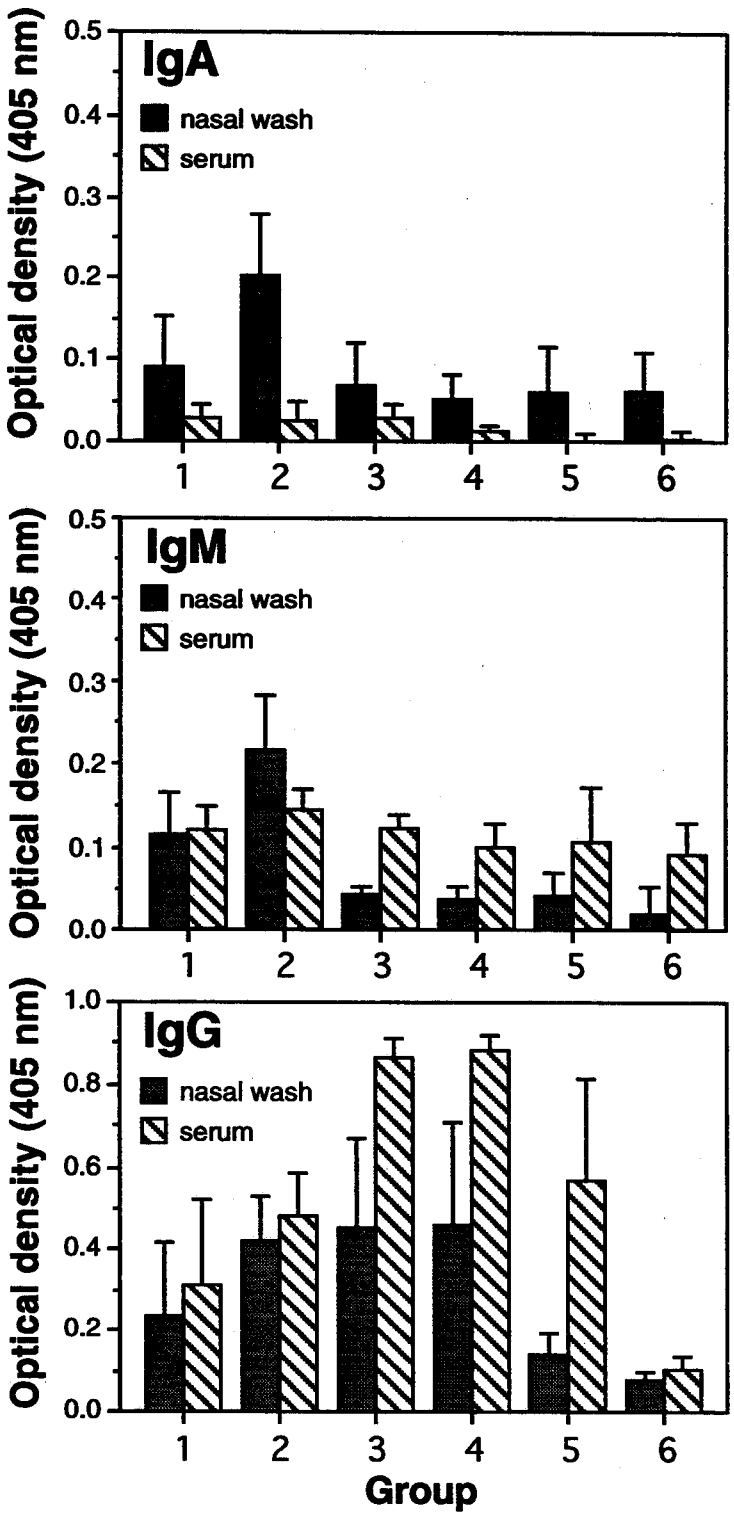

Fig. 1. Anti-NDV specific antibodies in the nasal washes and the sera of vaccinated chickens. Vaccination procedure of each group is described in the text. Samples from 5 chickens of each group were obtained before virus challenge. IgA, IgM, and IgG antibodies in the samples of individual birds were detected by ELISA as described in materials and methods. Results were expressed as the mean±SD optical density of 1:4 (nasal washes) or 1:16 (sera) dilution of the samples.

vaccination with inactivated virus (Table 2). While all of the control birds died, 4 of the 12 birds vaccinated intranasally three times with inactivated NDV alone (group 1) survived. When CTB was used as an adjuvant (group 2), the majority (12/14) of the birds survived without showing any disease signs, as was the case with those vaccinated subcutaneously three times (groups 3 and 4). Although the birds vaccinated subcutaneously once (group 5) produced serum HI antibodies of similar level to those of the group 2 birds, only 4 of the 10 birds were protected. Single intranasal vaccination with inactivated NDV alone did not confer protective immunity on the birds. None or 1 of the 5 birds vaccinated intranasally once with inactivated virus alone or together with CTB, respectively, survived after the challenge (data not shown).

Virus recovery from the sinus and blood of chickens after virus challenge

To examine whether the virus replication in the respiratory tract and subsequent viremia occurred, 5 chickens of each group were sacrificed for virus isolation from the sinus and blood 1 and 3 days post challenge. As shown in Fig. 2A, virus titers in the sinus of unvaccinated birds were $10^{3.3}$-$10^{4.3}$ PFU/g of tissue one day post challenge. No virus was recovered from the tissues of the all 5 birds or 1 of the 5 birds vaccinated intranasally with inactivated virus alone (group 1) or together with CTB (group 2), respectively. On the other hand, virus was recovered from the tissues of 5 of the 10 birds (groups 3 and 4) and 4 of the 5 birds (group 5) vaccinated subcutaneously, but at lower titers than those of control birds. On day 3 post challenge (Fig. 2B), no virus was detected in the tissues from 4 or 2 of

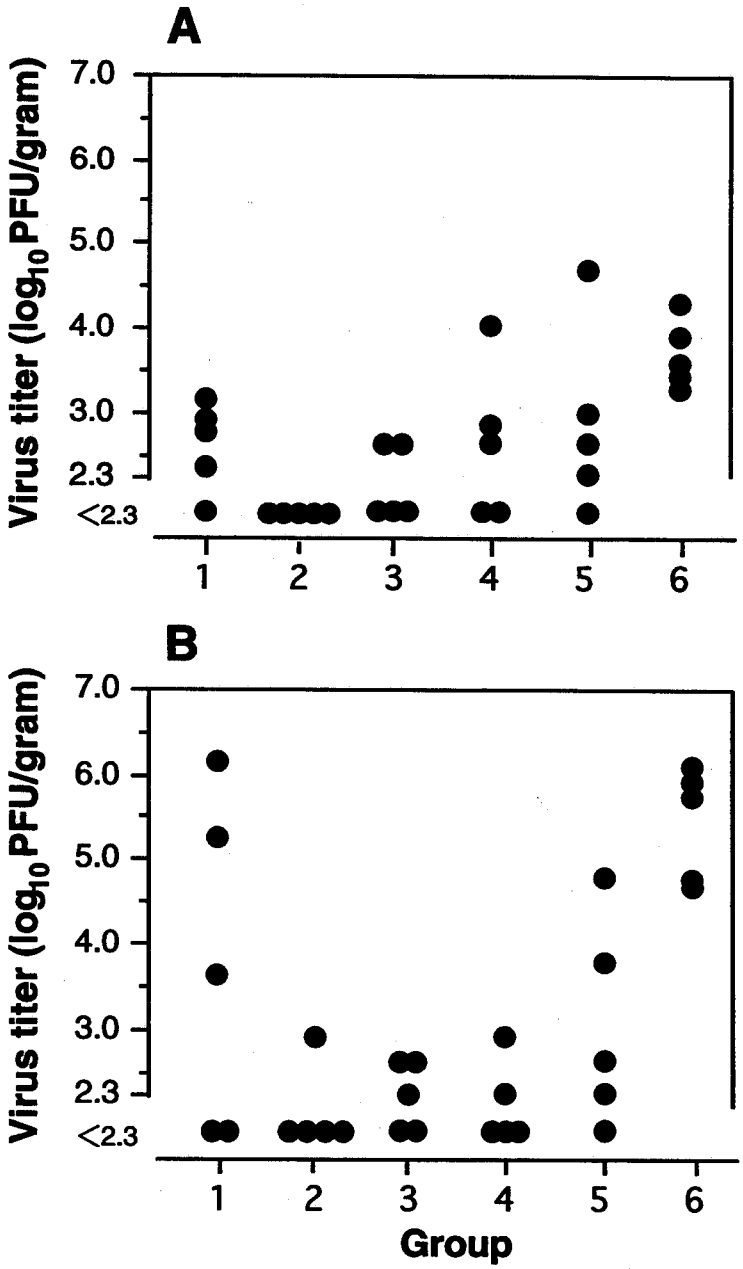

Fig. 2. Virus isolation from the sinus 1 day (A) and 3 days (B) post challenge. Five chickens of each group were sacrificed and virus titers were determined. Each point represents a single bird. The limit of detection in this assay was $10^{2.3}$ PFU/g of tissue. Chicken grouping is the same as both in Table 2 and Fig. 1.

the 5 birds of groups 2 or 1, respectively. On the other hand, virus was recovered from the sinus of 5 of the 10 chickens of groups 3 and 4, and 4 of the 5 birds of group 5, although the titers were lower than those of control birds.

No virus was detected in the blood samples collected from any bird 1 day post challenge. Three days post challenge, the virus was recovered from the blood samples of control birds at titers of $10^{3.1}$ -$10^{4.9}$ PFU/ml of tissue (data not shown). In contrast, virus was not detected in the blood samples from the birds of groups 2, 3, 4, and 5. From 2 of the 5 birds of group 1, virus was recovered at titers lower than those of control samples (data not shown).

Discussion

In the present study, the majority of the chickens vaccinated intranasally with inactivated NDV together with CTB survived challenge that was lethal for control birds. IgA, IgM, and IgG antibodies to NDV were detected in their nasal washes which neutralized virus infectivity. Furthermore, infectious virus was recovered at very low titers from the sinus of these birds. In contrast, subcutaneous vaccination induced only IgG antibodies in the nasal secretions, allowing the challenge virus to replicate in the sinus. These findings indicate that local IgA and IgM antibodies, rather than IgG, on the mucosal surface of the respiratory tracts of intranasally vaccinated birds play a critical role for inhibition of primary

replication of the virus, resulting in protection of the birds from subsequent systemic infection. Several reports also indicated the importance of the local antibodies as a primary barrier against viral infection on the mucosal surfaces (Nedrud *et al.*, 1987; Tamura *et al.*, 1988; Israel *et al.*, 1992; Lehner *et al.*, 1994). The present results also suggest that local antibodies in the mucosal secretions play an important role not only in protection of the challenged birds but in inhibition of shedding of the virus. On the other hand, subcutaneously vaccinated birds of which serum IgG antibody levels were markedly higher survived the virus challenge, although virus recovery was made from the respiratory tissues of some of the birds. This finding indicates that high levels of serum IgG antibodies might contribute to the protection from lethal systemic infection, although those were not effective to inhibit infection on the mucosal surfaces. Hence, there is a possibility that subcutaneously vaccinated chickens, possessing serum IgG but lacking secretory IgA or IgM antibodies may still shed the infectious virus, though they are immune against lethal infection.

IgA and IgM antibodies were detected in the nasal washes of chickens vaccinated intranasally with inactivated virus together with CTB (group 2), but not significantly above background in the sera (Fig. 1), suggesting that these antibodies were locally produced in the mucosal lymphoid tissues, including the Harderian glands which are thought to be the major source of the plasma cells producing local antibodies in the nasal secretion, tears and saliva in chickens. IgG antibody levels in the nasal washes were not distinctive among the birds of groups 2, 3 and 4. On the other hand, serum IgG levels of the birds of group 2 were much lower than those in the samples

of subcutaneously vaccinated birds (groups 3 and 4). Moreover, in the nasal washes of the birds vaccinated subcutaneously once (group 5), IgG antibodies were not detected, while the serum antibody levels were similar to those from the birds of group 2. These results suggest that IgG as well as IgA antibodies in the nasal washes of intranasally vaccinated birds were produced locally. Russell and Koch (1993) and Russell and Ezeifeka (1995) showed that oculotopical inoculation with live ND vaccine induced IgA, IgM, and IgG antibody-producing cell responses in the Harderian glands. On the contrary, IgG antibodies detected in the nasal secretions of chickens vaccinated subcutaneously three times may have been transudated from the serum.

In the present study, it was shown that vaccination with inactivated virus via the mucosal surface induced local antibody responses and protected birds from lethal challenge with virulent virus. The local antibodies, especially IgA inhibit primary replication of the virus at the site of entry. In addition, the present results demonstrate that the use of CTB as an mucosal adjuvant was also effective in this avian species. NDV infection would be controlled by the development of a vaccination strategy designed to stimulate not only systemic but also mucosal immune responses by administration of noninvasive antigens which have the advantage of safety and convenience.

Brief Summary

Intranasal vaccination of chickens with inactivated Newcastle disease virus (NDV) induced both local and systemic antibody responses, resulting in protection against intranasal challenge with a lethal dose of a virulent NDV strain. The immune response was enhanced by the use of cholera toxin B subunit (CTB) as an adjuvant and only small amounts of the challenge virus were recovered from the birds vaccinated together with CTB. On the other hand, subcutaneous vaccination with the same antigen induced only a serum antibody response in chickens, allowing the challenge virus to replicate in the sinus. The present results indicate that secretory antibodies induced on the respiratory mucosal surface by intranasal vaccination with inactivated NDV protected chickens from lethal infection by inhibiting virus replication at the portal of entry for the virus.

Part 2

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

Introduction

Parenteral vaccination of pigs against ADV infection with live or inactivated virus induced neutralizing antibodies in the blood, sensitized lymphocytes and consequently suppressed the severity of disease, but conferred only a partial protection (Crandell, 1985; Martin *et al.*, 1986). Studies on vaccination against ADV infection in dogs (Pensaert *et al.*, 1980) or cattle (Bironf *et al.*, 1982) also showed that parenteral vaccination did not confer satisfactory protection on the animals against the virus challenge. In order to prevent invasion of ADV into the host animals, it is a prerequisite to induce production of secretory antibodies that act as a barrier to primary infection of the virus on the mucosal surfaces.

As is the case for other secondary host species for ADV, the mouse is a 'terminal' host, unable to survive acute virus replication (Crandell, 1985), and thus mouse virulence has been used as a marker for characterization of the ADV strains (Platt *et al.*, 1980; Chong and Forster, 1987). In mice, the initial replication of the virus in the epithelial cells results in the lethal infection, meningoencephalitis (Babic *et al.*, 1994). Therefore, the mouse is a good model animal to evaluate protective effects of vaccines against

ADV infection. In this part, it was examined whether or not intranasal administration of inactivated ADV induced 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 cloned porcine kidney (CPK) cells were kindly provided by Dr. M. Shimizu of the National Institute of Animal Health (Tsukuba, Japan). CPK cells were cultured in 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 hour, EMEM was added and the cultures were incubated at 37℃ for 2 days. From the culture fluids harvested, the virus was purified by differential centrifugation and sedimentation through a 10-50% sucrose gradient (Kida and Yanagawa, 1979) and inactivated with 0.1% formalin.

Mice

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

Vaccination and protection test of mice

Mice were anaesthetized by an intraperitoneal injection with sodium

pentobarbital (0.5-0.75mg) and then vaccinated by intranasal administration or subcutaneous injection with the required dose of inactivated ADV alone or together with CTB (Sigma Chemical Co.) 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 LD_{50} ($10^{5.7}$ PFU) of live virus in 5 µl of PBS under anesthesia.

Nasal and trachea-lung washes were collected as described by Nedrud *et al.* (1987). 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 ELISA (Kida *et al.*, 1982). 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 and Bio-Rad Laboratories, respectively).

Western blotting was performed as described by Towbin *et al.* (1979). ADV proteins of the purified virus were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) 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.) 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 (pH 7.5) containing 4-chloro-1-naphtol (0.5mg/ml), H_2O_2 (0.015%), and 50 mM NaCl.

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 LD_{50} 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), a three-dose vaccination formula was examined.

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 3. Both IgA and IgG antibodies specific to ADV were demonstrated in the nasal and trachea-lung washes of

Table 3. Immune response of mice vaccinated with inactivated ADV intranasally or subcutaneously[a)]

Route[b)]	Vaccine[c)]	Antibody titer[d)]						Survival rate
		Nasal wash		Trachea-lung wash		Serum		
		IgG	IgA	IgG	IgA	IgG	IgA	
IN	V	3.0±1.1	1.8±0.4	2.4±1.4	2.2±1.0	6.4±0.4	1.2±1.5	100% (10/10)
	V+CTB	4.8±0.4	3.0±0.6	4.8±1.4	4.8±0.4	8.6±0.5	1.8±1.6	100% (10/10)
SC	V	0.4±0.5	—	3.2±0.5	—	7.8±0.4	0.8±1.0	40% (2/5)
	V+CTB	0.4±0.5	—	3.2±0.4	—	8.0±0.6	1.2±2.4	20% (1/5)
Control		—	—	—	—	—	—	10% (1/10)

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 LD_{50}).

b) IN: Intranasal, SC: Subcutaneous.

c) V: Inactivated ADV, CTB: Cholera toxin B subunit.

d) Antibody titer was determined by ELISA and expressed as $\log_4$ of the reciprocal of the end point sample dilution (mean±SD). —: Each of individual titers of the 5 mice was less than 1.0.

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 LD_{50}) 7 days after the last vaccination. Survival rates of these mice after the challenge are also shown in Table 3. All of the mice vaccinated intranasally with inactivated ADV, regardless of the use of CTB, 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 LD_{50} of virus, surviving

mice were again challenged with a higher dose of live virus (100 LD_{50}). 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. 3). 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 155 kDa protein of ADV (Fig. 4).

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, it was 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 LD_{50} or 100 LD_{50} of virus. These mice showed dose-dependent defensive responses (Table 4). Against the challenge with 10 LD_{50} 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

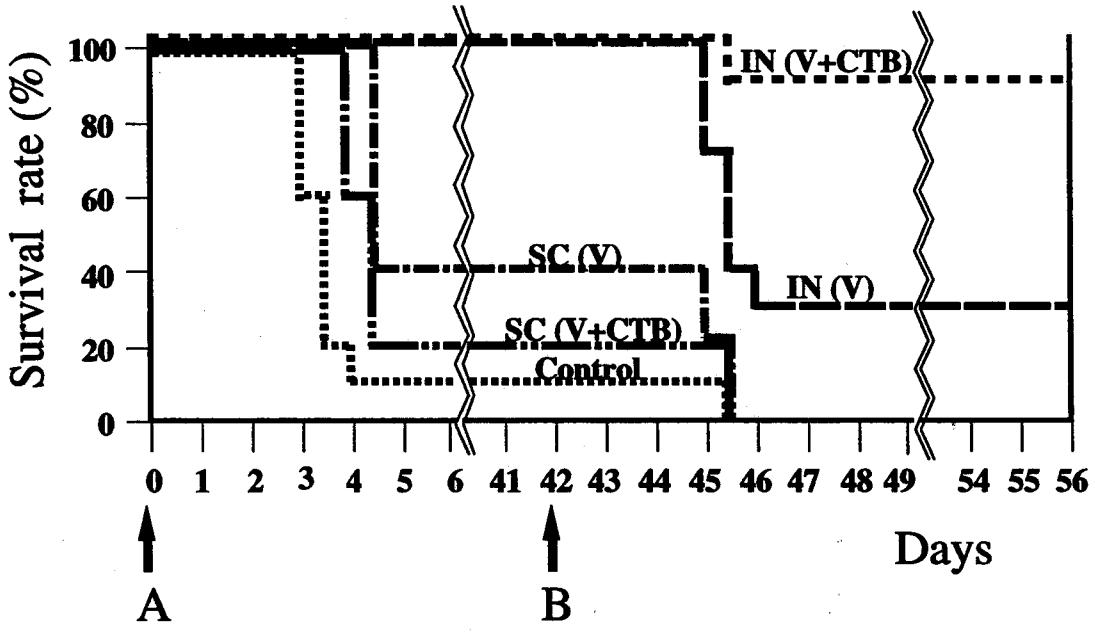

Fig. 3. Survival rates of mice after the challenge with virulent ADV. Mice were vaccinated subcutaneously (SC) or intranasally (IN) with inactivated ADV alone (V) or together with CTB (V+CTB). The arrows indicate intranasal challenge with 10 LD_{50} (A) or 100 LD_{50} (B) of live virus.

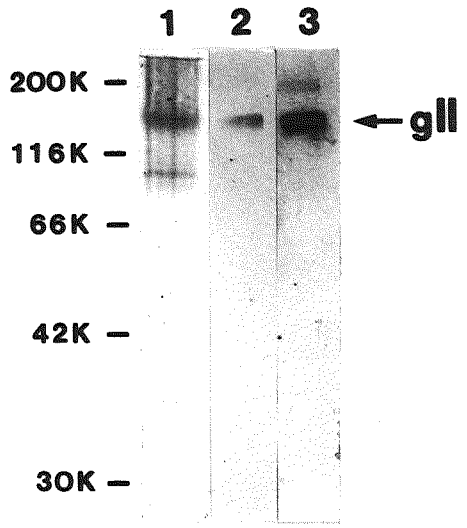

Fig. 4. Western blot analysis of ADV proteins to which antibodies in the nasal wash of intranasally vaccinated mice bound. Proteins of purified virions were separated in 10% SDS-PAGE under nonreducing conditions and transferred to nitrocellulose sheets. Proteins were stained with Amidoblack (lane 1). The sheets were soaked in the appropriately diluted nasal wash. After incubation with anti-mouse IgA (lane 2) or IgG (lane 3) antibodies conjugated to peroxidase, the sheets were soaked in the substrate solution.

Table 4. Survival rates of mice vaccinated intranasally with inactivated ADV after virus challenge

Challenge dose[b]	Vaccine dose[a] (μg/mouse)			
	40	20	10	0
10 LD_{50}	100% (5/5)	100% (5/5)	80% (4/5)	0% (0/5)
100 LD_{50}	40% (2/5)	20% (1/5)	0% (0/5)	0% (0/5)

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 LD_{50} of live virus after the last vaccination.

μg of inactivated virus showed complete protection against the challenge with 100 LD_{50} of virus (20 and 40% survival rates, respectively). None of the mice vaccinated with 10 μg of inactivated virus survived the challenge with 100 LD_{50} of virus.

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 155 kDa protein of ADV in the present study. By SDS-PAGE of structual proteins of strain Phylaxia, molecular weight of gII complex was estimated as 155 kDa under nonreducing condition (Lukács *et al.*, 1985). On the other hand, Nakamura *et al.* (1990) showed that gII complex of strain Indiana S migrated as

molecular weight of 230 kDa 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 (Wölfer *et al.*, 1990). It was shown that monoclonal antibodies to envelope glycoprotein gII neutralized the virus infectivity and inhibited its adsorption to the cells (Hampl *et al.*, 1984). The gII protein is essential for virus penetration including fusion function (Rauh and Mettenleiter, 1991). Intraperitoneal immunization with purified gII glycoprotein together with adjuvant gave a protective effect in mice and pigs (Nakamura *et al.*, 1993). 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 4). Mice vaccinated intranasally with inactivated ADV alone showed only partial protection against the challenge with 100 LD_{50} 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 LD_{50} of virus (Fig. 3). 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 stimulate 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.

Brief Summary

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.

Part 3

Induction of Protective Antibody Responses against Aujeszky's Disease Virus by Intranasal Vaccination with Glycoprotein B in Mice

Introduction

As described in part 2, intranasal vaccination of mice with inactivated ADV induced local secretory IgA and IgG antibody responses specific to envelope glycoprotein II (glycoprotein B, gB) resulting in complete protection of the animals against intranasal challenge with lethal doses of the virus. This gB is one of the major glycoproteins of ADV, consisting of a complex of three glycoproteins which are covalently linked by disulfide bonds (Hampl *et al.*, 1984; Lukács *et al.*, 1985), and shares homology with glycoprotein B of herpes simplex virus 1 (Robbins *et al.*, 1987). It has a major role for the entry of ADV into cells (Rauh *et al.*, 1991; Rauh and Mettenleiter, 1991). Monoclonal antibodies to ADV-gB neutralized virus infectivity and inhibited its adsorption to the cells (Hampl *et al.*, 1984; Nakamura *et al.*, 1990). Parenteral vaccination with purified gB conferred protection on mice against intraperitoneal challenge with a lethal dose of the virus, but only a partial protection on pigs against the challenge by the respiratory route (Nakamura *et al.*, 1993). In this part, therefore, the effect of intranasal vaccination of mice with gB of ADV was examined for

the ability to induce protective antibody responses.

Materials and Methods

Virus and cell culture

ADV strain, YS-81 was used. CPK cells were cultured in EMEM as described in part 1. Propagation and purification of the virus were carried out as described in part 1.

Purification of gB

Hybrid cell lines producing monoclonal antibodies to ADV were obtained according to the method of Kida *et al.* (1982). The specificity of the antibodies to gB was determined by Western blotting as described by Towbin *et al.* (1979). Monoclonal antibody 19/4 bound to the gB complex which has a molecular weight of 155 kDa under nonreducing conditions in SDS-PAGE (Lukács *et al.*, 1985). Monoclonal antibody 19/4 in the ascitic fluid was purified by fractionation with ammonium sulfate, and coupled to cyanogen bromide-activated Sepharose 4B beads (Pharmacia Biotech). Using the affinity column, gB was purified from ADV-infected CPK cells solubilized by 1% Triton X-100. The purified protein gave a single band with molecular weight of 155 kDa under nonreducing conditions in SDS-PAGE and was confirmed as gB by Western blotting.

Vaccination and protection test of mice

Ten or fifteen 6-week-old female ddY mice (Shizuoka Laboratory Animal Center) were vaccinated by intranasal administration with different doses (5, 0.5, or 0.05 μg) of the purified gB alone or together with 1 μg of CTB (Sigma Chemical Co.) as an adjuvant in 20 μl of PBS under anesthesia with sodium pentobarbital. After 2 weeks, a second vaccination was carried out, followed by a third vaccination a week later. One week after the last vaccination, 5 mice of each group were sacrificed to obtain nasal washes and sera.

Antibody assays

Nasal washes were collected as described by Nedrud *et al.* (1987). Anti-virus antibodies in those samples were measured by ELISA with disrupted viral antigen prepared from purified virus as described in part 1. Neutralization tests were done by plaque reduction assay with pooled nasal washes or sera. The samples were incubated with 100 PFU of the virus in the presence of 5% guinea pig serum as a source of complement.

Results

Intranasal vaccination with purified gB induced both local and systemic antibody responses to ADV. Both IgA and IgG antibodies were detected in the nasal washes of mice vaccinated intranasally together with or without CTB (Fig. 5). In the sera of these mice, IgA antibodies were scarcely

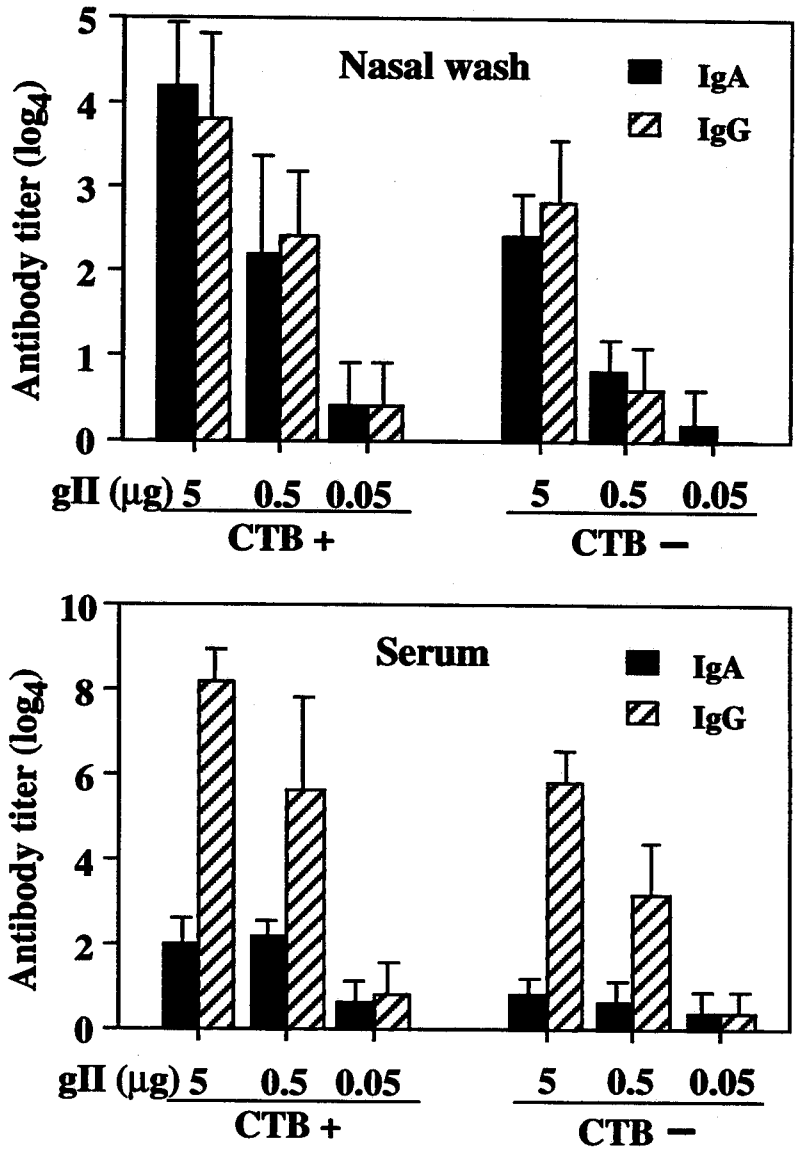

Fig. 5. Antibody response of mice vaccinated with purified gB of ADV. IgA and IgG antibodies in the nasal washes and the sera were measured by ELISA. The antibody titer is expressed as $\log_4$ of the reciprocal of the end-point sample dilution. Each bar represents the geometric mean of a group (±standard deviation) .

detected while high titers of IgG antibodies were demonstrated. The levels of these antibodies were dependent on the vaccine dose. Antibody titers both in the nasal washes and the sera of mice vaccinated with the gB together with CTB were significantly higher than those of mice vaccinated with gB alone. Undiluted nasal wash of the mice vaccinated with 5 μg of gB together with CTB gave 40% reduction of plaques in the presence of complement and the serum neutralization titer of these mice was approximately 1:160 (data not shown). Administration of CTB alone did not induce any detectable antibody response to ADV (data not shown). The specificities of the antibodies were verified by Western blot analysis (Fig. 6A). Both IgA and IgG antibodies in the nasal washes and IgG antibodies in the sera of mice vaccinated with purified gB together with CTB bound only to gB, while IgG antibodies in the antiserum to ADV bound to the three major glycoproteins, gB, gC, and gD of ADV.

To evaluate the protective effects of intranasal vaccination with purified gB, the remaining 5 or 10 mice were challenged intranasally with a lethal dose of virulent ADV (10 LD_{50}) in 5 μl of PBS under anesthesia 7 days after the last vaccination. Survival rates of the mice after the virus challenge are shown in Table 5. Against the challenge, these mice showed vaccine dose-dependent defensive responses. Survival rates of mice vaccinated with 5, 0.5, and 0.05 μg of the gB together with CTB were 100, 90, and 40 %, respectively. On the other hand, vaccination with gB alone gave partial protection; 60 and 40 % of the mice vaccinated with 5 and 0.5 μg of gB, respectively, survived. Vaccination with 0.05 μg of gB alone did not show any protective effect. To confer sufficient protective immunity

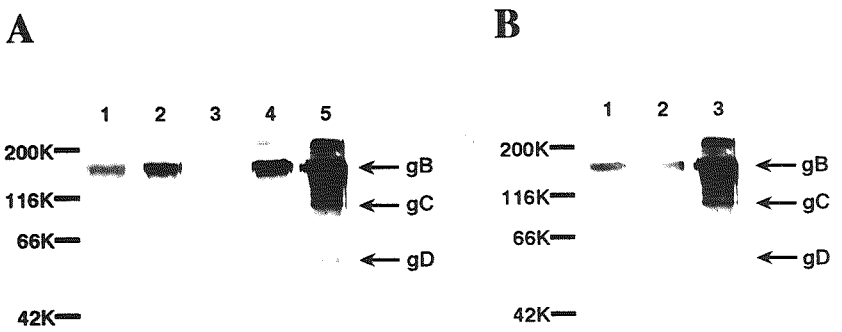

Fig. 6. A. Western blot analysis of ADV protein to which IgA and IgG antibodies bound. Proteins of purified virions were separated in 10% SDS-PAGE and transferred to nitrocellulose sheets. The sheets were soaked in appropriately diluted nasal washes (lanes 1 and 2) or sera (lanes 3 and 4) of mice vaccinated intranasally with gB together with CTB, or antiserum (lane 5) which was obtained from mice immunized with inactivated virus. After incubation with anti-mouse IgA (lanes 1 and 3) or IgG (lanes 2, 4, and 5) antibodies conjugated to peroxidase, the sheets were soaked in the substrate solution. **B. Western blot analysis of ADV protein to which serum IgG antibodies bound.** Sera obtained from mice vaccinated with 5 μg of gB together with CTB before (lane 1) and after (lane 2) virus challenge, and antiserum (lane 3) were examined.

Table 5. Protective effects against intranasal challenge with the virus[a)]

Vaccine (μg)		Survival rate
gB	CTB[b)]	
5	1	100% (10/10)
0.5	1	90% (9/10)
0.05	1	40% (4/10)
0	1	0% (0/5)
5	0	60% (3/5)
0.5	0	40% (2/5)
0.05	0	0% (0/5)
0	0	0% (0/10)

a) Mice were challenged intranasally with 10 LD_{50} of live virus.

b) Cholera toxin B subunit.

on animals by mucosal vaccination with purified viral proteins, use of an adjuvant may be required, in accordance with the results of previous studies (Nedrud *et al.*, 1987; Tamura *et al.*, 1988; Israel *et al.*, 1992). Although vaccination with CTB alone did not confer protective immunity against lethal challenge with ADV, survival periods of the mice were prolonged by approximately 24 hours compared to control mice, suggesting that CTB may potentiate a nonspecific defense on the mucosal surface.

Four weeks after the virus challenge, five and four of the surviving mice which had been vaccinated with 5 or 0.5 μg of the gB, respectively, together with CTB were sacrificed and the serum antibody titers and specificity were examined. Antibody levels in these samples were similar to or lower than those collected before challenge (Table 6) and IgG antibodies specific only to gB were detected (Fig. 6B), suggesting that replication of the challenge virus did not occur at all in these mice.

Discussion

In the present study, intranasal administration of purified gB of ADV induced a specific antibody response in the secretion of the respiratory tract of the mice and the antibody levels were closely related to the protective effects. The present results showed that serum IgG antibodies were induced by this vaccination procedure but were not likely to contribute to protection. This finding is in accord with our previous results showing that IgG antibodies detected at high titers in the sera of mice vaccinated

Table 6. Serum antibody titers[a] of mice before and after virus challenge

Vaccine (μg)		Before challenge[b]		After challenge[c]	
gB	CTB[d]	IgA	IgG	IgA	IgG
5	1	2.0±0.6	8.2±0.8	1.4±0.5	6.6±1.4
0.5	1	2.2±0.4	5.6±2.2	0.8±0.4	4.5±2.3

a) The antibody titer is expressed as a $\log_4$ of the reciprocal of the end-point sample dilution (mean ± standard deviation).

b) The sera were collected from vaccinated mice 1 day before virus challenge.

c) The sera were collected from surviving mice 4 weeks after virus challenge.

d) Cholera toxin B subunit.

subcutaneously with inactivated ADV did not contribute to protection of mice against primary replication of the virus on the nasal mucosa. These findings, thus, indicate that local secretory antibodies to gB neutralized virus infectivity on the mucosal surface, resulting in complete protection of mice from viral invasion into the host. The present results, along with previous studies on immunization against ADV and other herpesviruses (Israel *et al.*, 1992; Riviere *et al.*, 1992; Gallichan *et al.*, 1993; Nakamura *et al.*, 1993; Leary *et al.*, 1994), suggest that gB and analogous proteins of herpesviruses are possible candidates for safe and effective mucosal vaccines.

In the present study, CTB was used as a mucosal adjuvant which enhance both mucosal and systemic antibody responses. It is well known that oral administration of protein antigens results in the induction of a state of systemic unresponsiveness or suppression (oral tolerance). While Elson and Ealding (1984) showed that oral administration of CT did not induce tolerance in mice and abrogated tolerance to an unrelated protein antigen, a recombinantly produced CTB was found to be also an effective transmucosal carrier for induction of peripheral tolerance when conjugated with foreign antigens (Sun *et al.*, 1994). Different effects on the ability to induce tolerance may be lead by contamination of CT (Sun *et al.*, 1994). Indeed, it was shown that CTB supplemented with a trace amount of CT could act as an adjuvant (Wilson *et al.*, 1990; Tamura *et al.*, 1994). Recently, Waldo *et al.* (1994) demonstrated that intranasal immunization with protein antigen also caused suppression of systemic immune response. Such undesirable immune suppression may be enhanced by the unsuitable

use of CT or CTB as an adujvant for intranasal vaccination. On the other hand, CT adjuvant induced production of IgE antibodies and allergic sensitization after oral or nasal immunization with protein antigens (Snider *et al.*, 1994; Tamura *et al.*, 1994). These studies suggest that a cautious approach is required for the use of CT or CTB as a mucosal adjuvant. Yet, explanation on the adjuvant effect of CT should provide useful information for the development of effective mucosal vaccines, which may also contribute to our knowledge of the mechanisms involved in regulating mucosal immunity.

The vaccination strategy for Aujeszky's disease should be designed to induce production of secretory antibodies that act as a barrier on the mucosal epithelial cells. Consequently, this leads to prevention of latent infections, the sequel of primary infection, by prohibiting initial replication of ADV in the mucosal tissue, which is the portal of entry for the virus, and hence control of the spread of ADV throughout the pig population.

Brief Summary

Intranasal vaccination of mice with glycoprotein B (gB) of Aujeszky's disease virus (ADV) induced specific IgA and IgG antibody responses in the secretion of the respiratory tract, resulting in protection of the animals against intranasal challenge with a lethal dose of virulent ADV. The immune response was enhanced by the use of cholera toxin B subunit as an adjuvant. The present results indicate that local vaccination with gB is a promising strategy to confer protective immunity on animals against ADV infection by inducing secretory antibodies on their mucosal surfaces where the primary replication of the virus occurs.

Conclusion

In the present study, immune responses induced by intranasal (mucosal) and subcutaneous (parenteral) vaccinations with viral antigens were compared. Protective effects of these vaccinations against intranasal challenge with lethal doses of relevant virulent viruses were also evaluated. The better protection to mucosal infection, achieved by mucosal vaccination, was related to the better response of local antibodies secreted on the mucosal surfaces, indicating that the local secretory antibodies play a critical role for inhibition of the virus replication at the mucosal tissues where the primary infection takes place. Indeed, neutralizing activity was found in the secretions of the respiratory tracts of intranasally vaccinated animals. The secretory antibodies must directly neutralize virus infectivity by blocking attachment of virions to host epithelial cells, or preventing viral assembly in the cells.

The sites of primary infection for NDV are usually conjunctiva, respiratory or alimentary tracts. After destruction of mucous cells at the site of entry, viremia occurs, followed by multiplication of virus in many organs. And thus, infection with a highly virulent strain of NDV is acute and lethal (McFerran and McCracken, 1988). As shown in the part 1, subcutaneous vaccination that induced markedly high levels of serum IgG antibody response was effective to protect birds from viremia which must lead to lethal infection, but virus was recovered from their sinus which was the portal of entry for the virus. In contrast to the subcutaneous vaccination, intranasal vaccination stimulated local secretory IgA and IgM

antibody responses, as well as systemic IgG. Although protective effect of intranasal vaccination was similar to or even lower than those of subcutaneous vaccination, infectious virus was scarcely recovered from the respiratory tissues. These findings indicate that primary infection of the virus at the mucosal tissues could be inhibited by local secretory antibodies, but not by serum neutralizing antibodies.

ADV initially infect epithelial cells present on the mucosal surfaces and then spread from cell to cell via intracellular bridges in the absence of viremia, followed by immediate disease and, in its natural host, establishment of a latent infection. Consequently, systemic immunity given by parenteral immunization with current live or inactivated Aujeszky's disease vaccines may moderate the severity of disease but not reliably prevent primary infection of the virus. In the present study, intranasal vaccination of mice with inactivated ADV induced local secretory antibody responses to the virus in the secretion of the respiratory tracts, resulting in complete protection of the animals against intranasal challenge with the virulent ADV. Intranasal vaccination with purified gB of ADV was also effective to induce protective immune responses. On the other hand, subcutaneous vaccination stimulated serum antibody response, but not local secretory antibody, resulting in only partial protection. These findings indicate that the absence of the local antibodies allowed the challenge virus to replicate at the mucosal tissues of the respiratory tracts. In addition, it is noted that ability to confer a protection on animals by vaccination with a single glycoprotein of the virus should permit to distinguish vaccinated animals from those naturally infected serologically. The present results

suggest that vaccination strategies designed to stimulate not only systemic but also mucosal immune responses enable to inhibit primary replication of the virus at the portal of entry and hence to prevent subsequent latent infection of ADV.

The present results showed that a complete protection of animals from viral infection on the mucosal surfaces could be attained by induction of a specific immune defense at the site of entry for the virus. Almost by definition, this means generating local antibodies in the secretions, which must coat and inactivate virions before they have a chance to infect cells. In addition, there is a number of practical advantages of mucosal vaccination with a non-invasive antigen, with respect to the simplicity that does not require syringes and needles or trained personnel, easy storage and delivery, and cost effectiveness, which should abolish the risks inherent to the use of live vaccines. The present study demonstrated a promising approach for vaccination strategy against viral infections, designed to generate local antibodies in the exocrine secretions which provide the host animals with the first line of immune defense against foreign microorganisms.

Acknowledgements

The author would like to acknowledge Prof. H. Kida, Department of Disease Control, Hokkaido University Graduate School of Veterinary Medicine, for his support and guidance during the course of this study. Appreciation is extended to Dr. C. Itakura, Department of Veterinary Clinical Sciences, Drs. M. Onuma and K. Okazaki, Department of Disease Control, Hokkaido University Graduate School of Veterinary Medicine, for reviewing the manuscript.

The author is extremely grateful to Dr. T. Ito, Department of Disease Control, Hokkaido University Graduate School of Veterinary Medicine, Dr. E. Ono, Laboratory of Animal Experiments, Institute of Immunological Science, Hokkaido University, and Dr. Y. Shimizu, Division of Veterinary Microbiology, Kyoto Biken Laboratories, for their invaluable support and advice.

Finally, the author would also like to thank his colleagues, S. Taharaguchi, A. S. Mweene, H. Takakuwa, S. Watanabe, M. Hatta, and H. Ozaki, for their excellent assistance.

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