Release of Newcastle Disease Virus Vaccine from Chitosan Microspheres *In vitro* and *In vivo**

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ABSTRACT : Newcastle disease vaccine (NDV)-loaded chitosan microspheres (NDV-CM) were prepared. Stimulatory effects of these NDV-CM on antibody response compared to free NDV were examined *in vitro* and *in vivo*. *In vitro* stimulation of macrophages with virus vaccine resulted in higher number of cells compared to saline-treated control. Both NDV and NDV-CM induced secretion of interleukin-1 (IL-1) in dose dependent manner and the secretion of IL-1 by NDV-CM was delayed compared to free NDV. Irrespective of vaccine formulation, NDV subunit antigen was not effective in preventing mortality of the birds after challenge. However, CM loaded with NDV made of whole viron had antibody responses and protection similar to those shown by ND-K, a commercial inactivated oil-emulsion vaccine. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 4 : 543-547)***

Key Words : Newcastle Vaccine, Chitosan Microspheres, Macrophage, Interleukin-1

INTRODUCTION

A new and improved method of vaccine delivery is urgently needed to decrease mortality due to infectious diseases (Rehmani and Spradbrow, 1995). Establishment of sufficient levels of protective, long-lasting immunity by inactivated antigens mostly requires a strong adjuvant and multiple immunizations. Present knowledge on the induction and regulation of immunity suggests that at least partially distinct mechanisms underlie targets for immune interference including immunostimulation. Consequently, adjuvants may have distinct effects on either primary or secondary responses. Although it is generally believed that major influence of the adjuvant is established during the first contact between antigen and host, the final outcome of multiple injections is the sum of effects on primary and secondary responses (Hllgers et al., 1998). One possible way of facilitating efficient vaccine delivery is to reduce the number of repeated administrations required for long-term protection.

Microcapsules or microspheres were the most common forms used for the controlled-vaccine delivery formulations (Alonso et al., 1994; Morris et al., 1994). The mechanism of antigen release from biodegradable microsphere is a combination of surface and bulk erosion of the polymer, diffusion of the antigen through the poymer matrix and penetration of the antigen through the pores. The ideal controlled-release vaccine preparation should deliver antigen in such a way that a long-lasting boosting effect is achieved with a single administration and provide effective antibody responses against an infectious organism.

Chitosan is a valuable excipient for oral drug delivery systems due to its biocompatibility, biodegradability, low cost and ability to open intercellular tight junctions (Illum, 1998). Also, it was reported that chitosan could induce cytotoxic T lymphocytes and macrophage activation (Nishimura et al., 1984; Nishimura et al., 1985). Therefore, chitosan can be used as immunological adjuvants or vaccine carriers. Mi et al. (1999) reported that sustained release of Newcastle disease (ND) vaccines from crosslinked chitosan microspheres could be achieved through the adsorption-desorption mechanism.

Many studies on vaccines for poultry have been concerned with the development of ND vaccines (NDV) for village chickens (Rehmani and Spradbrow, 1995). The ND is one of the most serious diseases in animal husbandry. It always causes considerable chicken death and economic loss. Until recently, there had been no effective drug to treat this disease except for immunotherapy. It was found that chicken could only raise protective antibody responses after being inoculated intrasubcutaneously with formalin-inactivated NDV repeatedly (Yusoff and Tan, 2001).

In this study, chitosan microparticles for ND vaccination were prepared and evaluated for the potential as NDV carriers *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

Chitosan 10 kDa (Degree of deacetylation: 90.8%) was donated by JAKWANG Co., Ltd. (Ansung, Korea). Tripolyphosphate (TPP, pentasodium salt anhydrous) was

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purchased from Sigma (Missouri, USA).

Preparation of Newcastle disease vaccine

SNU0202 (virulent) and Ulster (Live vaccine, Fort Dodge Animal Health Division, Kansas, USA) as ND virus strains were used in this study. These viruses were propagated in the allantoic cavity of 10 day-old embryonated hen's specific pathogen free (SPF) eggs at 35°C for 48 h and then purified from the allantoic fluids. For the vaccine preparation, the purified virus was inactivated by 0.05% formalin at 37°C for 16 h. Virus inactivation was confirmed by inoculation into eggs with the vaccine.

Preparation of NDV-loaded chitosan microspheres (NDV-CM)

A 0.25% (w/v) chitosan solution was prepared by dissolving 0.25 g of chitosan in 100 ml of 2% (v/v) acetic acid. This solution was dropped into 15% (w/v) TPP solution through the syringe with sonication of 5 Watt for 5 min. The chitosan microspheres (CM) formed were allowed to stand in this solution for 30 min and were washed with deionized distilled water. One milliliter of NDV (3 mg/ml) containing 50 mg of CM was kept at 25°C for 24 h with shaking to immobilize the NDV by adsorption. After incubation, the medium was centrifuged to remove the supernatant containing free NDV. The loading efficiency of SNU0202 and Ulster into CM, calculated by measuring the protein content of the supernatant by BCA protein assay (Pierce), was 34.9 and 42.3%, respectively.

Observation of microspheres by scanning electron microscopy (SEM)

A drop of NDV-CM solution was placed on a stud. After air-drying at room temperature, the sample was coated with gold using a JEOL JFC-110E Ion Sputtering device (JEOL, Japan). SEM observation was made using JSM 5410LV scanning electron microscope (JEOL, Japan).

Measurement of dynamic light scattering (DLS)

Particle sizes and distribution were determined using an electrophoretic light scattering spectrophotometer (ELS 8000, Otsuka Electronics, Ltd., Osaka, Japan) with a 90° scattering angle at 25°C.

Macrophage stimulation study

The NCDC mouse macrophage cell line (CCL 46.1) (American Type Culture Collection, MD, USA) was cultured in completed RPMI-1640 medium (Gibco-BRL, NY, USA) supplemented with 5% fetal calf serum (FCS) (Gibco-BRL), 1% of L-glutaimin (Gibco-BRL) and 1% of gentamicin (Gibco-BRL) in triplicate wells of 96 well microtiter plates at 2×10^4 cells per well. After 72 h, medium was replaced with fresh completed RPMI-1640 containing NDV, either SNU0202 or Ulstar strains in a serial dilution at final concentration of 32, 16 or 8 hemagglutination (HA:units/25 μl) and 16, 8 or 4 HA (units/25 μl), respectively. For positive control, cells were stimulated with ConA (200, 100 or 50 ng/ml) and for negative control, microspheres only at equivalent to 32 HA were used. Cells were further incubated for 24 h for proliferation assay or for 48 h for IL-1 secretion in supernatant.

Cell proliferation

Colorimetric assay (MTT assay kit) (Roche Molecular Biochemicals, Germany) was used for the quantitative determination of cellular proliferation and activation in response to external stimuli such as ConA or NDV (Vaccine control and NDV-CM). The assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells. This cellular reduction involves the pyridine nucleotide cofactors NADH and NADPH. The formazan crystals formed are solubilized and the resulting colored solution is quantified using a scanning multi-well spectrophotometer.

Determination of IL-1

Enzyme-linked immunosorbent assay (ELISA) was used to measure the concentration of IL-1 from culture supernatant. Monoclonal anti-mouse IL-1β (R & D Systems Inc.) antibodies were used and the assay was performed as described previously (Estrada et al., 1997).

Hemagglutination (HA) and hemagglutination inhibition (HI) test

HA is commonly used to identify certain antigens (mainly viruses such as NDV or Avian Influenza) that hemagglutinate chicken red blood cells. HI test is to quantitate serum antibody to a specific pathogen (e.g. NDV, Influenza and Mycoplasma), which inhibits hemagglutination.

For red blood cell suspension, chicken erythrocytes were obtained from SPF chickens, washed and re-suspended in PBS to make a 1% suspension. NDV was diluted with PBS to make a serial two-fold dilution. Fifty μl of each sample was inserted into the wells of 96-well microplate and 25 μl of 1% chicken erythrocyte suspension was added. The microplate was covered with a lid, gently swirled on plate-mixter, left at room temperature for 45 min, and HA was numerically observed as the reciprocal of the highest dilution of samples of the HA expressed (Thayer and Beard, 1998).

Anti-NDV antibody titers were determined by HI. Chicken serum was serially diluted in PBS and 20 μl of each diluted serum was inserted into the wells of 96-well microplate. 25 μl of 1% chicken erythrocyte suspension was

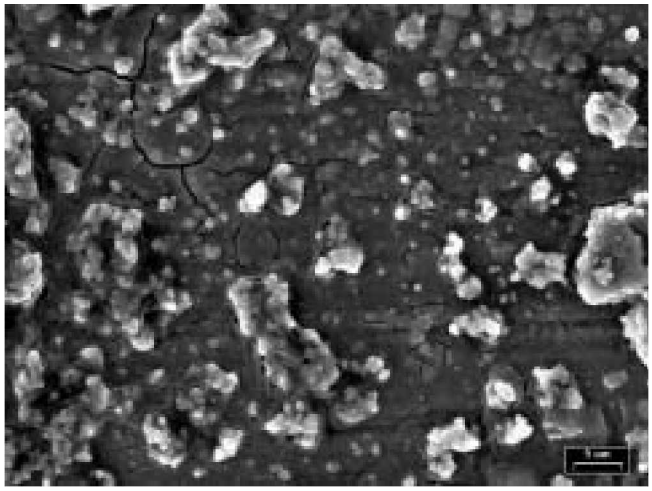

Figure 1. Scanning Electron Microscopic (SEM) photograph of NDV-CM (×2,000).

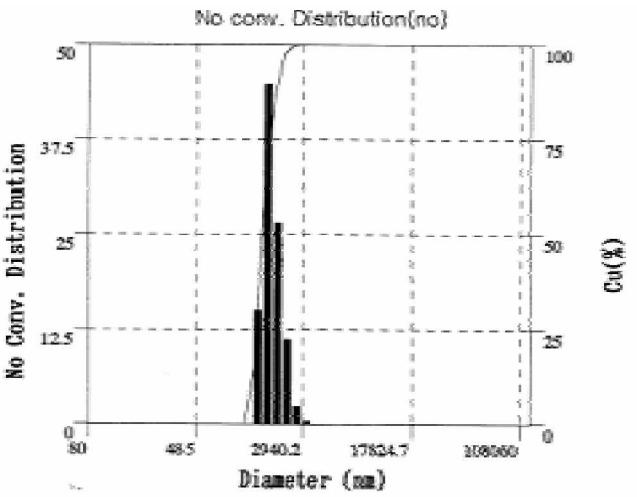

Figure 2. Average particle size and distribution of NDV-CM. Electrophoretic light scattering spectrophotometer (ELS 8000) was used with a 90 scattering angle at 25°C.

added to each serum. Eight HA units (eight unit/25 μl) of NDV were added to each well and plates were incubated for 15 min at room temperature. Then, 1% chicken erythrocyte suspension 25 μl was added and agglutination was detected after 1 h of shaking. The highest reciprocal serum dilution demonstrating HI was considered to be the titre and expressed as $\log_2$ value.

Animal experiments

Subunit antigen of NDV was prepared by treatment of diethylether with formalin-inactivated NDV. Diethylether treatment of NDV showed two- and three-fold HA titres of SNU0202 and Ulster, respectively, compared with untreated ones. SPF chickens at 14 days of age were vaccinated by subcutaneous injection with 500 μl of NDV-CM containing NDV subunit antigen diluted by 50 fold. Also, commercial ND inactivated oil-emulsion vaccine, ND-K (Fort Dodge Animal Health Division, Kansas, USA), was vaccinated by subcutaneous injection of 100 μl. Fourteen days after vaccination, the chickens were challenged with SNU0202. All the surviving chickens were sacrificed 14 days post challenge.

In the second experiment, 40 day-old chickens were divided into 4 groups of 10 birds each and chickens in three groups were vaccinated either with ND-K(500 μl/bird) or with NDV-CM(SNU0202 and Ulster strains, 500 μl/bird). The remaining group was served as a unvaccinated control. Four days after the first vaccination, the groups vaccinated with NDV-CM were revaccinated (500 μl/bird). Additional two groups of five birds each were vaccinated once with NDV-CM at 44 days of age. Blood samples for serology were taken before and after vaccination and HI antibody titres of the samples were determined.

RESULTS AND DISCUSSION

Loading of NDV into chitosan microspheres (CM)

Porous biodegradable chitosan microspheres were fabricated and used as antigen vehicles for the controlled release of the antigen of NDV. The NDV was immobilized in porous chitosan microspheres by being adsorbed onto their pores instead of the traditional gel entrapment methods. The chitosan microspheres had the higher quantity of antigen adsorption, because the antigen of the NDV in the culture medium is negatively charged and could strongly interact with the amino groups of chitosan based on the electrostatic attraction between the negatively charged substrate of the ND virus and the positively charged functional group (Mi et al., 1999). The loading efficiency of SNU0202 and Ulster into CM was 34.9 and 42.3%, respectively.

The morphology and particle sizes of NDV-CM

The chitosan microspheres coagulated in TPP solution displayed uniform spherical shapes with a diameter of around 2 μm. Even after vaccine loading, the morphology of the NDV-CM was not different from that of unloaded CM (Figure 1). The results similar to that of SEM measurement were observed in the dynamic light scattering (DLS) measurement and showed uni-modal distribution with narrow width (Figure 2).

Release of NDV from NDV-loaded CM *in vitro*

The release of NDV from NDV-CM *in vitro* was examined (data not shown). The release of NDV from NDV-CM rapidly occurred upto 70% at an initial stage (upto 2 days) and then, a few amount of release (10% of the

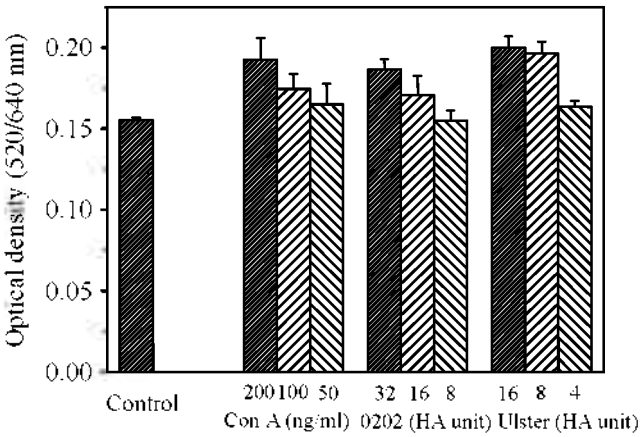

Figure 3. Proliferative responses in NCTC cells stimulated with 0202- or Ulster-NDV compared with negative control (microspheres only) and positive control (ConA). MTT assay was carried out to determine the effect of NDV (0202 and Ulster)-loaded CM using mouse macrophage cell line (NCTC). The figure is the representative of four experiments with similar results.

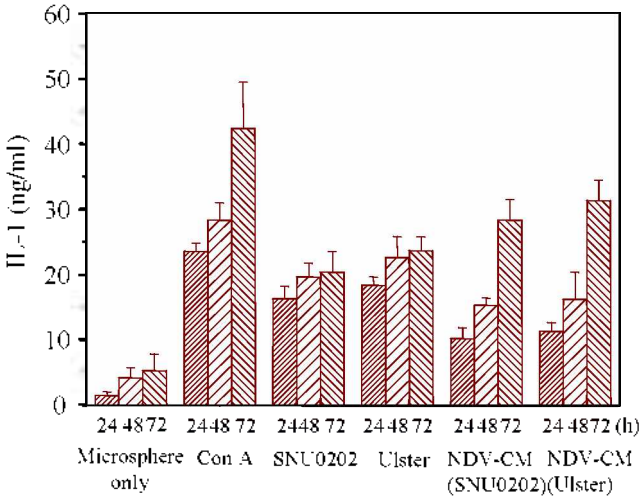

Figure 4. IL-1 secretion in NCTC cells stimulated with 0202- or Ulster-NDV compared with negative control (microspheres only) and positive control (ConA) IL-1 secretion was determined from supernatant of NCTC mouse macrophage cell line stimulated with NDV (0202 and Ulster)-loaded CM and compared with controls using ELISA. The figure is the representative of four experiments with similar results.

loaded NDV) was observed for 5 days (figure not shown). It is evident that strong interaction of NDV with chitosan resulted in a marked decrease in NDV release. The optimal formulation condition of NDV-CM should be found out for sustained release.

Macrophage activity and IL-1 secretion

The ability of inactivated ND virus vaccine to induce macrophage cells was assessed. As shown in Figure 3, *in vitro* stimulation of macrophages with NDV-CM resulted in higher number of cells compared to saline-treated control. As shown in Figure 4, both inactivated NDV and NDV-CM induced secretion of IL-1 in dose dependent manner. It is interesting to point out that the secretion of IL-1 by NDV-CM was delayed compared to the inactivated NDV. This must be an indication of slow release of NDV from NDV-CM and therefore, a cause for the pattern of delayed secretion. The amount of IL-1 secretion by NDV-CM at 72 h was even higher than NDV alone.

Protection of chickens from virulent ND viruses using NDV-CM

Protection against ND viruses is completely based on neutralizing antibodies against their fusion protein (Russell, 1988). To find out whether the NDV-CM containing SNU0202 or Ulster was able to protect chickens from challenge with a lethal dose of virulent ND viruses, we performed vaccination-challenge test in 14 or 40 day-old chickens.

None of the birds showed any signs of weight change after vaccination, indicating that the vaccines used were found to be safe in this experiment. Birds vaccinated with NDV subunit antigen did not give good results, irrespective of vaccine formulation and showed no HI values as shown in Table 1. Moreover, 50 fold dilution of the vaccines made all the chickens die after the challenge. This data suggested that vaccination with NDV subunit did not confer protective immunity on the chicken, irrespectively of oil emulsion or

Table 1. Haemagglutination inhibition antibody responses and protection of chickens vaccinated subcutaneously with NDV subunit-loaded chitosan microspheres (first animal experiment)

Type	Dose	No. of chickens vaccinated	14 days after vaccination		No. of survivors/ No. challenged (%)
			Weight (Mean±SD, g)	HI titer (log2)	
Control		10	232±29	0.0	0/10 (0.0)
SNU0202	1	7	252±32	0.0	1/7 (14.3)
	1/50	8	258±36	0.0	0/8 (0.0)
Ulster	1	7	270±33	0.0	2/7 (28.6)
	1/50	8	278±36	0.0	0/8 (0.0)
ND-K[1)]	1	7	250±31	0.0	3/7 (42.9)
	1/50	8	217±24	0.0	0/8 (0.0)

[1)] Commercial NDV vaccine (oil emulsion).

Table 2. Haemagglutination inhibition antibody responses and protection of chickens vaccinated subcutaneously with NDV whole virion-loaded chitosan microspheres (second animal experiment)

Type	HI titer (log2) (Mean±SD)		No. of survivors/ No. challenged (%)
	Challenge		
	Before	After	
Control	0.0±0.0	NA	0/10 (0)
SNU0202	0.6±0.8	6.8±2.9	9/10 (90)
	0.0±0.0	8.3±1.5	3/5 (60)
Ulster	0.9±1.1	6.8±1.4	9/10 (90)
	0.0±0.0	9.0±1.4	2/5 (40)
ND-K	2.2±1.7	8.3±1.6	10/10 (100)

chitosan microspheres. In the second experiment, NDV whole virions were used instead of NDV subunit. A single injection of ND-K elicited the highest antibody titre and all the vaccinated chickens survived after challenge. NDV-CM, SNU0202 and Ulster, also showed high antibody titres comparable to ND-K vaccination and nine out of 10 vaccinated chickens survived after challenge although two groups of five birds each vaccinated once with NDV-CM 44 days of age gave 60 and 40% protection as shown in Table 2. These data suggested that chitosan microspheres loaded with NDV whole virion had antibody responses and protection of chickens similar to those of the ND-K control, so almost all of vaccinated chickens survived after challenge.

CONCLUSIONS

Chitosan microspheres were incorporated with ND virus and used in order to characterize the efficacy of vaccine delivery carrier. NDV-CM showed the potential as vaccine carrier, comparable to the commercial product. Sustained release of vaccine from NDV-CM should be optimized for effective immune response of the loaded vaccine.

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