Characterization of haemagglutinin-neuraminidase glycoprotein of Newcastle disease virus expressed by a recombinant baculovirus

Masahiro Niikura [1], Yoshiharu Matsuura [2], Masakazu Hattori [1], Misao Onuma [1] and Takeshi Mikami [3]

[1] *Department of Epizootiology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060;*
[2] *Department of Veterinary Science, National Institute of Health, Tokyo 141; and* [3] *Department of Veterinary Microbiology, Faculty of Agriculture, The University of Tokyo, Tokyo 113, Japan*

Summary

A recombinant baculovirus containing a cDNA which encodes haemagglutinin-neuraminidase (HN) of Newcastle disease virus (NDV) was constructed. *Spodoptera frugiperda* cells infected with this recombinant virus produced a large amount of HN glycoprotein similar to the authentic HN in size. The recombinant HN glycoprotein was localized on the surface of the infected cells and conserved its haemadsorption and neuraminidase activities. The antigenic properties of the re-combinant HN glycoprotein seemed to be slightly different from the authentic one, as judging by the reactivity with a panel of monoclonal antibodies specific to the antigenic sites responsible for neutralization of viral infectivity. Chickens inoculated with the cells infected with the recombinant virus developed haemagglutination-inhibition and virus neutralization antibodies, and were completely protected from the NDV challenge.

Newcastle disease virus; Haemagglutinin-neuraminidase; Baculovirus vector

Correspondence to: M. Niikura, Department of Epizootiology, Faculty of Veterinary Medicine, Kita 18 Nishi 9, Kita-ku, Sapporo 060, Japan.

Introduction

Newcastle disease virus (NDV) is an enveloped single strand RNA virus belonging to the Paramyxovirus genus, and the causative agent of a major poultry disease. NDV possesses two glycoprotein spikes, haemagglutinin-neuraminidase (HN) and fusion (F) on its surface. These glycoproteins are known to be responsible for initiation and progress of infection. The cDNAs encoding HN glycoproteins of some NDV strains have been cloned and the nucleotide sequences were determined (Miller et al., 1986; Jorgensen et al., 1987; Gotoh et al., 1988; Schaper et al., 1988). The nucleotide sequence of cDNA encoding the HN glycoprotein of Miyadera strain predicts a polypeptide of 571 amino acids with a molecular weight (mol. wt.) of 62,529 and with five possible N-linked glycosylation sites.

Antibodies to HN glycoprotein of NDV were reported to have high virus-neutralizing activity (Nishikawa et al., 1983, Umino et al., 1984), and passive immunization of anti-HN polyclonal antibodies prolonged significantly the survival periods of NDV-infected chickens (Umino et al., 1987). Moreover, studies with human parainfluenza virus type 3 (PIV3) showed that immunization with HN glycoprotein of PIV3 reduced the virus recovery from PIV3-infected animals (Spriggs et al., 1987; Van Wyke Coelingh et al., 1987; Spriggs et al., 1988). However, to assess the importance of immune response to HN glycoprotein in the protection and the potential effectiveness of HN glycoprotein as a component vaccine against NDV infection, a large amount of HN glycoprotein without contamination of other NDV component is required. Therefore, we sought to express the HN glycoprotein of NDV using insect baculovirus vector of *Autographa californica* nuclear polyhedrosis virus (AcNPV) to obtain sufficient amounts of the native HN glycoprotein. We chose the AcNPV vector because of its success in high level expression of many foreign genes and its potential of post-translational modification such as glycosylation (Matsuura et al., 1987, 1988; Van Wyke Coelingh et al., 1987; Luckow and Summers, 1988; Kuroda et al., 1990). Although, Nagy et al. (1990) recently reported the results of successful expression of HN glycoprotein of NDV which is apparently authentic in its molecular property by a recombinant AcNPV, the detailed antigenic property and protective effect of the recombinant HN in vivo were still not examined. In this report, the expression of HN glycoprotein of NDV Miyadera strain with a recombinant AcNPV and the biological and immunological properties of the expressed glycoprotein are described.

Materials and Methods

Cells and viruses

AcNPV and recombinant AcNPV were grown in *Spodoptera frugiperda* (Sf) cells in Grace's medium (GIBCO, NY) supplemented with 10% fetal bovine serum. In experiments of ^{35}S-labeling of AcNPV proteins, TC 100 medium lacking methionine and tryptose was used (Kuroda et al., 1990). Plaque formation of AcNPV and

recombinant AcNPV was carried out as described previously (Brown and Faulkner, 1977). NDV Miyadera strain was provided by Dr. C. Morita, Research Center for Veterinary Science of the Kitasato Institute, Kashiwa (Arias-Ibarrondo et al., 1978), and passaged more than 10 times in the allantoic cavity of 10-day-old embryonated eggs before being used in the present experiments. NDV Sato strain was from the stocks of our laboratory. The viruses were grown in primary chicken embryo fibroblast (CEF) cells in Eagle's minimum essential medium (MEM) supplemented with 10% calf serum and 3 g/l of tryptose phosphate broth (Difco, MI). In experiments of ^{35}S-labeling of NDV proteins, methionine free-MEM was used.

DNA manipulations and constructions

Plasmid DNA manipulations were performed as described by Maniatis et al. (1982). Restriction enzymes and other modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan). The cDNA clone encoding HN glycoprotein of NDV Miyadera strain (Gotoh et al., 1988) was kindly provided by Dr. Y. Nagai (Nagoya University, Nagoya, Japan). The transfer vector of AcNPV, pAcYM1, was described previously (Matsuura et al., 1987).

Insertion of HN cDNA into the pAcYM1 transfer vector

The HN cDNA fragment was excised by digestion with Acc I and repaired with Klenow fragment of DNA polymerase. To the repaired ends Bam HI linkers were attached and the HN fragment was inserted into the Bam HI site of pBluescript II (Stratagene, CA). The poly(G) tail located upstream of the initiation codon of HN was deleted from Hind III site by Exonuclease III and Mung Bean exonuclease digestion. The deleted plasmids were subjected to sequence analysis by using a 7-deaza-sequencing kit (Toyobo, Osaka, Japan) and one clone that had no poly(G) tail and 19 base pairs (bps) of upstream sequence of the HN gene was selected. This plasmid was digested with Kpn I and repaired with T4 polymerase, then Bam HI linkers were attached to the repaired end. The deleted HN fragment was excised by digestion with Bam HI and inserted to the Bam HI site of pAcYM1 vector. The orientation of inserted HN gene was examined by sequencing the 5'-junction region (Fig. 1).

Transfection and selection of recombinant virus

Sf cells were transfected with a mixture of the purified infectious AcNPV DNA and transfer vector DNA as described previously (Matsuura et al., 1986). After 4 days incubation at 28°C, the supernatant fluids were harvested and subjected to plaque formation in Sf cell monolayers. Plaques exhibiting no evidence of occlusion bodies as determined by transmission light microscopy were recovered and then purified 3 times in Sf cells monolayers. High-titered ($> 10^7$ PFU/ml) stocks of recombinant virus were obtained using Sf cell monolayers.
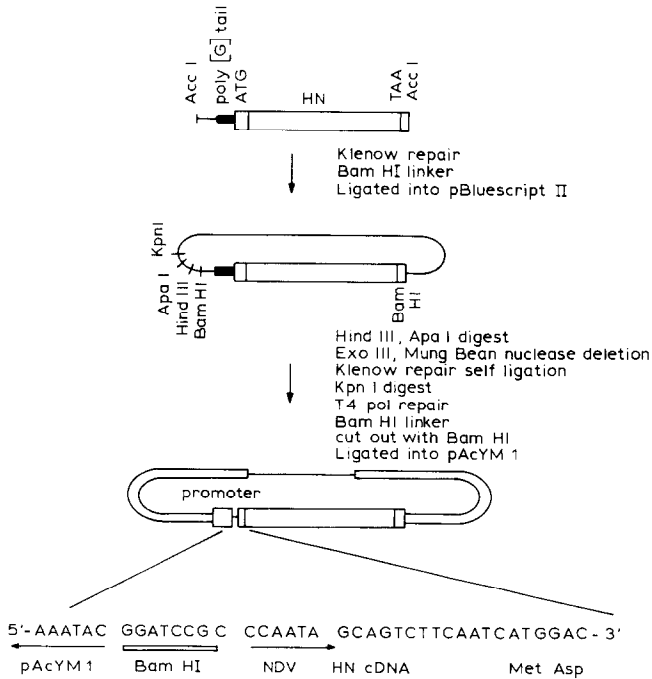
Fig. 1. Construction of transfer vector containing NDV HN cDNA. Details of the preparation of the recombinant transfer vector are described in the text.

Immunofluorescence analysis

Sf cells infected with recombinant or wild-type AcNPV at a m.o.i. of 1 were incubated at 28°C for 48 h. The infected cells were washed with phosphate-buffered saline (PBS) and incubated with a 1 : 100 dilution of a mixture of monoclonal antibodies (mAbs, cl.1 and cl.2) specific to NDV HN glycoprotein (Hoshi et al., 1983) for 1 h at room temperature. After washing with PBS, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Cappel, PA) for 1 h at room temperature. The cells were washed again with PBS and then examined for fluorescence.

Immunoprecipitation analysis

Sf cells (1×10^6) were infected with recombinant or wild-type AcNPV at a m.o.i. of 1 in 35 mm dishes. The infected cells were labeled with 50 μCi of TRAN [^{35}S]-LABEL (ICN, CA) in TC 100 medium lacking methionine and tryptose for 15 h before harvest. The cells were lysed with lysis buffer (0.5% NP-40. 0.15 M NaCl, 1 mM PMSF, 0.02% sodium azide in 50 mM Tris, pH 8.0) at indicated times and immunoprecipitated with the mixture of mAbs specific to NDV HN glycoprotein as described previously (Ikuta et al., 1981). CEF monolayers (4×10^6 cells per 35 mm

dish) that were infected with NDV Miyadera strain were labeled with 50 μCi of TRAN [^{35}S]-LABEL in methionine-free MEM for 15 h, as control experiments. The infected CEF cells and precipitates of supernatant fluids after centrifugation at 100,000 $\times$ g were lysed together and subjected to immunoprecipitation as the lysates of Sf cells. The precipitated proteins were analyzed on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Tunicamycin (TM) treatment of Sf cells infected with the recombinant AcNPV was carried out as described previously (Matsuura et al., 1987).

Enzyme-linked immunosorbent assay (ELISA)

Sf cells were infected with recombinant or wild-type AcNPV at a m.o.i. of 1 and incubated at 28°C for indicated hours. The infected cells were washed with PBS and lysed with 50 μl of lysis buffer per 10^6 or 3×10^6 cells. After centrifugation, the supernatants were diluted with 100 vol. of 50 mM carbonate buffer (pH 9.6) and absorbed overnight at 4°C to flat-bottomed ELISA plates (Sumitomo, Tokyo, Japan). Purified virions of NDV Miyadera strain were lysed and diluted with the same buffer as a control. The absorbed HN glycoprotein was detected with the mixture of mAbs specific to NDV HN glycoprotein at a 1 : 200 dilution as described (Nishikawa et al., 1983). In order to examine antigenic authenticity of the recombinant HN glycoprotein, a panel of four neutralizing mAbs that represent four antigenic sites (Nishikawa et al., 1986) were used.

Haemadsorption of recombinant AcNPV-infected cells

Monolayers of Sf cells in 35 mm dishes were infected with the recombinant or wild-type AcNPV at a m.o.i. of 1 and incubated at 28° C for indicated hours. The infected monolayers were washed with PBS and haemadsorption activities were examined with chicken red blood cells (RBC) as described previously (Morrison and McGinnes, 1989). The absorbed RBCs were lysed in 500 μl of 0.05 M NH$_4$Cl and released haemoglobin in the centrifugal supernatants were measured by OD$_{549}$.

Neuraminidase activity of recombinant AcNPV-infected cells

Monolayers of Sf cells in 35 mm dishes were infected with the recombinant or wild-type AcNPV at a m.o.i. of 1 and incubated at 28°C for indicated hours. The neuraminidase activity of the monolayers was examined with fetuin (Sigma, MO) as a substrate as previously described (Morrison and McGinnes, 1989). The amount of the released free sialic acid was measured by OD$_{549}$.

Antibody production in chickens

Chicks were hatched and reared in an isolator of our laboratory. Six-week-old chickens were subcutaneously inoculated with 10^7 cells infected with the recombinant AcNPV mixed with Freund's complete adjuvant (Day –7) followed by in-

traperitoneal inoculation with 10^7 infected cells (Day 0). Sera from the inoculated chickens were collected on Day 3, 6, 9 and 12, and subjected to haemagglutination-inhibition (HI) and virus-neutralization tests.

Serological tests

HI tests were done by the micromethod described by Sever (1962). Neutralization of viral infectivity was examined as described by Abenes et al. (1986). Neutralization titers were expressed as reciprocals of serum dilutions that could completely neutralize the infectivity of 100 $TCID_{50}$ of NDV Miyadera strain.

Protection of chickens from virulent NDV challenge by the recombinant HN

Four chickens (31-day-old, kindly provided by Dr. S. Ichikawa, Rakuno Gakuen University) which were serologically negative for NDV were used in the study. Two were subcutaneously inoculated with 10^7 of the recombinant AcNPV-infected cells mixed with Freund's complete adjuvant (FCA, Difco) on Day 0 or Day 22. The third chicken was inoculated with the same number of wild-type AcNPV-infected cells with FCA on Day 22. The fourth chicken was left as uninoculated control. All of the chickens were intratracheally challenged with 10^3 PFU of virulent NDV (Sato strain) on Day 27, and observed for 14 days after the challenge.

Results

Construction of recombinant baculovirus containing the NDV HN gene

The baculovirus transfer vector containing the NDV HN gene was constructed as described under Materials and Methods (Fig. 1). The nucleotide sequence of the 5'-junction region of the resultant plasmid was determined using an oligonucleotide 5'-AATGATAACCATCTCGG-3' that corresponds to a part of the AcNPV polyhedrin promoter (Matsuura et al., 1987) as a primer (Fig. 1). The sequence data indicate that this plasmid has proper orientation of HN gene for expression with 19 bps upstream sequence of HN initiation codon between the promoter and the open reading frame. The plasmid was cotransfected with purified infectious AcNPV DNA to Sf cells. One plaque without polyhedrin was selected from the supernatant fluid of the transfected cells, and after three cycles of successive plaque purification, stock of the recombinant virus was obtained.

Expression of HN glycoprotein in Sf cells

To confirm the expression of NDV HN glycoprotein in Sf cells infected with the recombinant AcNPV, immunofluorescence analysis was performed. The mixture of NDV HN glycoprotein-specific mAbs was reacted to the surface of the unfixed Sf cells infected with the recombinant virus (data not shown), indicating that the
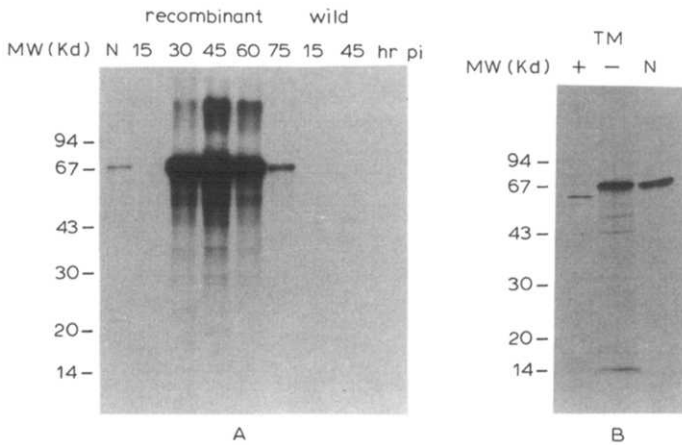
Fig. 2. (A) Radio-immunoprecipitation of HN glycoprotein expressed by the recombinant AcNPV. Cells were infected with the recombinant AcNPV at a m.o.i. of 1. After labeling for 15 h with 50 μCi of ^{35}S-amino acid mixture, the cells were harvested at 15, 30, 45, 60 and 75 h post infection and subjected to radio-immunoprecipitation using a mixture of mAbs specific to HN. (B) Effect of tunicamycin (TM) on the expression of HN glycoprotein by the recombinant AcNPV. Cells infected with the recombinant AcNPV incubated with (+) or without (−) TM were labeled with ^{35}S-amino acid mixture and subjected to radio-immunoprecipitation. Immunoprecipitate from CEF cells infected with NDV (lane N) is also shown. The positions of the mol. wt. markers are presented on the left of each panel.

recombinant AcNPV we obtained expresses NDV HN glycoprotein on the surface of the infected cells.

The expression of HN glycoprotein in the infected cells was also examined by radio-immunoprecipitation analysis (Fig. 2A). The infected cells were labeled with ^{35}S-amino acids for 15 h and harvested at 15, 30, 45, 60 and 75 h post infection. The NDV HN glycoprotein-specific mAbs precipitated a molecule with mol. wt. of 74.0 kd from the lysate of the recombinant AcNPV-infected cells but not from that of the wild-type AcNPV-infected cells. The precipitated molecule had apparently the same mol. wt. as that of HN glycoprotein of NDV Miyadera strain. From this result, we confirmed the expression of HN glycoprotein in the cells infected with the recombinant AcNPV. The recombinant HN glycoprotein in Sf cells appeared at 30 h post infection and reached to the maximum level during 30 to 45 h post infection. TM treatment of the cells infected with the recombinant AcNPV resulted in the reduction of the mol. wt. of HN protein from 74.0 to 63.0 kd which well corresponds to the mol. wt. of unglycosylated HN protein calculated from the nucleotide sequence (Fig. 2B).

Haemadsorption and neuraminidase activities of the expressed protein

We examined haemadsorption and neuraminidase activities of the expressed protein. The monolayers infected with the recombinant AcNPV at 45 h post infection expressed significant haemadsorption activity, although neither the monolayers infected with the recombinant AcNPV at 15 h post infection nor those
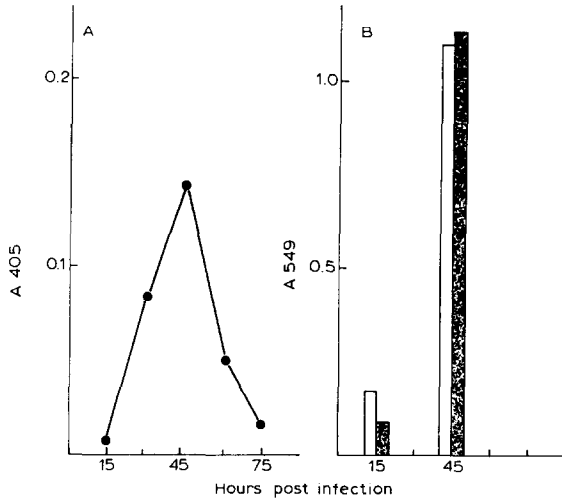
Fig. 3. (A) Time course-studies of expression of HN glycoprotein by the recombinant AcNPV. Cells infected with the recombinant AcNPV were lysed at 15, 30, 45, 60 and 75 h post infection and absorbed to microtiter plates. The absorbed HN was detected with a mixture of mAbs specific to HN by the procedure described in the text. (B) Haemadsorption and neuraminidase activities of HN glycoprotein expressed by the recombinant AcNPV. Haemadsorption activities (open bars) were expressed by OD values of released haemoglobin from chicken red blood cells absorbed to the cell monolayers infected with the recombinant AcNPV. Neuraminidase activities (black bars) were expressed by OD values of free sialic acids released from fetuin by the cell monolayers infected with the recombinant AcNPV. Background OD values from cells infected with wild-type AcNPV were subtracted.

infected with wild-type AcNPV expressed little or no such an activity (Fig. 3B). The neuraminidase activity of the expressed protein revealed to be significantly active in the monolayers infected with the recombinant AcNPV at 45 h post infection, although the activity of the monolayers infected with the recombinant AcNPV at 15 h post infection or those infected with wild-type AcNPV was hardly detectable (Fig. 3B). The time course of haemadsorption and neuraminidase activities well corresponded to the expression level of the recombinant HN glycoprotein detected by ELISA (Fig. 3A). Therefore, we concluded the expressed HN glycoprotein has authentic haemadsorption and neuraminidase activities.

Antigenic property of the recombinant HN glycoprotein

HN glycoprotein of NDV was reported to have four antigenic sites being responsible for neutralization of the virus (Nishikawa et al., 1986). We examined whether HN glycoprotein expressed by the recombinant AcNPV conserves those antigenic sites, by ELISA with four mAbs respectively specific to the four sites. Three of the four mAbs that are specific to the sites I, II and III reacted to the recombinant HN glycoprotein as to HN glycoprotein derived from purified NDV virion (Fig. 4). However, one mAb specific to site IV did not react to the recombinant HN glycoprotein, although it reacted to HN glycoprotein of NDV
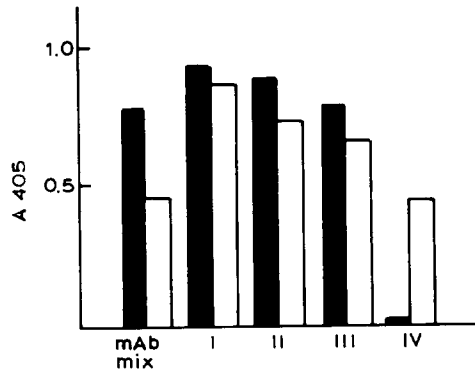
Fig. 4. Antigenic properties of HN glycoprotein expressed by the recombinant AcNPV. Reactivity of mAbs representing the four antigenic sites (I, II, III and IV) and the mAbs mixture is expressed by the OD values of ELISA. The OD values for the recombinant and the authentic HNs were expressed by black and open bars, respectively. Background OD values from cells infected with wild-type AcNPV were subtracted.

Miyadera strain used in the present study. It was reported that the mAb to site IV did not react to HN of Miyadera strain (Nishikawa et al., 1987). These results indicated that the HN glycoprotein expressed by the recombinant AcNPV is slightly different from that of NDV Miyadera strain used in this study, but similar to that of NDV Miyadera strain reported previously in their antigenicity. The difference in antigenicity of the two Miyadera strains might be attributed to the different passage history in two laboratories. However, the exact reason is not clear at this moment.

Antibody production in chickens inoculated with the recombinant HN

Antibody production in the recombinant HN-inoculated chickens was examined by HI and virus neutralization tests. As shown in Fig. 5, chickens inoculated with the cells expressing the recombinant HN produced HI and neutralizing antibodies. The HI and neutralization titers of the sera reached to 1024 and 256 respectively.

Protection of recombinant HN-immunized chickens from NDV challenge

Protective effect of the recombinant HN was examined in vivo. The chickens inoculated with the recombinant HN survived from the challenge by the virulent NDV without showing any clinical signs of Newcastle disease. On the other hand, the chicken inoculated with wild-type AcNPV-infected cells and the uninoculated control chicken showed typical clinical signs at 5 days after the challenge. The uninoculated control chicken died at 11 days after the challenge. The chicken inoculated with wild-type AcNPV-infected cells survived with clinical signs during the observation period. The HI titers of the chickens immunized with the recombinant HN on Day 0 and Day 22, at the time of challenge were 32 and 8, respectively,
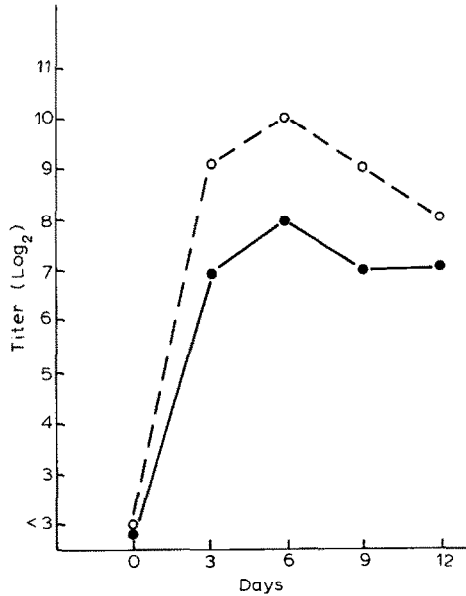
Fig. 5. Detection of virus neutralization and HI antibodies in the sera from chickens inoculated with the cells infected with the recombinant AcNPV. Chickens inoculated with the cells infected with the recombinant AcNPV were bled 3, 6, 9 and 12 days after the second inoculation (Day 0) and the sera were subjected to virus neutralization (●————●) and HI (○————○) tests. The antibody titers were expressed as reciprocals of dilutions on a logarithmic scale.

whereas the chicken inoculated with wild-type AcNPV-infected cells and the uninoculated control chicken showed undetectable level of HI titers (< 4) at that time.

Discussion

We constructed a recombinant AcNPV which expresses HN glycoprotein of NDV and examined functional and immunological properties of the expressed protein. The expressed protein had the same mobility on SDS-PAGE as the authentic HN glycoprotein, and TM treatment reduced the apparent mol. wt. of the expressed protein to the mol. wt. of unglycosylated HN protein which is calculated from the nucleotide sequence. These results indicated that the expressed HN glycoprotein in the insect cells infected with the recombinant AcNPV was glycosylated. These observations are similar to the previous ones by Nagy et al. (1990). In many cases, including HN glycoprotein of PIV3, foreign glycoproteins expressed by recombinant AcNPVs were glycosylated (Matsuura et al., 1987; 1989; Van Wyke Coelingh et al., 1987; Yoden et al., 1989; Kuroda et al., 1990).

The antigenic sites of HN glycoprotein of NDV related to virus neutralization were revealed to be localized to 3 or 4 (I, II, (III) and IV) sites by using mAbs and variant viruses (Nishikawa et al., 1986). Judging from the reactivities of the

representative mAbs, the HN glycoprotein expressed by the recombinant AcNPV conserves three (I, II and III) of four antigenic sites related to virus neutralization. Among the antigenic sites, the site I was shown to be conserved within a wide range of NDV strains and was considered to be immunodominant (Nishikawa et al., 1986). Moreover, mAb to site I was reported to have high HI and virus neutralizing activities (Nishikawa et al., 1986). The conservation of the site I on the recombinant HN glycoprotein may contribute to the production of virus neutralizing antibodies in birds inoculated with the recombinant HN glycoprotein, although Iorio and Bratt (1984) reported that all of the antibodies to four antigenic sites on HN glycoprotein are required for complete neutralization of NDV infectivity. Actually, chickens inoculated with the cells expressing the recombinant HN glycoprotein produced antibodies that inhibit the haemagglutination and neutralize the infectivity of NDV. Further, in the preliminary study of protection, the chickens were completely protected from the NDV challenge by the immunization with the recombinant HN. Spriggs et al. (1987, 1988) reported that a recombinant vaccinia virus expressing HN glycoprotein of PIV3 elicited more protective immune response than that expressing F protein of PIV3 in inoculated animals. Considering together, the HN glycoprotein expressed in insect cells is thought to be a good candidate for a component vaccine against NDV infection, although in a study with PIV3, immunization of both HN and F proteins is required to provide a significant level of protection (Ray et al., 1988).

The expressed HN glycoprotein of NDV in insect cells was transported to the cell surface and conserved its haemadsorption and neuraminidase activities, as in the case of Nagy et al. (1990). This indicates that the HN glycoprotein expressed by the recombinant AcNPV may be useful also in functional analysis of HN protein besides in immunological studies.

Acknowledgments

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