CHIMERIC NEWCASTLE DISEASE VIRUS NUCLEOCAPSID WITH PARTS OF VIRAL HEMAGGLUTININ-NEURAMINIDASE AND FUSION PROTEINS

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Summary. – The nucleocapsid (NP) protein of Newcastle disease virus (NDV) self-assembled in *Escherichia coli* as ring-like and herringbone-like particles. Several chimeric NP proteins were constructed in which the antigenic regions of the hemagglutinin-neuraminidase (HN) and fusion (F) proteins of NDV, *myc* epitope, and six histidines (a hexa-His tag) were linked to the C-terminus of the NP monomer. These chimeric proteins were expressed efficiently in soluble form in *E. coli* as detected by Western blot analysis. Electron microscopy of the purified products revealed that they self-assembled into ring-like particles. These chimeric particles exhibited antigenicity of the *myc* epitope, suggesting that the foreign sequences were exposed on the surface of the particles. Chickens inoculated with the chimeric particles mounted an immune response against NDV, suggesting the possibility of use of the ring-like particle as a carrier of immunogens in subunit vaccines and immunological reagents.

Key words: NDV; nucleocapsid; chimeric NP protein; protein assembly; molecular carrier

Introduction

NDV is the type species of the family *Paramyxoviridae* that poses a major world-wide problem for poultry industries. The viral nucleocapsid is enveloped by a lipid bilayer which is derived from the host cell plasma membrane. Embedded in and protruding out from the envelope are two types of surface glycoproteins, HN and F (Yusoff and Tan, 2001). These glycoproteins are highly immunogenic and their antigenic sites have been determined on the basis of sequence analysis of monoclonal antibody- (MAb) escape mutants (Yusoff *et al.*, 1988, 1999). Beneath the envelope is a layer of matrix (M) protein that interacts with the NP. The essential subunit of the NP is a single polypeptide of 489 amino acids (aa) of 53 K, which surrounds the viral genome, a single-stranded negative-sense RNA molecule (Kho *et al.*, 2001a). The nucleocapsid contains also phospho- (P) and large (L) proteins which are involved in the viral RNA synthesis (Hamaguchi *et al.*, 1983; Kho *et al.*, 2002).

The NP protein can be produced efficiently in *E. coli* where it self-assembles into ring-like and herringbone-like particles that closely resemble those isolated from intact virions (Kho *et al.*, 2001b). It is believed that at least three different conformations of NP monomers are held together by non-covalent forces to form the ring-like and herringbone-like particles (Kho *et al.*, 2001b). Insertion of a 29 aa peptide containing the *myc* epitope and a hexa-His tag into the C-terminus of the NP inhibited the formation of the herring-

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Abbreviations: BSA = bovine serum albumine; ELISA = enzyme-linked immunosorbent assay; F = fusion; HBcAg = hepatitis B core antigen; HN = hemagglutinin-neuraminidase; L protein = large protein; M = matrix; MAb = monoclonal antibody; NDV = Newcastle disease virus; NP = nucleocapsid; P protein = phosphoprotein; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate

bonelike particle but not that of the ring-like particle (Kho *et al.*, 2001b). In addition, this foreign peptide has been shown to be exposed on the surface of the ring-like particle (Kho *et al.*, 2001b).

In order to explore the potential of this particle, we constructed recombinant plasmids carrying the sequences encoding the antigenic regions of the HN and F proteins inserted into the 3'-terminus of the NP gene and the 5'-terminus of the coding regions of *myc* and hexa-His epitopes. These chimeric NP proteins were synthesized to high levels in *E. coli* as ring-like particles. Three of the six chimeras induced significant levels of antibodies against NDV in chickens.

Materials and Methods

Virus. NDV strain AF2240 was propagated in allantoic cavity of 8 to 9 day-old embryonated eggs as described earlier (Kho *et al.*, 2001b).

Construction of plasmids encoding chimeric NP proteins. Viral RNA was extracted from purified virions according to Kho *et al.* (2001b). Synthesis of cDNAs was carried out by reverse transcription according to Seal *et al.* (1995). The following forward primers specific for HN and F antigenic regions (1 μmol/l each) were used: for $HN_{272-307}$ 5'-GCC GAA TTC CAT GGA AGG TTA GGG-3', for $HN_{312-357}$ 5'-GCC GAA TTC TAC GGA GGG CTA AAA-3'; for $HN_{426-496}$ 5'-GCC GAA TTC GAA TAC CCT ATG ACA-3', for F_{57-100} 5'-CAG ACA GGG GAA TTC ATA GTC AAG TTG CTC-3', for $F_{144-183}$ 5'-GCC AAG CAG GAA TTC GCC AAC ATC CTC CGG-3', and for $F_{328-360}$ 5'-TCT GTG ATA GAA TTC CTT GAC ACC TCA TAC-3'). The cDNAs (6 μl) were used as templates in polymerase chain reaction (PCR) performed according to Seal *et al.* (1995). The same forward primers as above were used. The following reverse primers (0.5 μmol/l each) specific for HN and F antigenic regions were used: for $HN_{272-307}$ 5'-AAG CTT TCG TTG ATA ACA GAC CCA-3', for $HN_{312-357}$ 5'-AAG CTT GTA CGA AGA TTT AGC CAT-3', for $HN_{426-496}$ 5'-AAG CTT TTC ATC AAG CAT TG -3', for F_{57-100} 5'-AAG CTT ATC ACC AAG GGG GGT-3', for $F_{144-183}$ 5'-AAG CTT CTT CCC AAC TGC CAC TGC-3', and for $F_{328-360}$ 5'-AAG CTT ATA AAT ACC TGG AGA CAT GGG-3'). Reactions were carried out in a thermal cycler (PTC 200, MJ Research, USA) under the following conditions: 95°C for 2 mins, 25 cycles of 95°C/30 secs, 60°C/1 min, and 72°C/30 secs, and 72°C for 3 mins. The PCR poducts were precipitated and washed with 70% (v/v) ethanol, dried and dissolved in water (10 μl). As the PCR products contained an *Eco*R1 site at their 5'-termini, they were digested with *Eco*R1 (10 U) at 37°C for 4 hrs in a mixture (100 μl) consisting of 90 mmol/l Tris-HCl pH 7.5, 10 mmol/l $MgCl_2$, and 50 mmol/l NaCl, 2 μg of bovine serum albumine (BSA). After digestion, the mixtures were phenol extracted and ethanol precipitated. Prior to the ligation with the digested PCR products the vector pTrcHis2-NP_{cfus} (Kho *et al.*, 2001b) was first digested with *Eco*RI (5 U) and *Sna*BI (5 U) in the 1 X MULTI-CORE buffer (Promega) at 37°C for 4 hrs. The digested vector was extracted with phenol, chloroform and precipitated with ethanol. Ligation of the PCR products (100 ng) to the vector (50 ng) was carried out in the 1X ligase buffer (Promega) at 4°C overnight. The ligation products were introduced into *E. coli* Top 10 made competent by a heat shock at 42°C for 2 mins. Positive transformants were selected as ampicillin-resistant and tested by PCR. The inserts were subjected to nucleotide sequencing.

Purification of chimeric ring-like particles. E. coli cells which harbored recombinant plasmids encoding the chimeric NP proteins were grown at 37°C in LB broth. The cultures were induced with 1 mmol/l IPTG when reaching A_{600} of 0.6–0.8. After 5 hrs, the cells were harvested and lysed at 25°C for 30 mins (Kho *et al.*, 2001b). The cell extracts obtained as supernatants after centrifugation at 20,000 × g for 20 mins at 4°C were precipitated by ammonium sulfate (60% saturation). The precipitated proteins were recovered by centrifugation as above and dialyzed extensively against 50 mmol/l Tris-HCl pH 8.0 with 100 mmol/l NaCl. The dialysates were centrifuged on a 10–50% sucrose density gradient at 110,000 × g for 5 hrs at 4°C. After taking fractions those containing the chimeric NP proteins were saved, pooled and dialyzed.

Electron microscopy. After negative staining the purified proteins were observed under a Hitachi H-7100 electron microscope.

Immunization. Groups of five SPF chickens at 3 weeks of age were immunized with each of the purified proteins (50 μg) mixed with the complete Freund's adjuvant. A booster of the same immunization dose was given to the chickens 3 weeks later. NP_{cfus} containing only *myc* and the hexa-His tag (Kho *et al.*, 2001b) was used as a control. A total of 15 SPF chickens that were not inoculated with the proteins served as negative control. Chickens were bled at intervals of 10 days Blood samples were left at room temperature for 2 hrs, blood clots were removed and the remaining cells were spun down. The serum from each chicken was collected and stored at -20°C.

Enzyme-linked immunosorbent assay (ELISA) was carried out according to Makkay *et al.* (1999). Microtiter plate wells were coated with chimeric NP ring-like particles (10 ng per well) at 4°C overnight. The wells were then blocked and washed in a standard manner. A chicken anti-NDV serum (100 μl per well, 1:500 dilution in PBST) or an anti-*myc* MAb (100 μl per well, 1:500 dilution in PBST, Invitrogen) was added and the plates were incubated at 25°C for 1 hr. An anti-chicken (1:5000 dilution in PBST) or an anti-mouse (1:5000 dilution in PBST) IgG conjugated with alkaline phosphatase was added and the plates were incubated at 25°C for 1 hr. Phenyl-nitrophosphate (PNP, 1 mg/ml, 100 μl) was added and A_{405} was determined. The Newcastle Disease Antibody Test Kit (Kirkegaard and Perry Labs, USA) was used to measure the level of anti-NDV antibodies according to the manufacturer's instructions.

Western blot analysis was carried out according to Laemlli (1970). Partially purified recombinant HN and F proteins produced in *E. coli* were separated by polyacrylamide gel (12 %) electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and then electroblotted onto nitrocellulose membranes. After blocking the blots were probed with the prepared antisera and stained. The anti-NDV serum was used as a positive control. Individual steps of the Western blot analysis inclusive of staining were carried out in a standard way.

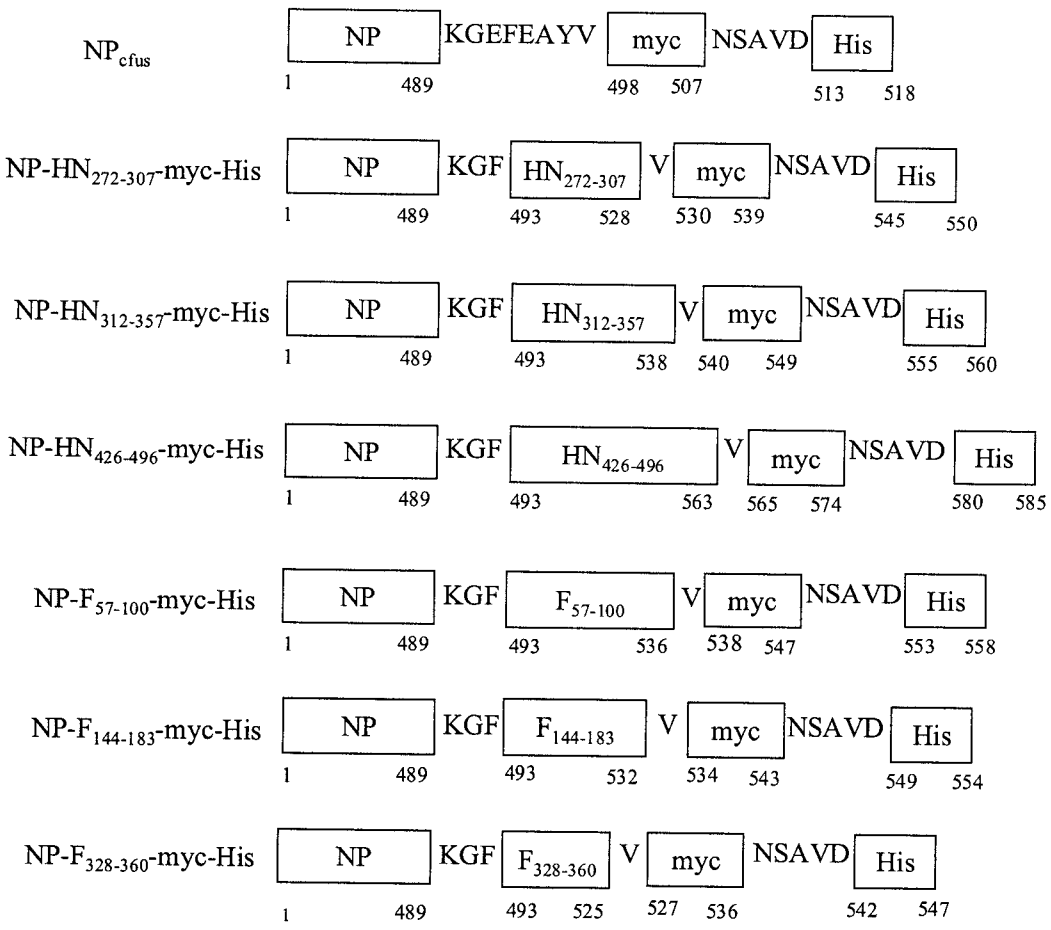

Fig. 1
Primary structures of chimeric NP proteins

Names of chimeric NP proteins are on the left. Subscript numbers indicate aa positions. The aa sequences (one-letter-symbols) are as follows: the *myc* epitope: ENKLISEEDL; $HN_{272-307}$: HGRLGFDGQHEKDLDTTVLFKDWVANYPGVGGGSV; $HN_{312-357}$: VWFPVYGGLKPNSPSDTAQEGRYVIY-KRYNNTCPDEQDYQIRMAKS; $HN_{426-496}$: YPMTVNNKTATLHSPYTFNAFTRPGSVPCQASARCPNSCITGVYTDPYPLVFHRNHTLRGVFG-TMLDDEQA; F_{57-100}: TGSIIVKLLPNMPKDKEACAKPPVEAYNRTLTTLLTPLGDSIR; $F_{144-183}$: AKQNAANILRLKESIAATNEAVHEVTDGLSQ-LAVAVGKMQ; $F_{328-360}$: SVIEELDTSYCIESDLDLYCTRIVTFPMSPGIY. His = the hexaHis tag.

Results

Construction of chimeric NP proteins

The plasmids expressing chimeric NP proteins were all derived from the plasmid pTrcHis2-NP$_{cfus}$ that encodes the NP protein fused with the *myc* epitope (10 aa) and the hexa-His tag (NP$_{cfus}$). The coding sequences (Tan *et al.*, 1995; Salih *et al.*, 2000) of the antigenic regions of HN and F glycoproteins (Yusoff *et al.*, 1988, 1989) were inserted between the C-terminus of the NP protein and the N-terminus of the *myc* epitope (Fig. 1). The nucleotide sequences of these chimeras were determined (data not shown).

Expression, purification and characterisation of chimeric NP proteins

The chimeric NP proteins were expressed in *E. coli* and subjected to Western blot analysis. The latter showed that all of the chimeric NP proteins could be detected by the anti-*myc* MAb as well as the chicken anti-NDV serum (Fig. 2). This suggests that the HN and F gene fragments were fused to the 3'-terminus of the NP gene correctly and formed an ORF producing recombinant proteins of larger size than the original NP$_{cfus}$. All of these chimeras were produced in high yields, were easily precipitated by ammonium sulfate and could be readily purified by sucrose density gradient centrifugation as reported by Kho *et al.* (2001b).

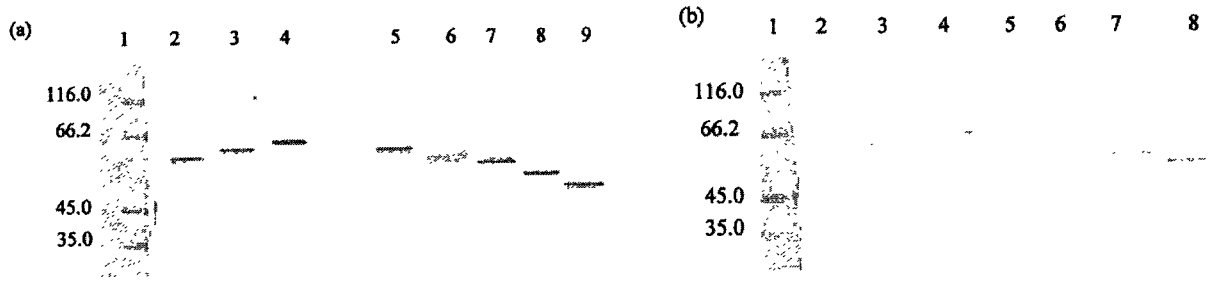

Fig. 2
Western blot analysis of chimeric NP proteins expressed in *E. coli*

(a): the chicken anti-NDV serum used as probe. Molecular size markers in K (lane 1); NP-$HN_{272-307}$-myc-His (lane 2); NP-$HN_{312-357}$-myc-His (lane 3); NP-$HN_{426-496}$-myc-His (lane 4); NP-F_{57-100}-myc-His (lane 5); NP-$F_{144-183}$-myc-His (lane 6); NP-$F_{328-360}$-myc-His (lane 7); NP_{cfus} (lane 8); NP (lane 9).
(b): the anti-*myc* MAb used as probe. Molecular size markers in K (lane 1); NP-$HN_{272-307}$-myc-His (lane 2); NP-$HN_{312-357}$-myc-His (lane 3); NP-$HN_{426-496}$-myc-His (lane 4); NP-F_{57-100}-myc-His (lane 5); NP-$F_{144-183}$-myc-His (lane 6); NP-$F_{328-360}$-myc-His (lane 7); NP_{cfus} (lane 8).

Electron microscopy of chimeric NP proteins

The full-length NP protein produced in *E. coli* self-assembled into herringbone-like particles, but appeared as ring-like particles by the addition of a foreign peptide containing 29 aa at its C-terminus (Kho *et al.*, 2001b). Therefore it was of great interest to investigate the structure of the purified chimeric NP proteins expressed in *E. coli*. Electron microscopy of these proteins revealed that they all assembled into ring-like particles similar to those formed by NP_{cfus} while no herringbone-like particles were observed (Fig. 3). The diameter of the ring-like particles ranged from 8 ± 2 nm to 16 ± 2 nm.

Antigenic and immunogenic properties of chimeric ring-like particles

In order to examine whether the chimeric NP proteins were antigenic, they were used as capturing antigens for the anti-*myc* MAb and chicken anti-NDV serum in ELISA. Fig. 4 shows that they all were detected by these antibodies thus indicating that the *myc* epitope as well as a part of the NP protein are exposed on the chimeric ring-like particles.

In addition, three out of the six chimeric NP proteins, namely NP-$HN_{272-307}$-myc-His, NP-$HN_{426-496}$-myc-His and NP-$F_{144-183}$-myc-His induced antibodies which could be detected by the intact NDV immobilized on the ELISA plate wells (Fig. 5). These antibodies were also capable of detecting recombinant HN or F proteins immobilized on the blots (Fig. 6). These results suggested that some of the HN or F antigenic sites present in the chimeric NP proteins were able to induce antibodies. The chimeric proteins NP-$HN_{312-357}$-myc-His, NP-F_{57-100}-myc-His, and NP-$F_{328-360}$-myc-His did not display any significant NDV reactivity.

Discussion

In the work described here the antigenic regions of HN and F proteins of NDV, *myc* epitope and hexa-His tag were fused to the C-terminus of the NP protein, expressed in *E. coli* in high yields and purified. They were found to self-assemble in *E. coli* into ring-like particles. This finding is in

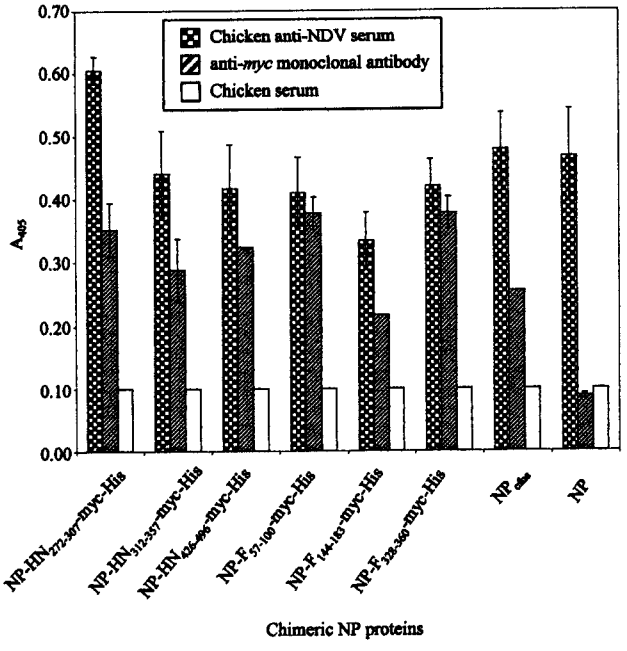

Fig. 4
Antigenicity of chimeric ring-like particles as tested by ELISA

Ordinate: A_{405} values. Abscissa: chimeric NP protcins. Histograms indicate means ± standard deviations from triplicate determinations.

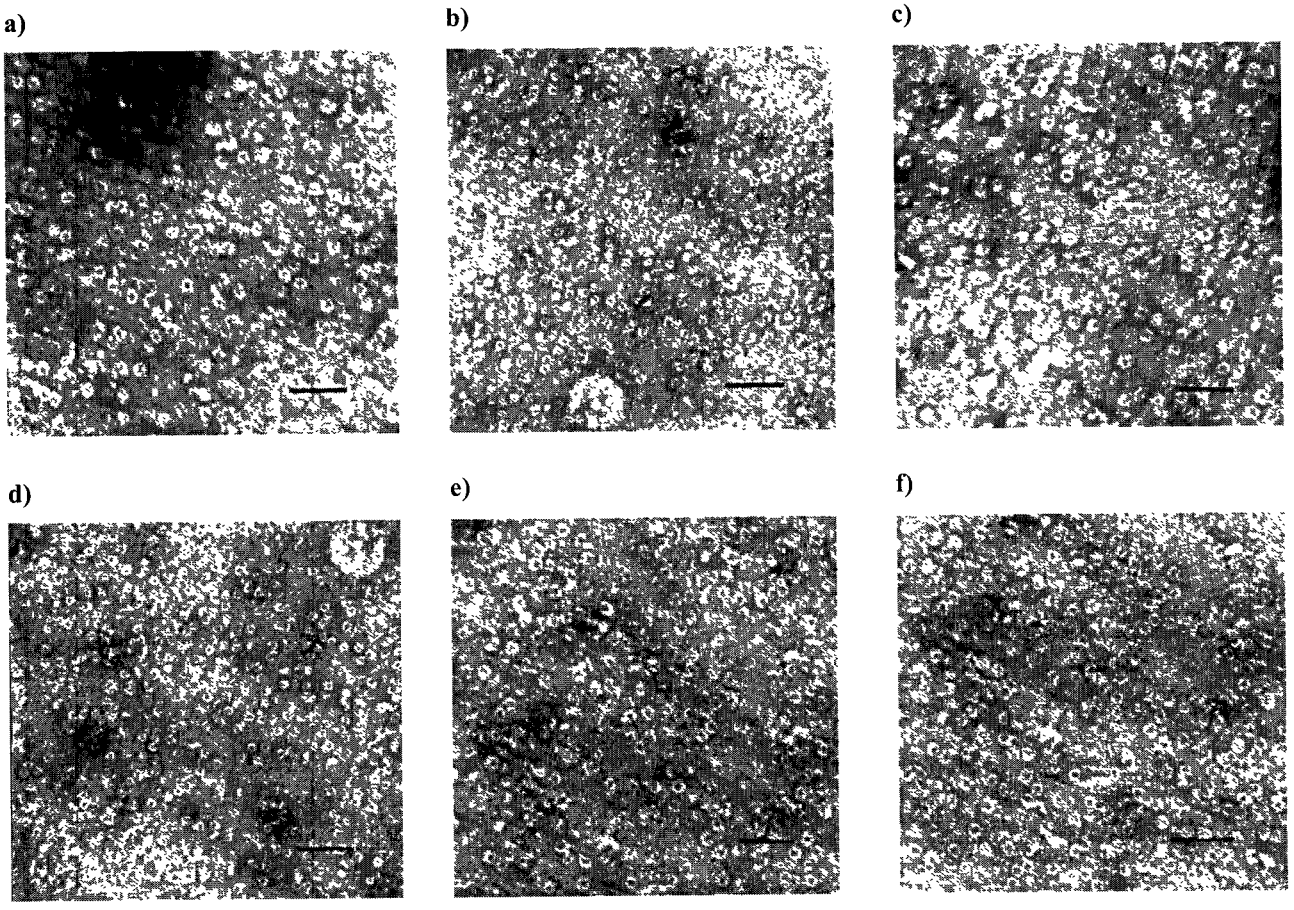

Fig. 3
Electron micrographs of chimeric NP proteins

NP-$HN_{272-307}$-myc-His (a); NP-$HN_{312-357}$-myc-His (b); NP-$HN_{426-496}$-myc-His (c); NP-F_{57-100}-myc-His (d); NP-$F_{144-183}$-myc-His (e); NP-$F_{328-360}$-myc-His (f). Negative staining. The bars represent 80 nm.

good agreement with that of the NP fusion protein (NP_{cfus}) harboring the *myc* epitope and hexa-His tag at its C-terminus (Kho *et al.*, 2001b). Furthermore, Kho *et al.* (2001b) have shown that the C-termini of the NP_{cfus} subunits forming ring-like particles are located around the edge of the central hole and are exposed on the surface of the ring. The heterologous peptides are thought to protrude out from the central hole and prevent the adjacent rings from stacking onto each other to form a long nucleocapsid-like particle. This feature allows attachment of additional immunogenic peptides to the major NP protein in developing multi-component vaccines and reagents for immunological studies.

All the antigenic components described here were attached to the C-terminus of the NP protein by insertion of relevant coding sequences at *Eco*RI and *Sna*B1 sites of the plasmid expressing the NP_{cfus} protein. The antigenic components were connected by a few amino acids resulting from translation of linker sequences introduced during the plasmid construction. The largest chimeric NP, NP-$HN_{426-496}$-myc-His had 96 amino acids attached at the C-terminus of NP and was still capable of self-assembling to ring-like particles. However, the size limit of the polypeptide that can be accommodated in ring-like particles is still unknown. In addition, the diameter of the ring-like particles varied. Whereas the largest diameter was 16 ± 2 nm (NP-$HN_{272-307}$-myc-His, NP-$HN_{426-496}$-myc-His, NP-$F_{144-183}$-myc-His and NP-$F_{328-360}$-myc-His), the smallest ones were 8 ± 2 nm (NP-$HN_{312-357}$-myc-His and NP-F_{57-100}-myc-His). Interestingly, the largest diameter of 16 ± 2 nm of chimeric ring-like particles is far smaller than that of the similar NP_{cfus} particles (24 ± 2 nm, Kho *et al.*, 2001b). The size of ring-like particles might depend on the number of NP monomers constituting the particles. Further work is being carried out to clarify this phenomenon.

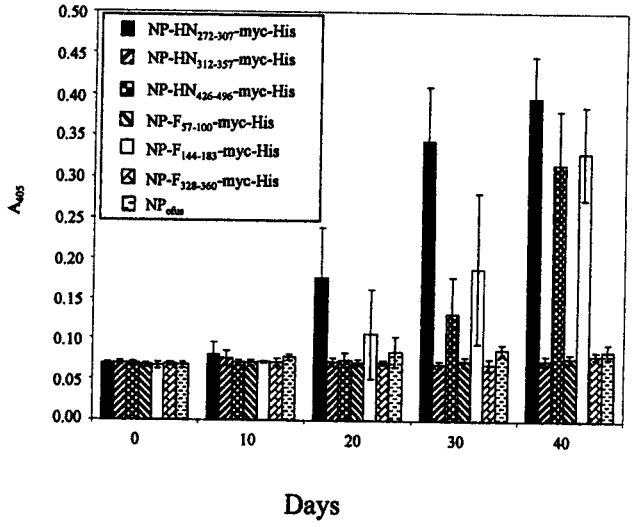

Fig. 5
Antibody response to chimeric NP proteins in chickens as tested by ELISA

Ordinate: A_{405} values. Histograms indicate means ± standard deviations from triplicate determinations.

The antigenic studies described in this article suggested that the foreign epitopes were displayed on the surface of the chimeric ring-like particles and were accessible to the anti-*myc* antibody directed specifically towards its linear epitope. The six antigenic regions of HN and F proteins that were fused to the NP protein are believed to be conformational (Yusoff *et al.*, 1988, 1989). Interestingly, chimeric ring-like particles carrying the linear polypeptides $HN_{272-307}$, $HN_{426-496}$ or $F_{144-183}$ did elicit production of antibodies that could be detected only after the booster. These antibodies were specific either to HN and F proteins since the anti NP-$F_{144-183}$-myc-His and the anti NP-$HN_{272-307}$-myc-His were not able to detect the recombinant HN and F protein, respectively. These observations indicated that the NP protein could serve as a carrier for immunogens. However, further studies have to be done to support this assumption. Apart from the NP protein of NDV, the envelope or coat proteins of other viruses possess a significant potential as powerful delivery systems for development of multicomponent vaccines. Heterologous sequences such as those of the immunodominant region of hepatitis B surface antigen (HBsAg) (Stahl and Murray, 1989; Shiau and Murray, 1997), the epitopes of the gp41 region of Human immunodeficiency virus 1 (Stahl and Murray, 1989), the capsid protein (VP1) of Foot-and-mouth disease virus (Clarke *et al.*, 1987), and the core protein (HCc) of hepatitis C virus (Yoshikawa *et al.*, 1993), have been fused to either N- or C-terminus of the hepatitis virus B core antigen (HBcAg) and in most cases the hybrids formed particles morphologically equivalent to HBcAg isolated from the liver of an infected individual (Cohen and Richmond, 1982).

The present study showed that the chimeric NP ring-like particles with parts of the viral HN and F proteins could serve as diagnostic reagents for the detection of antibodies to NDV in sera from the hosts infected with NDV or immunized with live viruses. Furthermore, the chimeric

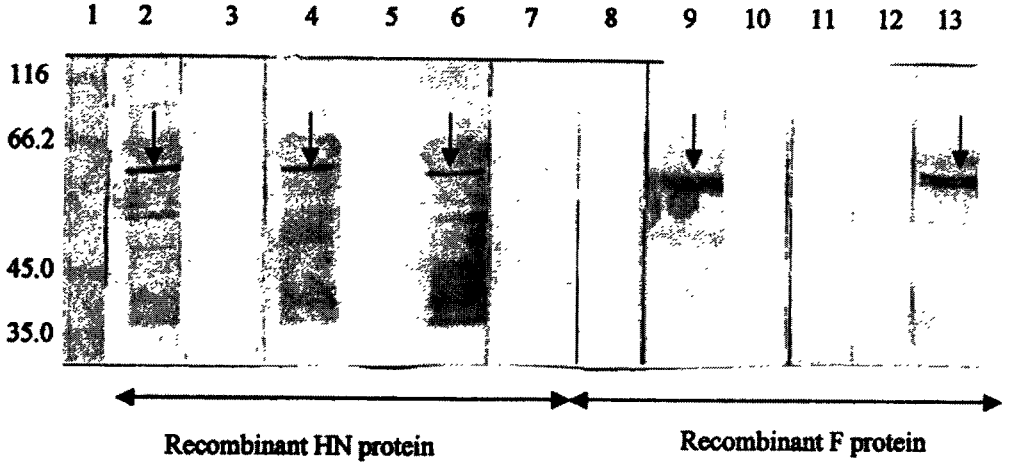

Fig. 6
Immunoblot analysis of chimeric HN and F proteins

Chimeric HN (lanes 2–7) and F proteins (lanes 8–13) probed with the following antisera: anti-NP-$HN_{272-307}$-myc-His (lane 2); anti-NP-$HN_{312-357}$-myc-His (lane 3); anti-NP-$HN_{426-496}$-myc-His (lane 4); anti-day 0 serum (lane 5); polyclonal anti-NDV serum (lane 6); anti-NP-$F_{144-183}$-myc-His (lane 7); anti-NP-F_{57-100}-myc-His (lane 8); anti-NP-$F_{144-183}$-myc-His (lane 9); anti-NP-$F_{328-360}$-myc-His (lane 10); anti-day 0 serum (lane 11); anti-NP-$HN_{272-307}$-myc-His (lane 12); polyclonal anti-NDV serum (lane 13). Protein size markers in K (lane 1). Arrows indicate the chimeric proteins.

particles can be used to raise anti-NDV antibodies. This application, in principle, can be exploited in designing novel antigens for other newly emerging diseases by simple attaching the relevant antigenic components to NP proteins. In addition, fusion of heterologous peptides to NP proteins could also provide a direct means to map linear antigenic or immunogenic epitopes on a particular protein.

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