Production and characterization of monoclonal antibodies to Newcastle Disease Virus

G Ravi Kumar1*, Shikha Saxena1, AP Sahoo, Uttara Chaturvedi, Satish Kumar, Lakshman Santra, GS Desai, Lakshyaveer Singh & Ashok K Tiwari
Molecular Biology Laboratory, Department of Veterinary Biotechnology, Indian Veterinary Research Institute, Izatnagar, 243 122, Uttar Pradesh, India

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Newcastle Disease (ND) is one of the major causes of economic loss in the poultry industry. Newcastle Disease Virus (NDV) is a single-stranded, negative-sense enveloped RNA virus (Fam. Paramyxoviridae; Order Mononegavirales). In the present study three monoclonal antibodies (MAbs) were produced by polyethylene glycol (PEG)-mediated fusion of lymphocytes sensitized to NDV Bareilly strain and myeloma cells. NDV possesses the ability to agglutinate erythrocytes of avian species. All the three MAbs designated as 2H7, 3E9 and 3G6 caused hemagglutination inhibition of NDV by specifically binding to NDV. The reactivity for all the 3 MAbs on indirect ELISA was found to be significantly higher than the antibody and antigen controls. On flow cytometry of HeLa cells infected with NDV using the MAbs as primary antibodies, there was a significant difference in the percentage of cells showing positive fluorescence compared to the mock control. One of the MAbs (3E9) was found to react with hemagglutinin-neuraminidase (HN) protein on western blot.

Keywords: HN gene, IFAT, NDV, Poultry, RNA virus

Newcastle Disease (ND), caused by Newcastle Disease Virus (NDV), has been one of the major causes of economic losses in the poultry industry1. It is a single-stranded, negative-sense enveloped RNA virus of the Paramyxoviridae family in the order Mononegavirales2 which includes avian paramyxovirus type 1 (PMV-1). The viral genome is 15186 nucleotides long3 and contains 6 genes encoding 6 major polypeptides: nucleocapsid protein, phosphoprotein, matrix protein, fusion (F) protein, hemagglutinin-neuraminidase (HN), and large RNA-dependent polymerase protein4. Co-transcriptional editing of P gene in NDV results in two more proteins — V and W5,6. Pathotyping in chickens is used to classify NDV strains into highly velogenic, intermediate or lentogenic strains. F protein, which is synthesized as non-functional precursor F0 and proteolytically cleaved to yield polypeptides F1 and F2 by host proteases7, is an important determinant of NDV pathogenicity8-10. Different pathotypes11 are characterized by differences in the amino acid sequences surrounding the F0 cleavage site, which hosts the molecular marker for virulence. Previous studies comparing the precursor F0 amino acid sequences of NDV varying in virulence for chickens showed that viruses that were virulent for chickens had the amino acid sequence 112 R/K-R-Q-K/R-R 116 at the C terminus of the F2 protein and phenylalanine at residue 117, the N terminus of the F1 protein12. Whereas, viruses of low virulence had the sequence 112 G/E-K/R-Q-G/E-R 116 at the C terminus of the F2 protein and leucine at residue 11712. The amino acid sequence in a virulent virus renders the F protein susceptible to cleavage by an omnipotent protease, resulting in a fatal systemic infection.

There are several methods for pathotyping and characterization of NDV viz., intra cerebral pathogenicity index (ICPI), intra venous pathogenesis index (IVPI) and mean death time (MDT) in SPF embryonated eggs13. These methods are cumbersome, time consuming, inhumane and sometimes indecisive. Different molecular techniques have been employed for detection14 and differentiation of NDV strains15. Application of one-step RT-PCR to various NDV samples, including wild-type virulent isolates and a virulent vaccine strains, demonstrated the potential for rapid identification of NDV isolates as well as the differentiation of virulent from non-virulent strains16-17. Recently, in order to rapidly detect and differentiate

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*Correspondence:
Tel/Fax: +91 581 2301584
E-mail: gandham71@gmail.com

1 contributed equally.
NDV isolates, a method based on real-time PCR SYBR Green I melting-curve analysis of the fusion (F) protein gene was also developed and used for this purpose\(^\text{18}\).

While methods are available for detecting NDV in animals, there remains a need for monoclonal antibodies, the hybridomas that produce them, and methods for differentiating high virulent NDV from the low virulent NDV used to produce vaccines. The present study is taken up to develop monoclonal antibodies against virulent NDV.

Materials and Methods

Cell lines

Myeloma (Sp2/0) and HeLa cell lines procured from the National Centre for Cell Sciences (NCCS), Pune, India, being maintained in Iscove’s Modified Dulbecco’s Medium (HiMedia) with 20% Fetal Bovine Serum (FBS) for hybridoma, 100 U Penicillin-Streptomycin under 5% CO\(_2\) at 37°C and in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS and antibiotics (Gentamycin 20 μg/mL), respectively were used in the present study.

Virus for immunization

NDV Bareilly strain grown in chicken embryo fibroblast (CEF) was used in this study. The primary cell cultures set up with 9-10 day old embryos were infected with virus stock available in the laboratory. The bulk virus grown was subjected to PEG precipitation, followed by ultracentrifugation. The purified virus was characterized to be NDV by Hemagglutination inhibition (HI) assay and by RT-PCR of HN and F genes. The purified virus was injected to immunize the Balb/c mice. The same NDV Bareilly strain adapted to grow in HeLa cells\(^\text{19}\) was used for screening of hybridomas supernatants by ELISA, Dot blot, Fluorescence activated cell sorting (FACS) and Hemagglutination inhibition (HI) assay. The HeLa cells adapted virus was also characterized to be NDV\(^\text{20}\).

Experimental animals

Balb/c mice (procured from CDRI, Lucknow) aged 4-6 wk were used for immunization with NDV virus antigen for the production of hybridomas. Six Balb/c mice were immunized using purified NDV antigen (100 μg). Three intraperitoneal inoculations were made: first (day 0) with Freund’s complete adjuvant, second (day 15), and third (day 21) with Freund’s incomplete adjuvant. Subsequently, three immunizations were carried out intravenously without adjuvant on three consecutive days (days 28, 29 and 30) prior to fusion (day 31). However, before fusion, the mice were test bled on the 25\(^{th}\) day of the immunization schedule to detect immune response against the inoculated antigen by both HI assay and ELISA.

Production of monoclonal antibodies

Monoclonal antibodies were produced by the standard protocols\(^\text{20-22}\) with some modifications as follows:

Preparation of Feeder cells

Feeder cells were prepared (Mouse peritoneal macrophages) 3 days before the fusion. Briefly, peritoneal macrophages were collected after washing the peritoneal cavity of the two Balb/c mice in sterile phosphate buffer saline (PBS). The collection tube is kept on ice to avoid adherence of the macrophages on the walls of the collection tube. The peritoneal washings were centrifuged at 1000×g for 10 min. The pellet containing mouse peritoneal macrophages was treated with chilled RBC lysis buffer (Qiagen) in order to remove red blood cells. The cells were resuspended in 100 mL of IMDM with 2x hypoxanthine aminopterin thymidine (HAT) having antibiotics and 20% FBS and seeded in 96-well cell culture plate 100 μL/well.

Preparation of spleen lymphocytes for fusion

The immunized Balb/c mice with the highest immune response were anaesthetized and sacrificed using volatile ether. The peritoneal cavity of the mice was opened and the spleen collected aseptically from the National Centre for Cell Sciences (NCCS), Pune, India, being maintained in Iscove’s Modified Dulbecco’s Medium (HiMedia) with 20% Fetal Bovine Serum (FBS) for hybridoma, 100 U Penicillin-Streptomycin under 5% CO\(_2\) at 37°C and in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS and antibiotics (Gentamycin 20 μg/mL), respectively were used in the present study.

Briefly, 1 mL of 50% PEG solution was added slowly to the mixture of cells under constant agitation through a period of 1 min which was further diluted with some modifications as follows:

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Characterization of MAb monoclonality of the hybridoma culture supernatants. Cloning was repeated at least twice so as to prove the appropriate stage of the growth. Single cell cloning was performed by ELISA, Dot blot, FACS, HI assay and western blot method for NDV+ve hybridomas using the limiting dilution followed by single-cell cloning and subcloning of antibodies. The positive clones were amplified, tested by ELISA and HI for the presence of antibodies. The clones: 2H7, 3E9, 3G6 by indirect ELISA, HI, Flow Cytometry and western blot.

Characterization of MAbs

The specificity of the MAbs was determined by testing the hybridoma cell culture supernatants of the clones: 2H7, 3E9, 3G6 by indirect ELISA, HI, Dot-blot ELISA, Flow cytometry and western blot.

Indirect ELISA

Antibody titres of individual MAbs were determined using an indirect ELISA. Briefly, the ELISA plates (Nunc, Maxisorp, Raskilde, Denmark) were coated with NDV antigen 50 ng/well. The plates were incubated at 4°C for overnight. Unbound antigen was washed three times using PBST (1X PBS with 0.2% Tween-20). About 100 µL of blocking buffer (1X PBS with 3% bovine serum albumin (BSA)) was added to each well for 2 h at 37°C. Further, 50 µL of hybridoma culture supernatants were added and incubated for 2 h at 37°C and washed as before. Anti-mouse horse radish peroxidise (HRPO) conjugate (1:1000 dilution) in blocking buffer was added to each well. Plates were further incubated for 1 h at 37°C, washed and 0-phenyl diamine (OPD) containing H₂O₂ was added to each well. The development of colour in well was observed for 10 min, followed by stopping of the reaction with an equal volume of 1M H₂SO₄. The absorbance of the colour in each well was recorded at 492 nm in an ELISA Reader.

HI assay

HI of both purified NDV and HeLa-adapted NDV were performed using 1% chicken RBC. The virus was confirmed to be NDV by HI assay using NDV antiserum. For this, serial two fold dilutions of serum were made in 25 µL and 4 haemagglutination (HA) units of NDV in 25 µL was added to each well. The plate was incubated at room temperature (25°C) for 30 min and then 25 µL of 1% (v/v) chicken RBCs were added to each well, mixed by gentle shaking, and incubated for 40 min at 25°C. For characterizing the clones, the cell culture supernatant was added in the place of serum into the well.

Dot-blot ELISA

About 10 µL dots of antigen (NDV) were put on the Nitrocellulose membrane (NCP). A dot of 1X PBS acted as negative control. The membranes were kept in blocking buffer (3% BSA) overnight at 4°C, washed thrice with washing buffer (1X PBS with 0.2% Tween-20), treated with monoclonal supernatants (1:1) in 3% blocking buffer and kept at 37°C for 2 h. The membranes were washed thrice and treated with anti-mouse HRPO conjugate (1:1000 dilution) and kept at 37°C for 1 h. Finally, the membranes were washed and colour was developed with di aminobenzamide (DAB) solution (Bangalore genei).

Flow Cytometry

HeLa cells showing 60-70% confluency in a 6-well culture plate (Nunc) were infected with NDV parental stock (diluted 1:20 in DMEM). Cells along with NDV were incubated at 37°C for 1 h with intermittent shaking. Thereafter, DMEM containing 2% FBS was added and incubated again at 37°C under 5% CO₂. After 48 h, when cytopathic effect (CPE) was observed, cells were pelleted, washed with PBS (pH 7.5) twice, and fixed in 4% paraformaldehyde (PFA) for 20 min. at room temperature (25°C). Cell pellets were washed again twice with PBS, permeabilized by treating with 0.2% Triton X-100 in PBS for 5 min, washed twice with PBS for 5 min and subsequently blocked with 2% BSA dissolved (in PBS) at 37°C. After 2 h of incubation the cell pellets were washed twice with PBS for 5 min and incubated with monoclonal supernatant for 2 h at 37°C. Finally, the cells were washed as previously, treated with anti-mouse HRPO conjugate for 1 h at 37°C, washed, resuspended in 1X PBS and analyzed by flow cytometer using FL1 filter. Uninfected HeLa cells was used as mock control.
Western Blot

HeLa cells showing 60-70% confluency in 25 cm² flask (Nunc) were infected with NDV as described earlier. After 48 h post infection (p.i.) the cells were scraped out, pelleted at 4000 rpm for 10 min. and protein was extracted with the help of cell lysis buffer (Invitrogen). These proteins were run on 10% SDS-PAGE gel for 2 h and transferred on nitrocellulose membrane, treated independently with specific MAb (culture supernatant, 1:1) for 1 h at 37°C, washed thrice with PBS with 0.05% Tween 20 and treated with anti-mouse HRPO conjugate (1:2000 dilution) for 1 h at 37°C. Finally, the membranes were washed and colour was developed with DAB solution.

Results and Discussion

The virus (NDV) was characterized before immunizing the mice by HA, HI and amplification of specific HN and F gene fragments on RT-PCR (Fig. 1a and b) followed by sequencing. The HA titre of the purified virus from CEF was found to be $2^{11}$ and that of the HeLa adapted virus supernatant was found to be $2^8$. The purified virus was used to immunize the mice. The HeLa adapted supernatant was used to screen the hybridoma supernatants by indirect ELISA. Hemagglutination inhibition with polyclonal NDV-specific antisera is used for differential diagnosis of NDV. The Hemagglutination inhibition of the virus using NDV specific hyperimmune serum raised in chickens confirmed that the virus in the present study is NDV.

Sequence analysis of RT-PCR amplified 1767 bp fragment of HN gene coding for 571 amino acids (aa) (Fig. 1c) suggested that the NDV virus is velogenic. This was supported by mean death time (data under...
An HN protein precursor of 616 aa has been found in avirulent but not in virulent NDV strains, whereas an HN protein of 571 aa can be detected in highly virulent strains only. An HN protein of 577 aa is present in virulent and avirulent strains. The sequence at the fusion protein cleavage site is a major determinant of NDV pathogenicity. The analysis of 675 bp sequence covering the F gene cleavage site also confirmed that the virus is velogenic. The F protein, synthesized as the non-functional precursor F0, must be cleaved into disulfide-linked F1 and F2 polypeptides by host proteases to become fusogenic and is an important determinant of the pathogenicity of NDV. The F gene cleavage site was found to have the sequence 112 R-R-Q-R-R 116 at the c-terminus of F2 protein and phenyl alanine at residue 117 (Fig. 1d).

Production of monoclonal antibodies

A significant increase in indirect ELISA signal after immunization of Balb/c mice on day 45 of immunization schedule indicated production of antibodies in the immunized mice. The mouse with the highest ELISA titer was sacrificed to produce hybridomas. The hybridoma clones were considered reactive clones, on screening the supernatants by HI and ELISA at appropriate stage. In all, out of 18 hybridoma clones initially identified on the basis of the reactivity, only 10 could be successfully amplified. Primary clones were subjected to single-cell cloning and sub-cloning. The limiting-dilution method of hybridoma clones was adopted for this purpose. Monoclonality of a clone was accepted only when all the wells of a microtitre plate with growing cells gave a positive reaction in indirect ELISA after repeated subcloning. Finally, 3 clones were characterized for the presence of monoclonal antibodies in the culture supernatants. The 3 clones 2H7, 3E9, 3G6 were characterized by indirect ELISA, Dot blot, FACS, HI and Western Blot.

Characterization of monoclonal antibodies

Indirect ELISA

Indirect ELISA is considered highly sensitive for detecting NDV antibodies. In the present study on indirect ELISA the absorbance at 492 nm was found to be significantly higher in culture supernatants of all the three clones than the antigen and antibody controls. This indicated that the culture supernatants have antibodies specific to NDV virulent Bareilly strain (Fig. 2a).

Fig 2.—Characterization of monoclonal antibodies. (a) Histogram depicting the results of indirect ELISA. The hybridoma culture supernatants of significantly high absorbance (A 492) values (twice) in comparison to AgC (antigen control), AbC (Antibody control) and CB (coating buffer); (b-d) Flow cytometry analysis of culture supernatants. A shift to right indicated that all the Mabs - 2H7, 3E9 and 3G6, are specific to NDV; (e) Western Blot with 3E9 culture supernatant; and (f) monoclonal antibodies of the clone 3E9 reacted with HN protein - 75 kD protein.
**FACS (Flow cytometry)**

Flow cytometry is faster and more accurate than any other method currently used for the direct detection and quantitation of virus particles. On FACS using the culture supernatant as the primary antibody, a significant shift to right observed in comparison to mock control suggested the presence of antibodies in the culture supernatants of all the three clones (Fig. 2 b-d).

**Dot blot ELISA**

An enzyme-linked immunosorbent assay using nitrocellulose blotting membrane (dot blot ELISA) was developed for the detection of antibodies against Newcastle Disease Virus (NDV) in chickens. On Dot blot the reactivity of the culture supernatants with the virus and no reactivity in the negative control confirmed the presence of antibodies in the culture supernatants of all the three clones (Fig. 2e).

**Hemagglutination inhibition**

The culture supernatants of all the 3 clones inhibited hemagglutination by the virus proving the specificity of the culture supernatants to the NDV virus. HI titre of the clones was found, 2H7- (2^4), 3E9- (2^4) and 3G6- (2^5) of NDV.

**Western Blot**

Out of the three clones, one clone 3E9 was found to react with HN protein of NDV (Fig. 2f).

The reactivity of the three hybridomas on ELISA, FACS and flow cytometry to NDV virus proved the specificity of the monoclonal antibodies to NDV. One of the clones (3E9) produced antibodies specific to HN protein. The non-reactivity of other monoclonals in western blot to any of the NDV proteins suggested that the monoclonal produced may be against conformational epitopes and not against linear epitopes. The hybridomas and monoclonal antibodies produced in this study are specific to velogenic Bareilly strain of NDV.

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**References**


