Short communication

Preparation of Monoclonal Antibody to Fusion Glycoprotein of Newcastle Disease Virus Isolated from Iran

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Summary

Ten Hybridomas secreting monoclonal antibodies directed against the F-glycoprotein of MK13 (Iranian isolate) strain of Newcastle disease virus (NDV) have isolated which 6 of them showed positive reaction in different tests. All of these antibodies are IgM class with κ chain which showed sharp and single band on F-Protein (56000 Dalton) in western blot assay. All these antibodies reacted with same epitope on competitive ELISA. On cross reactivity analysis with different avian viruses all were specific to NDV only and did not show any reaction with other virus. The importance and application of these antibodies would be on specific identification of NDV, highly purification of F-protein of NDV specially on preparation of recombinant F-protein on subunit vaccine, developing anti-idiotype vaccine and also blocking the cleavage site of F-protein which resulting in decreasing pathogenicity of virus.

Key words: monoclonal antibody, Newcastle disease virus, F-protein

Introduction

Newcastle disease virus (NDV) is the causative agent of a major disease of poultry. In spite of apparently adequate protection measures vaccination of birds, the disease

still recurs periodically. A wide range of strains has been isolated which differ markedly in pathogenicity. In comparative studies on these strains it was found that the glycoproteins of pathogenic strains were readily activated by proteolysis (Nagai *et al* 1976). NDV classified as avian paramyxovirus–1, contains a single strand, negative sense RNA genome coding for six viral proteins (Millar *et al* 1988). There are two surface glycoproteins in the envelope of the virus. The haemagglutinin neuraminidase (HN) glycoprotein mediates the attachment of the virus to cell surface receptors and the fusion (F) glycoprotein is responsible for cell–to–cell fusion, haemolysis and virus penetration (Merz *et al* 1981). Due to important role of F-protein in penetration of virus in to target cells, other investigators in which preparation of monoclonal antibody was also among them had done so many studies. These antibodies were applied for antigenic mapping, pathogenically antigenic variants of NDV, mutations located on both F1 and F2 subunits of the virus, determination of glycosylation and reactivity, location of neutralizing epitopes on the F protein, characterization of NDV and analysis of F protein expresse in insecte cells (Abenes *et al* 1986, Neyt *et al* 1989, Yusoff *et al* 1989, Murakami *et al* 1994, Nishikawa *et al* 1987). In other study (Masashi *et al* 1998) the protective efficacy of the recombinant Mareks disease virus type 1 which constructed and expressed the F-protein of NDV (NDV-F) was examined. It was proved that NDV-F is required for virus cell fusion and is responsible for vaccine immunity (Umino *et al* 1990). In the present study monoclonal antibody to F-protein of MK13 strain of NDV have been prepared and characterized.

Materials and Methods

Virus and purification. The MK13 strain of NDV was isolated from an outbreak in Khorasan province, Iran in April 1996. The isolate was completely neutralized by NDV positive serum in hemagglutination-inhibition (HI) test and placed in velogenic group based on its virulence. A 10^{-5}virus dilution was inoculated to 9-10 day-old specific pathogen free (SPF) (Lohman, Germany) embryonated eggs. The allantoic fluid was harvested and clarified by centrifugation at 8000rpm (Sorval OTD–T 865)

for 30min. Virus was then concentrated at 18000rpm for 2h (Sorval OTD, SW 50L). The pelleted virus was suspended in TNE buffer (0.01M Tris HCl, [pH7.4], 0.1M NaCl, 0.001M EDTA). One ml of suspended virus was layered onto 30-60% sucrose density gradient and centrifuged at 18000rpm for 4h (Beckman, 41Ti). The virus band was taken out and diluted with equal volume of TNE buffer and pelleted at 18000rpm for 2h (Beckman, 41Ti), followed by suspension in 2ml of TNE buffer. Purification of the virus was done according to Kianizadeh *et al* (1999) description.

Monoclonal antibodies. Five 8–week-old female BALB/c mice were immunized by subcutaneously injection of 200µg of purified virus with complete Freund's adjuvant (Weir 1978). Three additional booster injections with 100µg each of the same antigen were given subcutaneously one week interval with incomplete Freund's adjuvant and one additional injection was given intraperitoneally with saline three to four days before fusion. Spleen cells from immunized mice which it's sera showed high titre antibody (1/1600) fused with SP_2/O, myeloma cells (Razi institute collection) at a ratio of 5:1 and PEG (1300–1700 Sigma) was used for fusion. Fused cells were cultivated in RPMI-1640 medium containing HAT (Watanabe *et al* 1983). Culture fluid from colonies was tested by ELISA (see below) for the presence of virus specific antibody, cells from positive wells were cloned by limiting dilution in 96-well microtitre plate.

Enzyme-linked immunosorbent assay (ELISA). The Kimura–Kuroda and Yasui (1983) procedure was modified and used. Briefly purified virus was diluted in coating buffer (0.05M $NaHCO_3/Na_2CO_3$, pH9.6) to a concentration of 8µg protein/ml and dispensed at 100µl/well and allowed to adsorb to the plastic over night at 4°C. Then the wells were blocked for non specific binding by incubation with 1% BSA in PBS-0.05% Tween20 for 1h at 37°C. The hybrid supernatant was added (100µl/well) and allowed to react for 1.5h at 37°C. The plates washed three times with washing buffer (PBS–0.05% Tween20) and 100µl of Horseradish peroxidase labeled goat anti-mouse whole Ig (Sigma) was added then incubated for 1.5h at 37°C. The plates were washed four times and developed using 0.5mg/ml *O*-phenylenediamine (OPD)(Sigma) and 0.02% H_2O_2 in 0.05M citrate phosphate buffer (pH5.0). The reaction continued for

10min in dark and was stopped with 50µl of H_2SO_4, 2N. The optical density (OD) at 490nm was recorded with an ELISA reader (Merck, Dynatech Lab).

Concentration and isotype determination of monoclonal antibodies. The culture supernatant of high titer positive hybrid was collected and concentrated by $(NH_4)_2SO_4$ precipitation (50%) and dialysed against PBS. Their immunoglobulin subclass were determined by isotyping kit (Isostrip Roche, Germany).

Protein estimation of antibodies. Protein determination were done by the method of Lowry *et al* (1951) with bovine serum albumin (BSA) as standard.

Immunoblotting analysis of antibodies. The purified virus was run on sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) through 10% slab gel. After electrophoresis, protein was transferred to nitrocellulose sheets according to the procedure of Towbin *et al* (1979). The blots were incubated with monoclonal antibodies and bound antibodies were visualized by using Horseradish peroxidase cojugated goat anti- mouse whole Ig (Sigma). The reaction was developed using 3mg/ml chloronaphtol in methanol with 5 volume PBS containing 0.01% H_2O_2.

Determination of antibodies specificity. The specificity of each monoclonal antibody was determined by ELISA among different avian viruses caused infectious bronchities, avian influenza, egg drop syndrome, infectious bursal disease and Newcastle diesease which were used as antigens.

Epitope mapping by competitive binding ELISA. Peroxidase (10,000 unit, Merck) was conjugated to immunogloublin concentrated by ammonium sulphate precipitation by method of Wilson and Nakane (1978). The diluation of peroxidase–conjugated antibody in direct ELISA was determined. Procedures for competition were similar to those described previously (Umino *et al* 1985) except for adding separately antibody and conjugate within 30min with a solid phase purified NDV antigen in the present study.

Results and Discussion

ELISA test for antibody screening. Two of five immunised mice were died during bleeding. The sera titres of rest of them were checked inwhich the highest titre of one

of them was 1/1600 and same mouse was selected for fusion. The other mouse showed the titre upto 1/400 and due to low antibody titre couldnot be used. The supernatant screening of clones were done by same ELISA method and two single clones 3D5 (1.571OD) and 4E11 (1.367OD) due to their higher OD were selected among other clones. For monoclonality these two clones were taken for limiting dilution also which the screening of new selected clones are shown in table 1.

Table 1. *ELISA test for limiting dilution of highly positive clones*

3D5 Clones	OD*	4E11 Clones	OD
3D5L1G5	1.425	4E11L1C5	1.408
3D5L1D10	1.233	4E11L1D9	1.381
3D5L1G7	1.262	4E11L1E5	1.41
3D5L1E4	1.007	4E11L1B4	1.273
3D5L1F6	0.941	4E11L1D6	1.314
3D5L1C9	1.003	4E11L1C11	1.087
3D5L1F8	1.078	4E11L1D8	1.502
MEDIA	0.082	4E11L1G8	1.406

*Optical Density

Isotyping of antibodies. Due to limitation of isotyping kit only three clones 4E11L1C5, 4E11L1D9 and 4E11L1C11 were used and all three clones showed a single band on IgM class with κ chain.

SDS-PAGE and western blot analysis. Immunoblot analysis of five clones (4E11L1C5, 4E11L1D9, 4E11L1C11, 4E11L1D8 and 4E11L1B4) was examined by SDS-PAGE. Protein bands of the purified virus transferred to nitrocellulose paper, which showed a single and sharp band on F-protein zonewith 56,000 D molecular weight. This sharp and single band by itself proves the monoclonality of above clones that clearly can be seen on figures 1 and 2.

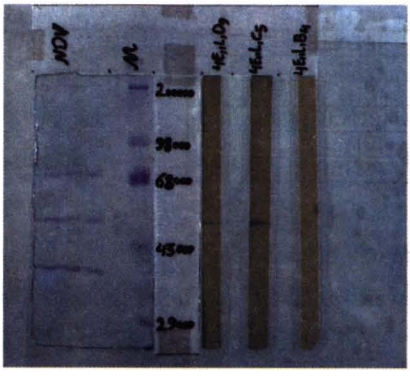
Antibodies cross reactivity by ELISA. The cross reactivity of the antibodies with different avian viruses were checked by ELISA in which high OD of antibodies reaction with NDV proved their specificity toward NDV only (Table 2).

Epitope analysis. The resultes of competitive ELISA on all antibodies were checked with pure NDV as antigen showed that there is only one epitope on all antibodies.

Table 2. *ELISA test for cross–reactivity with different virus*

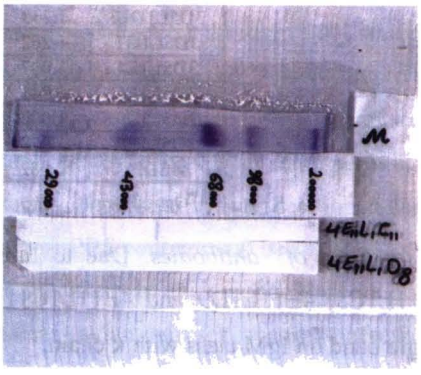
Clones	NDV	IBV	AIV	EDS	IBDV
3D5L1G5	1.545	0.127	0.128	0.145	0.179
3D5L1G7	1.515	0.146	0.134	0.129	0.195
3D5L1D10	1.387	0.162	0.146	0.135	0.188
4E11L1C5	1.441	0.138	0.145	0.129	0.197
4E11L1G8	1.528	0.146	0.144	0.141	0.187
4E11L1D9	1.485	0.147	0.135	0.128	0.195
BLANK	0.103	0.143	0.13	0.142	0.146
MEDIA	0.123	0.146	0.136	0.145	0.148

NDV:Newcastle disease virus
IBV:Infectious bronchitis virus (M41 strain)
AIV:Avian influenza virus (A/chicken/Iran/259/1998/H9N2)
EDS:Egg drop syndrome (EDS–76 strain)
IBDV:Infectious bursal disease virus (D78 strain)



MW: Molecular weight marker
Positive Clones:4E11L1D9, 4E11L1C5, 4E11L1B4

Figure 1. *Western blot analysis of positive clones*



MW:Molecular weight marker
Positive clones:4E11L1C11, 4E11L1D8

Figure 2. *Western blot analysis of positive clones*

Monoclonal antibodies against NDV have made previously (Toyoda *et al* 1988) aiming at comparsion, antigen mapping and functional analysis. In the present study, we have prepared and characterized monoclonal antibodies specific to the F-protein of MK13 strain of NDV. The importance of preparing antibodies against F-protein is due to this fact that virus–induced membrane fusion is thaught to be mediated by the N-terminus of the F-protein because of the findings that this region is extremely hydrophobic (Blumberg *et al* 1985) and that cell fusion, viral infectivity and hemolysis could be inhibited by oligopeptides whose amino acid sequences resemble that of the N-terminus of the F-protein (Richardson *et al* 1983). Recently ,other

hydrophobic sites between the transmembrane domain and the N-terminus of the F-protein have been identified and it was suggested that this sites too may be available for interaction with membranes during fusion. In several studies evidences suggesting the existence of cellular receptors for the F-protein have been obtained (Markwell *et al* 1985). Previously, it had also been suggested that there may be different receptors for the F-protein because different oligopeptides could inhibit fusion of the same cell type by different paramyxoviruses (Richardson *et al* 1980). Based on these observations preparation and characterization of our antibodies to F-protein of MK13 as an Iranian isolate would be very important for future studies. The specificity of these antibodies to F-protein can be seen in figures 1and 2 which a single and sharp band clearly proved. The same results can be seen in other investigator works (Murakami *et al* 1994, Hulslander *et al* 1997). Preparing antibodies against F-protein of MK13 will be useful for analysing this isolate and compare it with standard one.

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