PRODUCTION OF A PANEL OF MONOCLONAL ANTIBODIES AGAINST THE NUCLEOCAPSID PROTEIN OF HEAT RESISTANT NEWCASTLE DISEASE VIRUS

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ABSTRACT : A panel of six monoclonal antibodies against the nucleocapsid protein (NP) of Newcastle disease virus (NDV) strain AF2240 was produced by immunization of Balb/c mice with purified NP. Isotyping test revealed three of the monoclonal (mAbs) were IgG1 and the others were IgG2a. All of the mAbs recognized linearized NP epitopes. The reactivity of these mAbs with local isolates revealed that three mAbs recognized conserved antigenic sites and the others bind to antigenic sites which undergone considerable changes.

Keywords*: Monoclonal antibody; nucleocapsid; Newcastle disease virus.*

1. INTRODUCTION

Newcastle disease virus (NDV) is the only member of the new genus *Avulavirus* of the family *Paramyxoviridae* (Mayo, 2002). Initially, NDV was grouped in the *Paramyxovirus* genus. Later in 1993, it was placed within the genus *Rubulavirus* [1] together with simian virus 5, mumps virus, human parainfluenza virus type 2, type4a and 4b. However, the lack of hydrophobic (SH) protein gene which is present in all other members of *Rubulaviruses* [2] causes it to be classified in the new genus.

NDV infects many avian species and results in great economic lose in poultry industry. It can be divided into 3 pathotype groups based on the severity of the disease that the virus caused. Highly virulent NDV that causes viscerotropic and neurotropic symptoms are grouped as velogenic strains. Mesogenic strains caused milder respiratory diseases which are not usually fatal. The third group which is termed the lentogenic strain, causes inapparent respiratory disease [3].

NDV contains a single-stranded non-segmented negative-sense genomic RNA of approximately 15.2 kb [4]. The genome encodes six major proteins namely the nucleocapsid protein (NP), the phosphoprotein (P), the large protein (L), the fusion glycoprotein (F), the heamaglutinin-neuraminidase glycoprotein (HN) and the matrix protein (M) [5]. The genome is encapsidated by the NP protein forming helical nucleocapsid and it protects the genome from nuclease activities. NP protein is the major

component of NDV. Each virion contains approximately 2600 NP [6]. Under electron microscope, it resembles a herringbone morphology which is in common with other members of *Paramyxoviridae* familiy. It plays a critical role in the replication of the virus in the host cells. Together with the viral genome, the complex acts as a template for genome synthesis and transcription during viral infection.

NP was highly immunogenic in nature that it induces significant antibody responses in rabbits, mice and chickens however it fails to confer immunoprotection. Challenge of NP immunized chicken with virulent NDV strains result in mortalitys of the chicken within 5 days [7]. In contrast, rabies virus NP alone can induce protective immunity against lethal infection in mice and dogs [8, 9]. However, due to its immunogenic property, NP of NDV is used for diagnostic purposes in which NP-based immunoassays are used mainly to monitor vaccination programs and as a diagnostic test in differentiating between vaccinated and infected animals in conjunction with subunit vaccines [10]. MAbs against NP of NDV are not extensively studied except by a study carried out by Panshin and his collegues (2000) [23]. Most studies in many laboratories were focused on mAbs against HN and F proteins of NDV [11, 12, 13, 14, 15]as these glycoproteins have important roles in virulence and their antibodies conferred protective immunity to avian species [16, 17].

In this study, we described the production of a panel of mAbs against the NP and characterized the mAbs by isotyping, Western blotting and reactivity test towards different NDV strains.

2. MATERIALS AND METHODS

2.1 Viruses and cells

The NDV strains used in the cross-reactivity assay were lentogenic (4989/92 P3, 5270, 5731/88 P4, 8820/92 P3); mesogenic (3147/89 P2) and velogenic (6270/92 P3). They were obtained from Veterinary Research Institute (VRI), Ipoh, Malaysia (Table 1). The viruses were propagated in embryonated chicken, harvested and semi-purified as described previously (Yusoff *et al*., 1996). The SP2/0-Ag14 myeloma cells (ATCC, USA) were used as fusion partners in the production of hybridoma cells. The cells were maintained in DMEM (Gibco, USA) with 10% fetal bovine serum, FBS (Gibco, USA) and were incubated at 37^{o}C with 5% of CO_2.

2.2 Mouse immunization and mAbs production

Five to six weeks female Balb/C mice were immunized with 45 μg of purified NP full-length protein for production of mAbs. A panel of mAbs against NP was prepared by fusing spleen cells from the immunized Balb/C mice with Sp2/0-Ag14 mouse myeloma cell line as described by [18]. The screening of the hybridoma producing antibodies was carried out by indirect ELISA using the purified NP as the antigen according to Hornbeck (1991) [19]. The cloning of the selected positive clones was perfomed by limiting dilution that was repeated three times to ensure monoclonality.

2.3 Isotyping

MAb isotyping was carried out on hybridoma culture supernatants using the Mouse Monoclonal Antibody Isotyping Kit (Pierce, USA).

2.4 SDS-PAGE and Western blot

Purified NDV or NP proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). After the electrophoresis, the fractionated proteins were blotted onto a nitrocellulose membrane (Osmonics, USA) and Western blotting was performed according to Harlow and Lane (1988) [20] with some modifications. The blotted membranes were incubated in culture fluids of mAbs instead of diluted serum before incubation with secondary antibody conjugated to alkaline phosphatase. Nitro-blue tetrazolium chloride (NBT; Amresco, USA) and bromochloroindolyl phosphate (BCIP; Amresco, USA) were used as a substrate solution. Polyclonal antiserum against NDV or NP was used as a positive control.

2.5 Cross-reactivity assay

Reactivity of the mAbs with different NDV isolates was tested by indirect ELISA with six local isolates collected from various outbreaks in Malaysia (Table 1). The indirect ELISA was conducted as described by Hornbeck (1991) except that 500 ng of semi-purified virus which acts as antigen was adsorbed to the microtitre plates. Positive reactivity was considered when the mAbs supernatant produce an OD_{405} two fold higher than the control culture (SP2/0-Ag14).

Table 1: Reactivities of mAbs with the local NDV isolates using indirect ELISA

			MAbs against purified NP of NDV strain AF2240					
			a2	a2s	b2	b4s	c1	b3
Viruses	Year[a]	Pathotype[b]						
3147/89 P2	1989	mesogenic	+[c]	+	+	-	+	-
4989/92 P3	1992	lentogenic	+	+	+	-	-	-
5270	Not available	lentogenic	+	+	+	+	+	-
5731/88 P4	1988	lentogenic	+	+	+	+	+	-
6270/92 P3	1992	velogenic	+	+	+	-	-	-
8820/92 P3	1992	lentogenic	+	+	+	+	+	-
AF2240	1974	velogenic	+	+	+	+	+	+

[a] Year of isolation of the local NDV isolates
[b] pathotype as determined by mean death time of embryonated eggs.
[c] +, Optical density at 405 nm (OD_{405}) of the mAbs were two fold higher than the OD_{405} of a SP2/0-Ag14 culture supernatant in indirect ELISA; -, OD_{405} of the mAbs were less than twice the OD_{405} of a SP2/0-Ag14 culture supernatant in indirect ELISA.

3. RESULTS AND DISCUSSION

3.1 Production of mAbs against NP

After the fusion, 91 wells from 468 seeded wells contained hybridomas producing NP-specific antibodies. In order to optimize the selection, these hybridomas were then pooled

into two portions and cloned by 3 cycles of limiting dilution. Based on their high OD_{405} in indirect ELISA, five clones namely mAbs a2, a2s, b2, b3, b4s were obtained from one original portion and one clone, c1was from the other portion. MAb isotyping demonstrated that three of the mAbs (a2, a2s and b2) were IgG1 whilst the remaining mAbs (b4s, b3 and c1) were IgG2a (Table 2). All of the mAbs possess a kappa light chain but not lambda light chain.

Table 2: Biological properties of mAbs against NP of NDV strain AF2240.

MAbs	Isotype	Light chain	Western Blot analysis	
			NDV[a]	NP[b]
a2	IgG1	kappa	strong	strong
a2s	IgG1	kappa	strong	strong
b2	IgG1	kappa	strong	strong
b4s	IgG2a	kappa	medium	medium
c1	IgG2a	kappa	medium	medium
b3	IgG2a	kappa	medium	medium

[a] Western blot was carried out using purified NDV proteins blotted on nitrocellulose membrane
[b] Western blot was carried out using purified NP blotted on nitrocellulose membrane

3.2 Specificity of mAbs

Western blotting was carried out to further determine the specificity of the mAbs. The results obtained revealed that all the mAbs bound to the purified NP proteins from *E. coli* (Figure 1a, lanes 3-8). These mAbs also bound specifically to NP (53 kDa) of the virus lysate and did not cross-react with other proteins of NDV (Figure 1b, lanes 3-8). However, one protein band with molecular mass smaller than the NP was also detected by the mAbs (Figure 2a, lanes 6-8; Figure 1b, lanes 4 and 5). The smaller fragment was full-length NP which had undergone proteolytic degradation. Similar observation on NP degradation was also observed by Hamaguchi *et al*. (1983) [21]. The biological properties of the mAbs were summarized in Table 2.

The ability of all the mAbs to bind to the heat-denatured NP in the Western blot indicates that these antibodies recognize sequential or linear epitopes of the NP protein. None of the mAbs produced in the present study recognized conformational epitopes unlike two separate studies performed by Russell *et al*. (1983) [22] and Panshin *et al*. (2000) [23]who managed to obtain mAbs which recognized conformational epitopes in the NP protein. Perhaps during selection by limiting dilution, only those hybridoma clones which produced mAbs that bound to linear epitopes were selected.

3.3 Reactivity of mAbs with different isolates

The results demonstrate that mAbs a2, a2s and b2 reacted with all NDV isolates tested (Table 1). Hence, these mAb should recognize at least one highly conserved antigenic site of NP which present in all NDV isolates tested. Based on this result, indirectly it revealed that some antigenic sites of the NP protein from the local isolates remained conserved in spite of their pathogenicity and years they were isolated. The binding site for mAbs a2, a2s and b appeared to be highly conserved in lentogenic, mesogenic and velogenic NDV

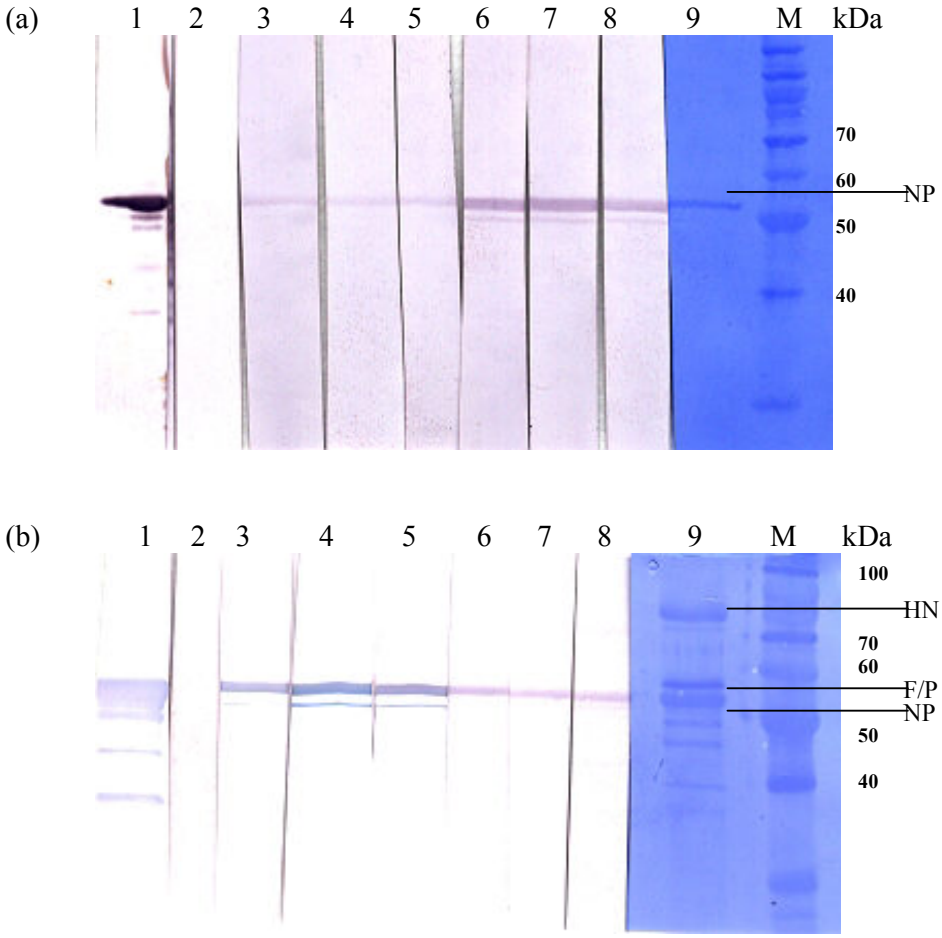

Figure 1: Specificity of MAbs against NP.
(a) Immunologic detection of NP after separation of purified NP by SDS-PAGE, electrophoretic transfer to nitrocellulose membrane (lane 9) and probed with mAbs b3, c1, b4s, b2, a2s and a2, (lane 3,4,5,6,7 and 8 respectively), polyclonal NP mouse serum (positive control, lane 1) and SP2/0-Ag14 culture supernatant (negative control, lane 2). The NP appeared as a sharp band of about 53 kDa when probed with various mAbs against NP and polyclonal against NP mouse serum. No band was detected when probed with SP2/0-Ag14 culture supernatant.
(b) Purified NDV (lane 9) was probed with mAbs a2s, b2, a2, b3, c1 and b4s (lane 3,4,5,6,7 and 8 respectively), polyclonal NP mouse serum (positive control, lane 1) and SP2/0-Ag14 culture supernatant (negative control, lane 2). NP, nucleocapsid protein; F/P, fusion/ phosphoprotein; HN, hemagglutinin-neuraminidase, M, protein molecular weight.

strains and they were probably similar to that site recognized by mAbs 38 generated by Russell *et al*. (1983) [22] and mAbs 401 and 402 produced by Nishikawa *et al*. (1987) [24] since these antibodies cross-reacted extensively with all NDV strains tested. On the other hand, although mAbs 479 (Russell *et al*., 1983) [22], 18d2 and 12f8 (Panshin *et al*., 2000) [23] exhibited full reactivity with all NDV strains tested, the conserved antigenic sites bound by these antibodies differs from the present study because they recognized conformational epitopes only.

On the other hand, mAb b3 did not react with any of the isolates except for the strain AF2240 which was used to immunize the mice for the fusion step. MAbs b3 obtained from this study appeared to be specific for the isolate (AF2240) used to prepare the antibody. Although this antibody has the potential to be used as a diagnostic reagent in detecting NDV strain AF2240, further tests with a larger panel of NDV isolates will be needed in order to validate the ultimate specificity. Previous study has shown that mAbs 3 and 43 (mAbs against NP of NDV) that reacted specifically against NDV strain 617/83 from 18 other NDV isolates in one study reacted with other isolates when further tested with a larger panel of NDV isolates of 818 in another study [25, 26].

The other two mAbs (b4s and c1) bound to NDV isolates with varying reactivities. For example, mAb b4s reacted with three of the six isolates (isolates 5270, 5731/88/P4 and 8820/92/P3) and mAb c1 bound an additional, mesogenic isolate, 3147/89/P2. These antibodies seem to be not specific to viral virulence as they react to both lentogenic as well as velogenic isolates. In addition, mAb c1 also reacted with the mesogen. Probably, the regions recognized by both of the antibodies could have undergone some structural changes or the epitopes bound by them are not conserved amino acid sequences and it varies in different isolates.

4. CONCLUSIONS

In the present study, a panel of six monoclonal antibodies against the NP of a heat-resistant virulent NDV strain AF2240 was produced by immunization of Balb/c mice with purified NP protein. All of the mAbs showed specificity against NP protein only and did not cross-react with other proteins of NDV. The ability of all the mAbs to bind to the heat-denatured NP in the Western blot indicates that these antibodies recognize sequential or linear epitopes of the NP protein. The reactivity test revealed that some of the antigenic sites of the NP protein from the local isolates remained conserved in spite of their pathogenicity and years they were isolated. While, some other sites were subjected to moderate and remarkable changes.

5. ACKNOWLEDGEMENT

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