

Production and Characterization of Monoclonal Antibodies against Formalin-Inactivated Nipah Virus Isolated from the Lungs of a Pig

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ABSTRACT. Eight clones of monoclonal antibodies (Mabs) to Nipah virus (NV) were produced against formalin-inactivated NV antigens. They reacted positive by indirect immunofluorescent antibody test, and one of them also demonstrated virus neutralizing activity. They were classified into six different types based on their biological properties. These Mabs will be useful for immunodiagnosis of NV infections in animals and further research studies involving the genomes and proteins of NV.

KEY WORDS: monoclonal antibody, Nipah virus.

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Nipah virus (NV) was isolated in March 1999 and subsequently identified as the etiological agent responsible for an outbreak of severe respiratory disease in pigs and fatal viral encephalitis in pig farm workers in Peninsular Malaysia [1–3].

The outbreak was controlled only after the new virus etiology was identified. Pigs were identified as the source of the virus infecting humans, and more than 1 million animals in the affected areas were culled (Centers for Disease Control and Prevention. 1999, MMWR Morb. Mortal. Wkly Rep. 48: 265–269). This outbreak was devastating because not only did it incur the loss of human life but it also resulted in great economic loss. The disease in swine was primarily a febrile respiratory illness with epistaxis, dyspnoea and coughing in weaner and young animals. Some pigs, particularly sows, exhibited generalized neurological signs such as ataxia, paresis, seizures and muscle fasciculation [1]. NV was isolated from both swine lung tissue and human brain tissue and is currently classified in the new genus *Henipavirus* within the family *Paramyxoviridae* provisionally [2,3,16]. NV shared antigenic cross-reactivity with only Hendra virus (HV), and its sequence was found to be more homologous with HV than with any other paramyxoviruses [3, 8].

The epidemiological features of the outbreak indicated that the infection in humans was due to close contact with live infected pigs and the spread of the infection was due to movement of the infected pigs (Centers for Disease Control and Prevention. 1999, MMWR Morb. Mortal. Wkly Rep. 48: 265–269 and 335–337). However, the origin of NV causing the outbreaks in Malaysia is suspected to be bats (order *Chiroptera*), particularly pteropid bats (flying foxes), a species of which are the probable natural host of HV virus in Australia [4–7,9,13]. How the virus was introduced into pigs is still unclear [6], and more study is needed to examine the participation of other animals as part of the source of the virus spill-over [6, 11]. Although the outbreak has been

under control, there is an urgent need to identify the natural reservoir of the virus to obtain a better understanding of the mode of its introduction into livestock and to prevent future reintroduction. Serological surveillance of wildlife animal species for evidence of NV infection is an integral part of the outbreak investigation [9, 13].

The usefulness of monoclonal antibodies (Mabs) has been well documented, in terms of their specificity, homogeneity and ability to be produced in unlimited quantities. Mabs have been an essential research tool and are in demand as immunodiagnostic reagents [12]. There is no report of Mabs against the NV. In this study, we developed hybridomas secreting Mabs specific to NV and characterized them for use in the improvement of diagnostic techniques and epidemiological research of NV infection in animals.

The virus used for the preparation of Mabs was the VRI-ASI strain, a Malaysian pig isolate of NV [1]. NV replication and titration were carried out in Biohazard Cabinet Class III in a Biosafety Level 3 Laboratory at the Veterinary Research Institute, Ipoh.

The NV antigens were made safe for handling by inactivation with formalin. NV infected cells in culture flasks were covered with plastic bags, and frozen and thawed three times. Formalin was directly added at a final concentration of 0.1%, and the flasks were left for 72 hr at 4°C. The virus infectivity of the materials was determined by inoculating them into Vero cells. NV antigen was then prepared as follows. The supernatant was collected by low-speed centrifugation (3,500 rpm for 30 min) and concentrated by ultracentrifugation (35,000 rpm for 120 min). The pellet was partially purified by discontinuous sucrose (30–60%) gradient centrifugation (27,000 rpm for 90 min). The fraction of the virus was collected and dialyzed against PBS. To confirm the antigenicity of the prepared NV antigen, rabbits were inoculated with the inactivated NV that had been emulsified in Freund's complete adjuvant according to the Canadian Council on Animal Care guidelines on immuno-

logical procedures, and the antiserum that had a NV neutralizing antibody titer of $\times 7,000$ by 50% plaque reduction test [18] was obtained.

BALB/c mice were immunized subcutaneously with 120 micrograms of formalin-inactivated purified NV antigen that had been emulsified in Freund's complete adjuvant. Two weeks later the mice were intraperitoneally boosted with the same antigen emulsified in Freund's incomplete adjuvant, and the antibody levels were monitored by indirect fluorescence antibody test (IFAT). Two to four weeks after the second injection, the mice were given a third injection of the inactivated virus intraperitoneally. After 3 days, mouse spleen cells were fused with P3-X63-Ag8-U1 myeloma cells using 50% polyethylene glycol (SIGMA HYBRI MAX P-7181). The hybridoma cells were selected with HAT medium, and the antibody-positive hybridomas were screened 2 weeks after the fusion.

Hybridomas secreting NV-specific Mabs were selected by the IFAT using acetone-fixed NV-infected Vero cells on multi-well slides. Positive hybridomas were cloned three times by the limiting dilution method and intraperitoneally inoculated into pristine-primed BALB/c mice to produce ascitic fluid containing Mabs. The hybridoma culture supernatant and ascitic fluid were utilized for subsequent experiments.

We tested several properties of the eight cloned positive hybridomas using the IFAT. To determine the class of immunoglobulin (Ig) of the Mabs, we used the mouse monoclonal antibody isotyping kit (Roche Diagnostic Co.). The reactivity of the Mabs was examined by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) [10] followed by Western blotting (WB) [14] using the formalin-inactivated purified NV antigen. The neutralizing activity against NV was tested by 50% plaque reduction test, and the ELISA was done by the ordinal method [12].

The results are shown in Table 1. Two different patterns of positive fluorescent reaction were observed in the IFAT. Five of the Mabs mainly reacted with granular antigens in the NV-infected cell cytoplasm (G type), while three of them showed positive fluorescence on the cell membrane part of the infected cells (M type) (Fig. 1). And one of the Mabs showed strong virus neutralizing activity. On the

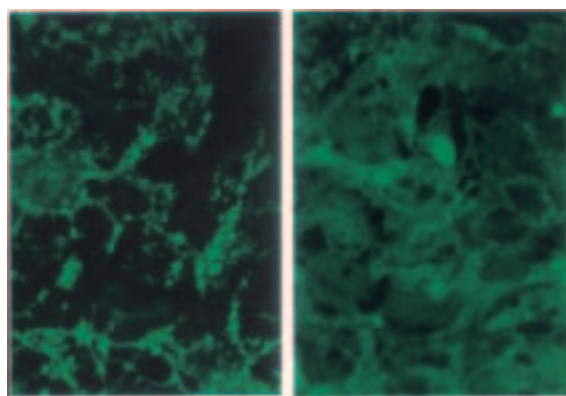


Fig. 1. NV antigens in Vero cells stained with monoclonal antibodies, 36 hr after infection. Those on the left were stained with 12D7 (G type), and those on the right were stained with 18C4 (M type). Magnification, $\times 360$.

other hand, WB immunostaining revealed four different patterns (Fig. 2). As a result, it appeared that there were at least 6 different types of Mab according to their biological properties (Table 1): (1). Mab 11F6 showed a G type positive reaction by the IFAT and recognized a 100-kDa protein by WB, although did not demonstrate neutralizing activity. (2). Mab 13A5 showed the same reactivity as 11F6 but recognized a 60-kDa protein. (3). Mab 11E11 showed the M type positive reaction and had strong neutralizing activity, but no NV proteins were immunostained by WB. (4). Mab 18C4 showed M type positive reaction but no neutralizing activity. It recognized a 60-kDa protein. (5). Mab 13C10 showed M type positive reaction, no neutralizing activity and negative WB immunostaining. (6). Mabs 12A5, 12D7 and 13C4 showed G type positive reaction, and they recognized 48- and 60-kDa proteins by WB without neutralizing activity.

WB immunostaining analysis revealed that three Mabs (12A5, 12D7 and 13C4) showed the same pattern and appeared likely to react with F protein according to the molecular weight and the data of the major structural proteins of NV [15,17]. Similarly, 11F6 appeared to recognize the P protein of NV, and 13A5 and 18C4 appeared to react

Table 1. Characterization of monoclonal antibody by indirect immunofluorescence test (IFAT), neutralization test (NT) and Western blotting (WB) with NV VRI-ASI strain

Clone name of Mab	Classification of Mabs	Isotype of Mab	ELISA OD of Supernatant	Antibody titer of IFAT			Antibody titer of NT**		kDa with WB***
				Supernatant	Ascitic	IFA pattern*	Supernatant	Ascitic	
11F 6	(1)	G1, κ	1.20	64	not	G	<2	not	100
13A 5	(2)	G1, κ	0.96	128	32,000	G	<2	<10	60
11E11	(3)	G1, κ	1.09	128	32,000	M	64	>10,000	ND
18C 4	(4)	G1, κ	1.27	32	not	M	<2	not	60
13C10	(5)	G2a, κ	0.37	32	8,000	M	<2	<10	ND
12A 5	(6)	G1, κ	0.92	256	not	G	<2	not	48, 60
12D 7	(6)	G2a, κ	1.02	64	16,000	G	<2	<10	48, 60
13C 4	(6)	G1, κ	0.87	256	32,000	G	<2	<10	48, 60

*: Immunofluorescence picture : G means granular antigens type, M means membrane antigens type.

: 50% plaque reduction; "not" means not tested. *: "ND" means not determined.

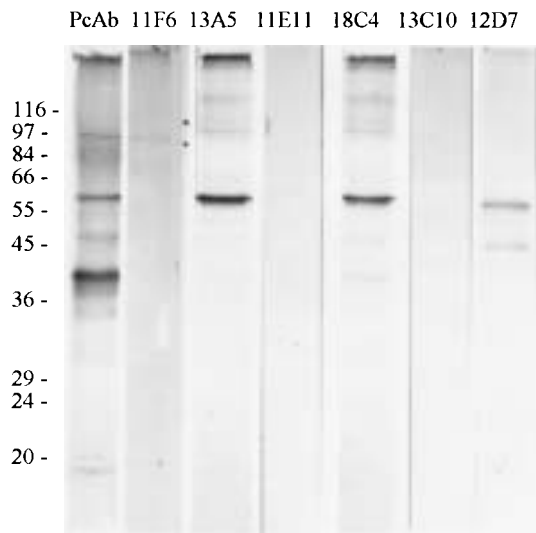


Fig. 2. Western blot analysis of NV antigens. Shown is the pattern of immunostaining with polyclonal and monoclonal antibodies. The molecular weights of standard marker proteins are shown on the left.

with the N protein of NV. There is a large variation in the G protein of paramyxoviruses, and G and F proteins of many paramyxoviruses, including NV, induced neutralizing antibodies [15]. Therefore, 11E11 may recognize an epitope on the G or F protein, however, it did not immunostain any protein of NV by WB. This suggested that 11E11 recognizes a conformational epitope on the protein that might be altered by the effect of SDS. As the 13C10 also did not react with the NV antigens by WB, its behavior could not be estimated.

These Mabs were also tested at the Australian Animal Health Laboratory, where researchers noticed that 11E11 was able to neutralize NV but not HV, and by IFAT that 11E11, 11F6 and 13A5 only reacted with NV-infected cells and that the 12A5, 12D7, 13C4 and 18C4 reacted with both NV- and HV-infected cells (Dr. John White, personal communications). The cross-reactivity of these Mabs should be further examined in the future.

Although we were not able to identify all of the characteristics the proteins of NV reacted with these Mabs, the set of these Mabs will be useful for immunodiagnosis of NV infections in animals and in further research studies involving the genomes and proteins of NV. Further study to characterize these Mabs by an ELISA or any other appropriate techniques will be conducted. The ultimate aim of this research is to obtain those Mabs that can recognize the epitopes for different functions of the proteins (e.g., Mabs that can differentiate NV especially from HV and other paramyxoviruses) and that can be used as epidemiological tools in determining the persistence of NV in the environment, such as in the development of a competitive ELISA that will enable the detection of antibodies of all species of wild animals.

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