Arch Virol (2008) 153: 273–281
DOI 10.1007/s00705-007-1079-x
Printed in The Netherlands

Archives of Virology

Original

Monoclonal antibodies against the nucleocapsid proteins of henipaviruses: production, epitope mapping and application in immunohistochemistry

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Received 5 July 2007; Accepted 14 October 2007; Published online 5 November 2007

Summary

Four monoclonal antibodies (mAbs) were generated by immunizing BALB/C mice with recombinant nucleocapsid protein (N) of Nipah virus (NiV) and Hendra virus (HeV) expressed in *E. coli*. Two mAbs each were obtained for the HeV N and NiV N, respectively. All four mAbs displayed specific reactivity with the recombinant N proteins of both viruses by western blot, which was further confirmed by immunofluorescent antibody assay using fixed insect cells infected with recombinant baculoviruses expressing either the HeV or NiV N protein. Epitope mapping using a 12-mer random peptide phage display library revealed two linear antigenic sites of the henipavirus N proteins, KLxR (aa 17–20) and FKREM (aa 446–450), which have not been reported previously. Two of the mAbs were able to specifically recognize HeV antigens by immunohistochemical staining of lung tissue sections of a horse experimentally infected with HeV. These reagents will be a useful addition to the collection of tools essential for further research and improvement in diagnosis of henipaviruses.

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Introduction

Two newly emerged paramyxoviruses, Hendra virus (HeV) and Nipah virus (NiV), were identified in Australia and Malaysia in 1994 and 1999, respectively [3, 15]. The broad species tropisms and the ability to cause fatal disease in both animals and human have distinguished HeV and NiV from all other known paramyxoviruses, which formed the basis for their classification as Biosafety Level 4 (BSL4) agents [6, 12]. Their novel virological properties also led to the classification of these two viruses in a separate genus *Henipavirus*, in the family *Paramyxoviridae* [7, 14, 22]. It has been identified that bats in the genus *Pteropus* are the natural reservoirs of HeV and NiV [4, 10].

HeV and NiV have a non-segmented negative-sense RNA genome of approximately 18.2 kb, which is substantially larger than the genomes of previously known paramyxoviruses [6, 23]. Henipaviruses contain two envelope glycoproteins: the G protein, which is responsible for binding the cellular receptor Ephrin B2 [1, 16]; and the F protein

which mediates membrane fusion [2]. Lying beneath the viral envelope is the matrix (M) protein, which interacts with both the glycoproteins and the nucleocapsid or ribonucleoprotein (RNP) complex. The latter is made up of many copies of nucleocapsid protein (N) that encapsidate the RNA genome. Associated with the RNP is the phosphoprotein (P) and the large protein or RNA polymerase (L). These three components form the functional machinery responsible for replication and transcription of the viral RNA [13].

The N protein is the most abundant structural protein of paramyxoviruses and the essential component of the helical nucleocapsid [13]. Although antibodies to the N protein of HeV and NiV showed no neutralization activity [19], the recombinant N protein can be a sensitive diagnostic marker for evidence of infection [8]. Limited studies indicated that the main antigenic region of NiV N is located at the C-terminal end and contained an epitope covering aa 503–509 [9, 20]. However, there is a lack of detailed antigenic studies of henipavirus N proteins due to the shortage of mAb variety available.

In this study, the N protein of HeV and NiV were expressed in *E. coli* and Sf9 insect cells, and four different mAbs were produced which recognize both NiV and HeV N proteins. Using a combination of phage display technology and overlapping recombinant fragments, the binding sites for all four mAbs were determined. The findings and reagents generated from this work will facilitate future work in development of better diagnosis and in better understanding of N protein antigenic structure.

Material and methods

Expression of recombinant N proteins in E. coli and baculovirus

The protein-coding regions of NiV and HeV N genes were obtained by PCR using the following primers: 5′-GC*GGATCC*ATGAGTGATATCTTTGAAGAGGCGG-3′ (NNF) and 5′-GC*GTCGAC*TCACACATCAGCTCTGACGA AATC-3′ (NNR) for NiV N; 5′-GG*GAATTC*ATGAGTGA TATATTTGACGAGGCGGC-3′ (HNF) and 5′-GG*GTCGAC* CACGTCTGCTCTAACAAAGTCC-3′(HNR) for HeV N. The PCR fragments were cloned into pET28-a(+) (Novagen, U.S.A.) for expression. After confirmation by sequencing the cloned genes, N protein expression was conducted using three different *E. coli* strains (all obtained from Promega, U.S.A.): BL 21(DE3), BL 21(DE3)PlysS and JM109(DE3).

For expression in baculovirus, the N gene fragments were cloned into the pFastBac-Mels-6His vector (Invitrogen, U.S.A.), which fuses the melittin signal peptide [21] and the His_6 tag to the N- and C-termini, respectively, of the protein to be expressed. The recombinant bacmid DNA was transfected into insect cells (Sf9) using the Lipofectamine 2000 reagent (Invitrogen, U.S.A.), resulting in recombinant baculoviruses rAcNPV-HN and rAcNPV-NN, which express HeV N and NiV N proteins, respectively.

Purification of N proteins expressed in E. coli

Recombinant His-tagged proteins from *E. coli* culture were purified under denaturing conditions using the Talon-NX metal resin (Clontech, U.S.A.) following protocols provided by the supplier. The quality and purity of the eluted proteins were determined by SDS-PAGE.

Western blot, ELISA and immunofluorescent antibody test (IFAT)

For western blot analysis, protein samples were separated on a 12% SDS-polyacrylamide gel, and then transferred onto a PVDF membrane (Millipore, U.S.A.). After blocking with PBS containing 1% BSA, the membranes were incubated with appropriately diluted rabbit anti-HeV and anti-NiV antibodies generated at the Australian Animal Health Laboratory (AAHL) using inactivated whole viruses (unpublished results). After thorough washing with PBS containing 0.05% Tween-20, the membrane was incubated with HRP-conjugated secondary antibodies. Signals were developed by enhanced chemiluminescence (ECL) using the Supersignal West Pico Trail Kit (Pierce, U.S.A.).

For ELISA analysis, a 96-well Nunc plate was coated with *E. coli*-expressed N protein at 0.2 μg/well following by blocking with 200 μl PBS (pH 7.4) containing 5% fetal calf serum at 37 °C for 1 h. After washing, 100 μl of appropriately diluted primary antibodies was added to each well and incubated at 37 °C for 1 h. The same washing and incubation were carried out with HRP-conjugated secondary antibodies at a dilution of 1:10,000. Color development was carried out by adding to each well 100 μl of a substrate solution (0.04% ortho-phenylenediamine (Sigma, U.S.A.) in 0.1 M phosphate-citrate buffer with the addition of 15 μl 30% H_2O_2 for each 10 ml). All assays were conducted in duplicate. For inhibition ELISA, the inhibition ratio was calculated as follows:

$$\% \text{ inhibition} = (OD_{490\ \text{Positive}} - OD_{490\ \text{Phage Plus}}) / OD_{490\ \text{Positive}} \times 100$$

$OD_{490\ \text{Positive}}$ refers to readings for wells containing mAb solution only and $OD_{490\ \text{Phage Plus}}$ for wells containing mAb-phage complex solution.

For IFAT, the Sf9 cell monolayers (grown in 96-well plates) infected with rAcNPV-HN and rAcNPV-NN were fixed by cold acetone and probed with appropriately diluted primary antibodies. After incubation for 1 hr at 37 °C, FITC-conjugated secondary antibodies (Sigma, U.S.A.) were added to each well and the plate was incubated for 1 h at 37 °C. After washing, the wells were examined under a Zeiss Axioskop 40 fluorescence microscope (Zeiss, Germany). For each experiment, uninfected Sf9 cells and unrelated antibodies of the same species were used as negative controls.

Production and screening of hybridoma

Four-to-six-week-old female BALB/C mice (purchased from Changchun Bio-Product Factory, China), were immunized intraperitoneally with 5 μg of purified NiV or HeV N protein expressed in *E. coli* together with Freund's complete adjuvant (Sigma, U.S.A.). Two booster injections were given on days 14 and 28, with the same dose of antigens emulsified in Freund's incomplete adjuvant (Sigma, U.S.A.). Mice with the highest serum antibody titer (determined by ELISA and IFAT as described above) received a further intraperitoneal injection of 25 μg antigen without adjuvant. Three days later, the splenocytes of the immunized mice were isolated and fused with SP2/0 myeloma cells using 50% (w/v) polyethylene glycol (Sigma, U.S.A.). Hybridomas were screened for secretion of desired antibodies by ELISA and IFAT. Monoclonal antibody isotypes were determined using an Isotype Determination Kit (Roche, Switzerland). The ascites containing high concentrations of monoclonal antibodies were harvested and purified as described [17]. The specificity of the mAbs was further analyzed by western blot using baculovirus-expressed NiV and HeV N proteins.

Epitope mapping using phage display random peptide library

MAbs of IgG isotype were purified on protein A resin (Pharmacia, France) and those of IgM by the PEG method as previously described [17]. The concentration of purified mAbs was determined using a BCA Protein Concentration Determining Kit (Pierce, U.S.A.). Three rounds of panning were conducted with the following conditions: first round, mAb coated at 15 μg/well with 10 washes using buffer containing 0.1% Tween; second round, mAb at 1.5 μg/well with 15 washes and 0.3% Tween; third round, mAb at 0.15 μg/well with 20 washes and 0.5% Tween. A total number of 1.5×10^{11} phage clones of the PhD-12 Random Peptide Library (New England Biolabs, U.S.A.) were used for the first round of panning. The same amount of phage clones were used for subsequent rounds of panning. The panning procedures provided by the manufacturer were followed without modification. The eluted phage pool from each panning was titrated and the panning efficiency was calculated by output phage/input phage.

After 3 rounds of panning, the bound phage pool was eluted, amplified and titrated following manufacturer's instructions. To demonstrate the presence of selected phage clones in the pool, an aliquot of phage solution (containing 1.5×10^{11} clones) was mixed with 100 μl mAb solution at different dilutions (1:250, 1:500, and 1:1000, respectively), incubated overnight at 4 °C and then used in the IFAT as above.

For phage pools demonstrating a specific inhibition in the above IFAT, individual phage clones were picked, grown up and titrated. Similar to the inhibition assay by IFAT, 1.5×10^{11} phage particles were pre-mixed and incubated with mAbs at three dilutions before being tested in ELISA to demonstrate specific inhibition of mAb binding to recombinant NiV or HeV N proteins coated in the ELISA well.

Phage clones with different inhibition efficiency were selected for sequence analysis. Purification of phage DNA was carried out following the protocol of the supplied kit manual. Peptide sequence analysis and alignment with henipavirus N protein sequences were carried out using DNAStar software (DNASTAR Inc., Madison, U.S.A.).

Epitope mapping using overlapping protein fragments

In parallel to the phage random peptide library approach, mapping of mAb-binding regions was carried out using overlapping fragments of the N proteins generated by expression of GST-fusion proteins in *E. coli.* A total of five overlapping fusion protein fragments were produced to HeV N and NiV N, respectively. Gene fragments were obtained by PCR using primers with cloning sites incorporated, then gel purified and cloned into expression pGEX-6P-1 (Amersham, U.S.A.). Protein expression in BL21(DE3) was conducted as described above. The expression of fusion proteins with expected sizes was confirmed by SDS-PAGE and Coommassie Blue staining. The reactivities of four mAbs with each of the ten fusion proteins were then tested by western blot.

Immunohistochemistry (IHC)

Sections obtained from a paraffin block containing lung tissue from a horse experimentally infected with HeV [25] were mounted onto glass slides. Slides were dewaxed and antigen retrieval was achieved through incubation with 0.1% trypsin (w/v) in 0.1% aqueous $CaCl_2$, pH 7.8, with 0.1 M NaOH for 20 min at 37 °C. Slides were blocked with 10% H_2O_2 for 20 min at room temperature. After blocking, the slides were incubated for 1 h at 37 °C with antibodies appropriately diluted in TBS (10 mM Tris–HCl, pH 8.0, 150 mM NaCl) containing 1% (w/v) skimmed milk. The negative control mAb (NDV Q24.1.2 19-5-00), raised against the N protein of Newcastle disease virus (existing stock at the Australian Animal Health Laboratory (AAHL), Australia) was used at a dilution of 1:10; the HeV and NiV positive control sera (AAHL stocks) and the test mAbs were

diluted at 1:400. Slides were rinsed with dH_2O and labeled polymer-HRP anti-mouse or anti-rabbit (Dako, Australia) were added. Following incubation at 37 °C for 30 min, slides were stained with AEC+ Substrate Chromogen (Dako, Australia), rinsed, counter-stained in haematoxylin and Scott's bluing solution, and mounted. These were examined under a DM LB compound microscope and images recorded with a DC500 digital camera (both from Leica, Germany).

Results

Expression of N proteins in E. coli and baculovirus

After cloning of the N genes into expression vector pET-28(a), levels of expression were compared in three different *E. coli* strains. Although the genes were expressed in all three strains, the highest level of expression was achieved in JM109(DE3). Analysis by SDS-PAGE and densitometry indicated that the recombinant N protein accounts for 13.8% (for NiV N) and 14.6% (for HeV N) of total bacterial proteins. Both N proteins can be recognized by rabbit anti-HeV and anti-NiV sera in western blot (Fig. 1A).

The two N genes were also expressed in baculovirus using an expression system which allows secretion of recombinant proteins with a C-terminal His_6 tag. Analysis by SDS-PAGE showed that the highest expression level of N protein was achieved at 72–96 h post-infection, reaching approximately 2.3 mg/L of target protein. The recombinant HeV N and NiV N proteins were recognized in western blot by both anti-HeV and anti-NiV sera (Fig. 1B).

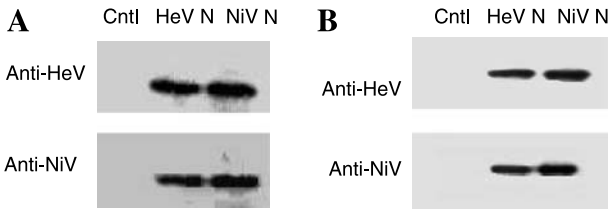

Fig. 1. Western blot analysis of recombinant HeV N and NiV N probed by rabbit anti-HeV and anti-NiV sera. (**A**) Purified recombinant N proteins expressed in *E. coli*. (**B**) Purified recombinant N proteins expressed in baculovirus. Negative control (*cntl*) samples were total protein lysate prepared from *E. coli* strain JM109(DE3) (**A**) and Sf9 insect cells (**B**), respectively

Preparation of mAbs against N protein of HeV and NiV

After screening and several rounds of cloning of hybridomas using ELISA and IFAT, four hybridomas stably excreting N-specific antibodies were obtained: two (N1H5, N4E2) from the immunization with NiV N, and two (H2F5, H3G5) from HeV N. Isotyping revealed that three mAbs were IgG1 and one (H2F5) was IgM. The titers of hybridoma supernatants were in the range of 1:64 to 1:256 by ELISA, whereas the ascites had a titer of approximately 1:200,000. All four mAbs reacted in western blots and were able to cross react with both NiV N and HeV N (Fig. 2).

Epitope mapping using a phage display random peptide library

To determine the binding sites of the four mAbs, a random 12-mer phage display peptide library was employed for panning. After three rounds of selection under different conditions it was evident that there was a gradual increase of selection ratio after each panning (data not shown). The specific selec-

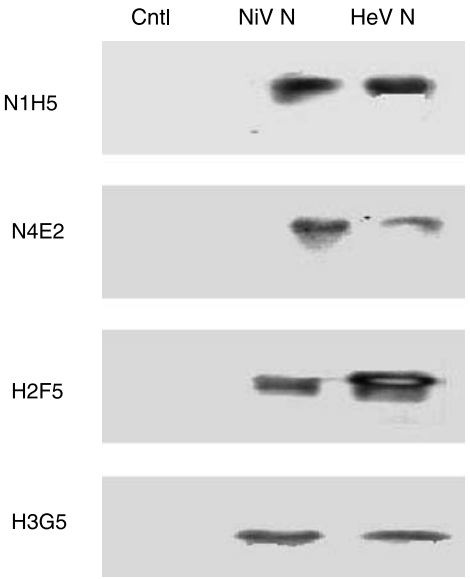

Fig. 2. Western blot analysis for confirmation of reactivity of mAbs. The same Sf9 cell lysate (used as negative control) and purified recombinant baculovirus-expressed HeV and NiV N proteins as in Fig. 1 were used for this analysis. Each mAb was used at a dilution of 1:1000

tion was further confirmed by a significant inhibition of IFAT signal on N-expressing Sf9 cells in the presence of pooled phage suspension (data not shown).

N1H5 (IgG)

	14											25	% Inhibition
NiV N		Y	Q	S	K	L	G	R	D	G	R	A	S	
HeV N		Y	Q	S	K	L	G	R	D	G	R	A	S	
C13	T	L	S	Y	K	L	Y	R	H	S	L	L		77.3
C14	T	L	S	Y	K	L	Y	R	H	S	L	L		74.6
C2		S	V	E	K	L	V	R	A	G	Q	V	S	71.5
C4		V	E	T	K	L	T	R	E	T	V	W	T	67.1
C12		P	V	S	K	L	S	R	A	S	G	V	G	67.0
C15		F	H	A	R	L	Q	R	L	P	P	H	R	63.9
C16	F	H	A	Y	S	K	L	T	K	S	L	L		56.9
C6		S	W	Y	K	L	V	H	G	D	A	Q	A	54.7
C17		S	P	L	K	L	S	R	S	S	M	Y	V	53.2
C18		T	P	N	K	L	K	K	P	P	V	P	Q	53.0
C8		W	T	Q	R	L	V	K	G	A	E	V	G	52.3
C9		D	T	E	R	L	Y	R	A	G	Q	E	V	50.4

N4E2 (IgG)

	442										453	% Inhibition
NiV N		Q	S	V	T	F	K	R	E	M	S	M	S	
HeV N		Q	S	V	T	F	K	R	E	M	S	I	S	
C22		R	T	T	D	F	K	R	E	M	S	T	S	81.5
C3		V	P	L	T	F	K	H	E	M	S	L	A	77.8
C1		V	L	P	T	F	K	R	E	M	T	L	V	73.6
C13			N	M	F	K	K	E	M	A	L	S	L D	73.8
C14			N	M	F	K	K	E	M	A	L	S	L D	74.6
C10		W	L	S	G	F	K	R	D	M	V	S	S	74.0
C12		G	Y	A	P	F	K	R	E	M	F	Q	N	72.0
C11		P	V	L	V	F	H	R	E	M	S	L	G	71.0
C15			N	H	W	K	M	E	M	S	L	P	S S	63.9
C7		D	Y	N	R	F	H	R	E	Y	V	V	S	57.0
C16			F	N	P	W	Q	W	E	M	T	F	P T	53.2

H2F5 (IgM)

													% Inhibition
C5		F	R	A	S	T	D	Y	P	V	F	G	F	78.1
C6		A	K	A	P	L	D	L	P	V	D	G	F	77.3
C1		A	K	A	P	L	D	L	P	V	D	G	F	76.2
C8			S	T	I	T	D	K	P	V	P	G	F G	68.8
C2			S	T	I	S	D	K	P	V	P	G	F G	63.2
C4		H	D	S	F	W	T	H	P	V	P	G	A	58.2

H3G5 (IgG)

													% Inhibition
C7		V	H	N	S	H	A	S	W	R	Y	T	G	70.4
C4		S	N	F	T	T	Q	M	T	F	Y	T	G	65.4
C6		V	Q	D	N	T	G	L	L	H	Y	T	G	64.0
C11		T	Q	L	M	W	P	N	L	I	Y	T	G	63.2
C3		S	N	F	T	T	Q	M	T	F	Y	T	G	63.1
C2			A	S	F	E	L	H	Q	W	Y	T	A T	61.3
C9		S	N	F	T	T	Q	M	T	F	Y	T	G	61.2
C10		F	T	S	S	N	V	P	L	R	Y	T	G	59.4
C14		S	V	T	T	L	P	A	Q	L	Y	T	G	58.3

Fig. 3. Identification of consensus motifs by sequence alignment. Conserved residues present in most peptide sequences were shaded. For each phage clone, the % inhibition on the binding of the corresponding mAb with recombinant N protein is shown at the right side. For mAbs N1H5 and N4E2, the sequence of the matching region in NiV and HeV N proteins are given at the top of the alignment with their aa positions indicated above

Individual clones from the pool of third-round panning were grown and tested by ELISA to further examine their ability to compete with mAb. For each mAb, at least ten different clones with significant inhibition were selected for sequencing analysis. The results are presented in Fig. 3. Consensus peptide sequences were obtained for each of the four mAbs: KLxR for N1H5, FKREM for N4E2, DxPVxGF for H2F5 and YTG for H3G5. Alignment of these consensus peptide sequences with the aa sequences of NiV and HeV N proteins revealed that the two motifs recognized by N1H5 and N4E2 matched aa sequences in the N proteins: KLxR at aa 17–20 and FKREM at 446–450, respectively (Fig. 3). However, the alignment failed to identify matching sequence motifs in the N proteins for the peptide sequences obtained for the other two mAbs.

Epitope mapping using overlapping protein fragments

To complement the approach of epitope mapping using phage display random peptide libraries, five

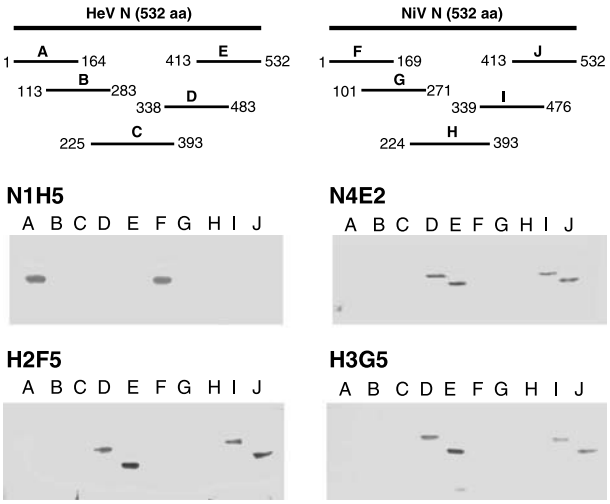

Fig. 4. Location of mAb-binding regions by Western blot analysis of overlapping protein fragments. The precise location (aa positions) of each of the ten recombinant protein fragments (*A*–*J*) was given in the schematic diagram at the top. The four panels below are the reactivity patterns of the four mAbs against each of the ten protein fragments. Each mAb was used at a dilution of 1:1000

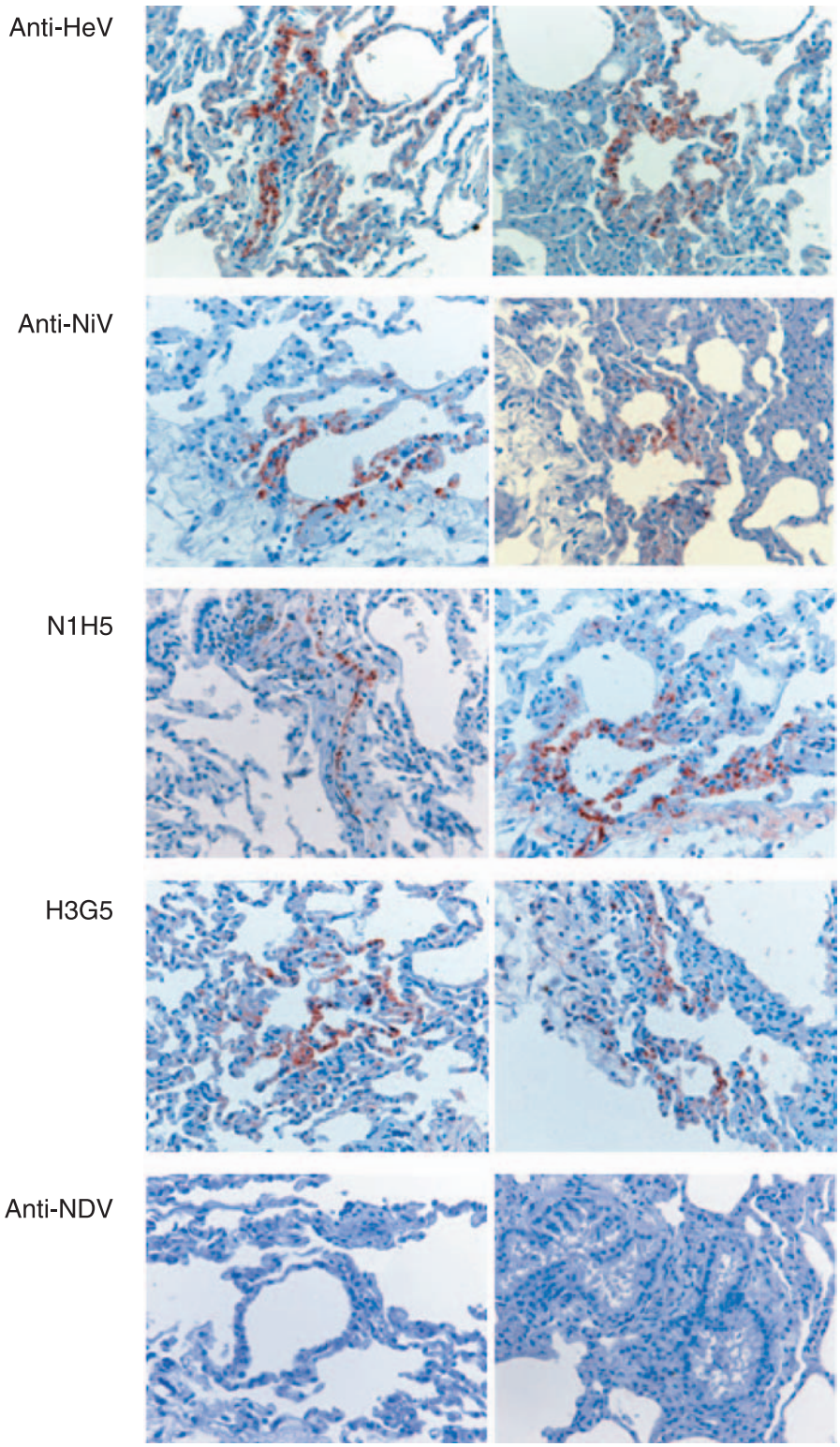

Fig. 5. Immunohistochemical staining of HeV-infected horse lung tissue sections. Antibodies used: anti-HeV, rabbit serum raised against inactivated whole virus; anti-NiV, rabbit serum raised against inactivated whole virus; N1H5 and H3G5, mAbs produced in this study; anti-NDV, a negative control mAb (IgG) specific for Newcastle disease virus. All antibodies were used at 1:400 with the exception of anti-NDV (used at 1:10). Due to the limited supply of the horse tissue sections left from previous experiments, it was impossible to use sections from the same location for testing different antibodies. Two different sections were therefore used for each antibody to improve reliability

overlapping fragments were produced in *E. coli* for HeV N (designated A–E) and NiV N (designated F–J), respectively (see Fig. 4). Each of the ten fusion proteins was tested for their reactivity against each of the four mAbs. N1H5 reacted with fragments A and F, whereas N4E2 reacted with D, E, I and J (Fig. 4), confirming the results obtained from above using phage peptide libraries. Interestingly, mAbs H2F5 and H3G5 also reacted with D, E, I and J, indicating that they bind to a 64-aa region present in all four fragments.

Application in immunohistochemistry (IHC)

Since the N proteins are the most abundantly expressed of all henipavirus proteins in infected cells, the antibodies against N proteins are therefore an ideal tool for specific detection of viral antigens in infected tissues. To determine whether any of these four mAbs would work in IHC, they were tested against existing lung tissues from a horse experimentally infected with HeV [25]. As shown in Fig.5, out of four mAbs, N1H5 and H3G5 were able to specifically stain HeV-infected cells in different sections of the infected horse lung tissues.

Discussion

In this study, the N genes of HeV and NiV were expressed in *E. coli* and baculovirus, and the recombinant proteins produced in both systems were recognized by rabbit sera raised against both inactivated HeV and NiV. The strong cross-reactivity between the N proteins of the two viruses was expected from their high level of sequence similarity [23]. The purified recombinant *E. coli* proteins were used to immunize mice for production of mAbs to be used as diagnostic and research tools for these deadly emerging viruses. A total of four different mAbs were obtained, two from HeV N and two from NiV N. All four mAbs specifically reacted with recombinant baculovirus N proteins of both HeV and NiV. Since the N proteins of two viruses share 91% amino acid sequence identity, it was not unexpected that all four mAbs showed cross-reactivity between the two N proteins.

The binding sites or epitopes for these mAbs were determined using phage display random peptide libraries. This technology, originally developed by the Smith group [18], has many advantages over other epitope mapping methods [5, 11, 24]. The specificity of the selection was monitored by the selection ratio and the inhibition of mAb-binding to N protein by specific phage peptide. Clones showing a 50% inhibition or higher were further analyzed by sequencing. For mAbs N1H5 and N4E2, the consensus sequence motifs matched regions present in both the HeV and NiV N proteins. N1H5 recognizes a KLxR (x = unspecified residue) motif which is located at aa 17–20 at the N-terminus of the N proteins. The motif recognized by N4E2, FKREM, is located between aa 446–450, towards the C-terminal end of the 532-aa N protein. The localization of the mAb epitopes was further confirmed by a parallel approach using western blot and overlapping truncated fragments. To our knowledge, these represent new epitopes of henipavirus N proteins which have never been reported previously.

In contrast, the motifs for H2F5 (DxPVxGF) and H3G5 (YTG) revealed from the mapping using phage display libraries did not match any sequence of the N proteins by direct alignment analysis. When analyzed by western blot of different truncated fragments, it was shown that both H2F5 and H3G5 bind to a 64-aa overlapping region present in the two fragments at the C-terminal region. This was unexpected considering that N4E2 also binds to this region. The failure to obtain a matching sequence consensus suggests that these mAbs recognize a different epitope from that of N4E2. The different sequence motifs obtained for H2F5 and H3G5 further suggests that they represented two independent hybridoma cell lines, rather than two subclones of the same parental hybridoma. It can therefore be concluded that the three mAbs represent independent hybridoma clones which recognize different structural features, albeit all in the 64-aa region.

One of the main applications for those mAbs will be to specifically detect henipavirus antigen in animal or human tissues suspected of infection. It is well known that not every mAb will work in IHC,

and there are many factors which can affect the performance of a given mAb in IHC. There is no reliable method to predict which mAb will work in IHC. In this context, we conducted preliminary testing on existing lung tissues from HeV-infected horses. Our findings were consistent with the unpredictable nature of mAb performance in IHC. While it was pleasing to see that both N1H5 and H3G5, known to bind two very different regions, were able to detect HeV antigen in lung sections, it is not easy to explain why N4E2 and H2F5 failed to work. The failure of H2F5 might be explained by the fact that this was the only antibody of IgM subtype, and IgM antibodies generally do not work well in IHC due to their large sizes. For N4E2, there is no obvious reason to account for its failure in IHC. It was originally raised against HeV N, its precise binding site has been mapped, and it worked in IFAT against both HeV and NiV N proteins expressed in baculovirus. It is possible that the particular epitope recognized by N4E2 is inaccessible to the mAb when N is present in its natural RNP state in infected tissues.

In summary, we have generated four mAbs against henipavirus N proteins. These mAbs, together with the recombinant N proteins produced in *E. coli* and baculovirus, will be useful tools for further development of diagnostic reagents for use in laboratories which lack access to BSL4 facilities. The fine mapping of two mAbs further enhanced the value of these mAbs as a research tool for studying this important group of emerging viruses. The preliminary testing in IHC indicated that at least two mAbs can be used for specific detection of viral antigen in infected tissues. Further testing in other animal species is required to determine their general suitability for application in IHC.

Acknowledgements

We thank John Bingham, Diane Green and Gail Russell for help with IHC analysis. This work was supported by grants from animal disease preventive platform program of the Ministry of Science and Technology of P.R. China (2004BA519A48), scientific and technological project of Guangdong Province of P.R. China (2004A20403001) and Australian Biosecurity Cooperative Research Center for Emerging Infectious Diseases (Project 1.013RE).

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