Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Virus Research, 11 (1988) 319–333
Elsevier

VRR 00453

Mapping of three antigenic sites on the haemagglutinin-neuraminidase protein of Newcastle disease virus

Khatijah Yusoff, Mark Nesbit, Hazel McCartney, Peter T. Emmerson and Anthony C.R. Samson

Department of Biochemistry and Genetics, Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne, U.K.

(Accepted 26 August 1988)

Summary

Nine neutralizing monoclonal antibodies (MAbs), each of which react with the haemagglutinin-neuraminidase (HN) glycoprotein of the Beaudette C strain of Newcastle disease virus (NDV), have been used in competitive binding assays to delineate three non-overlapping antigenic sites A, B and C. Epitopes within these sites have been identified on the basis of cross-reactivity of MAb-resistant mutants against the panel of MAbs, determined by plaque assays and Western blotting. Site A contains three non-overlapping epitopes (A1, A2 and A3). A1 is the only linear epitope; all remaining epitopes are conformational. MAbs which react with epitopes A2 and A3 inhibit neuraminidase activity (NA) when assayed with neuraminlactose. Site B contains three partially overlapping epitopes (B1, B2 and B3) and site C is represented by a single epitope (C1). HN gene sequence analysis of MAb-resistant mutants showed that they each had only single amino acid substitutions which range from amino acid residues 347–460 for site A, 284–325 for site B, and at 481 for the C1 epitope. The apparent molecular mass of the HN glycoprotein of one mutant was increased from 72 to 75 kDa. This correlates well with the creation of an additional potential glycosylation site in this mutant from Asn-Ser-Pro(325) to Asn-Ser-Ser(325).

NDV; Haemagglutinin-neuraminidase; Monoclonal antibody; Epitope mapping

Correspondence to: K. Yusoff, Department of Biochemistry and Genetics, Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH, U.K.

Introduction

Newcastle disease virus (NDV) is an avian paramyxovirus. Two surface glycoproteins project from the enveloped virion: the haemagglutinin-neuraminidase (HN) protein which contains both haemagglutinin (HA) and neuraminidase (NA) activities, and the fusion (F) protein which is involved in cell-fusion and haemolysis (Scheid and Choppin, 1973, 1974; Hsu et al., 1979). Polyvalent antiserum directed against either of these proteins is capable of neuralising viral infectivity (Umino et al., 1984).

Anti-HN monoclonal antibodies (MAbs) have been used to identify at least four antigenic sites on the HN protein (Iorio and Bratt, 1983; Nishikawa et al., 1986; Russell and Alexander, 1983). A fifth site which overlaps two of the above sites has been suggested (Iorio et al., 1986). Most of the antigenic sites have been shown to be highly conserved while some sites appear to be strain specific (Nishikawa et al., 1983; Russell and Alexander, 1983; Iorio et al., 1984; Meulemans et al., 1987). A highly conserved linear epitope has been located by Western blot analysis of expressed fragments of the HN gene produced in *E. coli* and confirmed by RNA sequencing of a MAb-resistant mutant of NDV which showed the involvement of a single amino acid substitution in the HN protein (Chambers et al., 1988).

Sequencing MAb resistant mutants has been used to locate functional antigenic sites on paramyxoviruses such as Sendai virus (Portner et al., 1987; Thompson and Portner, 1987), human parainfluenza virus type 3 (Coelingh et al., 1986, 1987) and, more recently, NDV (Gotoh et al., 1988).

We have studied nine anti-HN neutralizing MAbs and assigned them to three antigenic sites according to competitive antibody binding assays. The epitopes which are recognised by individual MAbs have been correlated with the amino acid changes in the HN primary structure and with various biological functions affected by the MAbs.

Materials and Methods

Virus

The NDV strain Beaudette C was used. Viruses were grown in the allantoic fluid of 10-day-old chick embryos at 37 °C. After four days, the allantoic fluid was harvested and virus purified as previously described (Chambers and Samson, 1980).

Production of murine hybridomas

BALB/c mice were immunised with solubilised glycoproteins and matrix proteins (Kohama et al., 1981). Mice were given an intraperitoneal injection of 100 μg of mixed viral proteins in 100 μl phosphate-buffered-saline (PBS) and 100 μl complete Freund's adjuvant, and boosted twice with 100 μg proteins in PBS given intraperitoneally at one month intervals, and sacrificed four days after the final booster.

NS1 cells were maintained in full growth medium [FGM: RPMI 1640 medium (pH 7.2) supplemented with 15% foetal calf serum and 1% (w/v) penicillin and streptomycin]. A monodisperse spleen cell suspension in RPMI was prepared from an immunised mouse and mixed with the NS1 cells at a ratio of 5 : 1. The cells were pelleted by centrifugation at $1000 \times g$ for 10 min. The pellet was disrupted and gently overlayed with 1 ml of 50% polyethylene glycol 1000 (BDH) in RPMI without additives. The suspension was gently mixed at 37 °C for 1 min, diluted to 25 ml over the next 5 min, and subsequently made up to 100 ml using FGM. One hundred microlitre aliquots of the cells (2×10^7/ml) were seeded into 96-well flat-bottom plates (Falcon 3072) and incubated at 37 °C for 24 h in a 5% CO_2/95% air incubator. To each well was added 100 μl double strength hypoxanthine-aminopterin-thymine (HAT) medium in FGM. Cells were grown in this medium for 12 days after which aminopterin was omitted from the medium.

Supernatants from potential hybridomas were screened for antibody activity using indirect immunoperoxidase assay (IIP) as described by Russell et al. (1983a) and enzyme linked immunosorbent assay (ELISA) using 4-chloro-1-naphthol as the substrate. Cells positive for both assays were cloned by limiting dilution and further assayed after 12 days. This cloning scheme was repeated and the hybridomas were expanded in FGM.

For ascites production, BALB/c mice were primed with 500 μl pristane given intraperitoneally. After 10 days, mice were injected with 1×10^7 hybridoma cells, and ascites fluid collected 2–6 weeks later. Ascites fluids were clarified at $5000 \times g$ for 10 min and stored at −20 °C.

Isotyping of immunoglobulins

Ouchterlony double immunodiffusion gels were used in conjunction with sheep anti-mouse specific antisera (ICN Immunobiologicals). Seventy-five microlitre of ascites fluid 1 : 500 in PBS was added to the central wells and 10 μl of antisera then added to the outer wells. Gels were then stored at room temperature for 24–48 h and isotypes determined from the pattern of precipitation arcs between wells.

Enzyme linked immunosorbent assay (ELISA)

The method was modified from that described by Engvall and Perlmann (1972). Purified virions (10 μg/ml) were U.V.-irradiated for 15 min (dose = 5.7 erg/mm^2/sec) and adsorbed to plastic microtitre wells (Dynatech, M129A) overnight at 4 °C in coating buffer ($NaHCO_3/Na_2CO_3$, pH 9.6). Between each application of reagents, wells were extensively washed with 0.05% PBS (v/v) Tween-20. Wells were then blocked with 1% bovine serum albumin (BSA) for 1 h at 37 °C and incubated with 50 μl of hybridoma supernatant for 2 h at 37 °C, and coated with a 1 : 1000 dilution of peroxidase-conjugated rabbit anti-mouse immunoglobulin (ICN Biomedicals LTD) for 1 h at 37 °C. The substrate, orthophenylene diamine (OPD) 0.5 mg/ml, was then added and the reaction was stopped by the addition of 3 M H_2SO_4. Plates were read in a BIO-RAD ELISA reader using a 492 nm filter. Clones were considered positive when they gave an absorbance three times higher than when PBS was used as a negative control.

Purification and peroxidase labelling of MAbs

MAbs were purified by affinity chromatography of ascites fluid using DEAE Affi-Gel Blue IgG (BIO-RAD). A stepwise salt gradient (0–2% (w/v) NaCl in 0.02 M Tris-Cl, 0.023 M NaCl, pH 8.0) was used to elute selectively the IgG which was dialysed extensively against sodium carbonate buffer (0.01 M Na_2CO_3, pH 9.5) at 4° C. Covalent attachment of MAb to horseradish peroxidase (HRP; 0.5 mg/ml, Sigma), by the periodate-oxidation method was carried out according to O'Sullivan and Marks (1981).

Western blotting

The procedure was as described previously (Samson, 1986).

Estimation of IgG concentration in ascites fluid

Fractions eluted from DEAE Affi-Gel Blue columns were assayed spectrophotometrically at OD_{260} and OD_{280}. Protein concentration (mg/ml) was determined by the formula: $1.11 \times OD_{280} - 0.755 \times OD_{260}$. Fractions were analysed by SDS-PAGE and ELISA, and those containing pure IgG were pooled.

Competitive binding assays (CBA)

The procedure was used as for ELISA except that peroxidase-labelled MAb was added immediately after the addition of serially diluted competitor MAb in ascites fluid.

Neutralization test (NT)

Purified virus (200 PFU in 100 μl PBS) was mixed with an equal volume of two-fold serially diluted ascites fluid initially adjusted to 200 μg protein/ml. After 1 h incubation at 37° C, 100 μl samples were plated onto 30 mm plates of chick embryo fibroblasts (CEF). Following a 30 min adsorption at 37° C the cells were washed in PBS and plaque assays were performed as described by Nishikawa et al. (1983). Antibody to the matrix (M) protein was used as a negative control. The neutralizing titre was taken as the reciprocal of the highest antibody dilution needed to reduce the PFU titre by 50%.

Haemagglutination inhibition (HI)

Serial two-fold dilutions of ascites fluid (25 μl) were mixed with an equal volume of virus (8 HA units) and 96-well V-shaped microtitre plates. After 30 min incubation at 37° C, 50 μl of human erythrocytes (1×10^8 cells/ml) were mixed and allowed to stand for at least 2 h at 4° C. The reciprocal of the highest dilution of the antibody which still had the ability to inhibit haemagglutination was taken as the HI titre.

Neuraminidase inhibition (NI)

Serial two-fold dilutions of ascites fluid (50 μl) were incubated with an equal volume of virus (200 μg protein/ml) for 30 min at 37° C. To these were added 80 μg of N-acetyl neuraminlactose (Sigma), and the mixture incubated for 1 h at 37° C.

The amount of N-acetyl neuraminic acid released was determined by the method of Aminoff (1961). Antibody to the matrix (M) protein was used as a negative control. The reciprocal of the highest dilution of antibody to inhibit NA activity by 50% was taken as the titre.

Haemolysis inhibition

Titres of haemolysis inhibition (HLI) were determined as described by Iorio and Bratt (1984). Two-fold serially diluted virus in PBS (50 μgl) was mixed with an equal volume of serially diluted ascites fluid. After 30 min at 37°C, 100 μl of human erythrocytes (7×10^8 cells/ml) was added to the mixture and the incubation was continued for 1 h. The reaction was stopped with 600 μl ice-cold PBS and the haemolytic activity was determined as described by Clavell and Bratt (1972). Specific haemolysis titre was taken as the reciprocal of the concentration of antibody required to cause a 50% shift in a plot of haemolytic activity as a function of virus concentration.

RNA sequence analysis

Viral RNA extraction was performed as described by Both and Air (1979). A set of 17-mer synthetic oligonucleotide primers complementary to the Beaudette C HN gene sequence (Millar et al., 1986), at intervals of approximately 200 nucleotides, was used in the dideoxy-chain terminating sequencing reactions. The procedure for sequencing was essentially that of Cavanagh and Davis (1988) except that 20 U of RNasin (Promega) and [α^{35}S]dATP (Amersham) were used in the reactions.

Results

Specificity of MAbs

A panel of nine neutralising MAbs was used in the study. MAb 445 was previously described by Russell et al. (1983b) and MAbs 4D6 and 8C11 were previously described by Le Long et al. (1986). The remaining six MAbs were isolated in this laboratory from a single fusion. All of these MAbs were shown to bind the Beaudette C virion by IIP and ELISA studies (not shown); and to the HN protein in the absence of the reducing agent 2-mercaptoethanol (Fig. 1a). When virions were boiled in the presence of the reducing agent, only MAbs 4D6, NHN-8 and NHN-10 continued to detect the HN protein (Fig. 1b) suggesting that the epitope(s) recognised by these antibodies are linear whilst the remaining MAbs in this study are conformational.

Competitive binding assay

To determine whether these MAbs define areas on the HN protein which are topologically distinct from each other, the MAbs were purified from the ascites fluids, labeled with peroxidase and used in reciprocal competitive binding assays. As shown in Table 1, three distinct patterns of reactivity were observed, indicating the presence of three binding sites: site A was defined by MAbs 4D6, NHN-2, NHN-3,

TABLE 1

COMPETITIVE BINDING ASSAY

Peroxidase labelled MAb	Unlabelled competitor MAb [a]									Antigenic site
	4D6	NHN-8	NHN-10	NHN-2	NHN-3	8C11	445	NHN-6	NHN-1	
4D6	100	87	84	95	99	0	0	0	0	A
NHN-8	102	100	100	99	99	0	0	14	0	A
NHN-10	103	101	100	99	101	14	3	18	6	A
NHN-2	103	103	96	100	105	19	0	18	19	A
NHN-3	97	97	86	99	100	6	2	11	13	A
8C11	0	0	0	0	0	100	91	40	0	B
445	0	0	14	0	0	107	100	114	10	B
NHN-6	0	0	0	0	0	37	83	100	0	B
NHN-1	9	13	12	0	0	2	11	4	100	C

[a] Percent inhibition of binding of peroxidase-labelled antibody was calculated as $100\{(A-n/(A-B)\}$ where A, B and n are OD_{490} in the absence of competitor, in the presence of homologous antibody at antibody excess, and in the presence of competitor, respectively (Nishikawa et al., 1983).

NHN-8, and NHN-10; site B was defined by MAbs 8C11, NHN-6 and 445, and site C was defined by MAb NHN-1. Site A antibodies showed complete competition with each other (approximately 100%) and no significant competition with the other antibodies. The fact that site A binds five out of the nine MAbs isolated suggests that the antigenic region recognised by these antibodies is immunodominant for the Beaudette C HN glycoprotein. MAbs 8C11 and NHN-6 exhibited reciprocal competition (approximately 40%) with each other and 445 showed complete competition with both 8C11 and NHN-6, suggesting that site B is a complex antigenic region. MAb NHN-1 did not show significant competition with any of the other MAbs and is the only MAb representing site C.

Biological properties of the MAbs

The biological activities of single isotypic MAbs, are summarised in Table 2. In all cases, serial two-fold dilutions of 200 μg of antibody was used. For the neuraminidase inhibition assays the low molecular weight substrate N-acetyl neuraminlactose was used. This substrate was chosen in preference to the higher molecular weight fetuin as it is less perturbed by steric hindrance and should better define the site at which antibody mediates inhibition. Only MAbs NHN-2 and NHN-3 inhibited neuraminidase activity suggesting that the epitopes for MAbs NHN-2 and NHN-3 are located close to each other and possibly also close to the catalytic site.

Haemolysis is thought to correspond to the fusion of the virus to the host plasma membranes, a function carried out by the F protein (Hsu et al., 1979). Recent studies have suggested a role for the HN protein in fusion (Ozawa et al., 1979; Portner et al., 1987). In the HLI assays the relative haemolytic activities were determined by comparing concentrations of the virus required to lyse the erythrocytes to the same extent rather than by direct comparison of lysis by a given

TABLE 2

Biological properties of monoclonal antibodies

MAb	IgG isotype	Titres [a]			
		HI	NI	HLI	NT
4D6	2A	256	< 2	ND [b]	1024
NHN-8	1	256	< 2	0.26	512
NHN-10	1	256	< 2	ND	512
NHN-2	1	512	294	0.26	1024
NHN-3	1	256	90.5	0.26	512
8C11	2A	128	< 2	0.13	2048
445	2B	128	< 2	0.26	2048
NHN-6	2A	8	< 2	0.06	16
NHN-1	1	256	< 2	0.26	512

[a] Titres were determined as described in Materials and Methods.
[b] Not determined.

concentration of virus, since haemolysis is not directly proportional to the amount of virus present (Clavell and Bratt, 1972). All of the nine MAbs inhibited haemolysis to some extent.

In HI assays, all the MAbs had titres which were at least 16-fold higher than MAb NHN-6, suggesting that the epitope for MAb NHN-6 may be located further from the haemagglutinin site than the other MAbs.

All nine MAbs neutralise the virus and could therefore be used to isolate resistant mutants, which could in turn be used to locate the MAb binding sites. MAb NHN-6 was the least efficient at neutralization and a higher concentration of it was required to isolate resistant mutants (Table 2).

Isolation and characterization of MAb resistant mutants

Distinct epitopes can be defined by the inability of MAb resistant mutants to bind to other MAbs. To identify epitopes located on the HN protein we isolated several independent mutants of Beaudette C that were resistant to members of the panel of MAbs. The frequency of isolation of these mutants was in the range of 10^{-3} to 10^{-4}.

Cross-reactivity tests using both Western blotting and plaque assays were performed among the various mutants and the nine MAbs to define the epitopes located within the three antigenic sites. Table 3 shows that the results from Western blotting entirely agreed with those from the plaque assays. As expected, all MAb resistant mutants failed to bind to their respective selecting MAb. Furthermore, all 4D6, NHN-8 and NHN-10 resistant mutants also failed to bind to MAbs 4D6, NHN-8 and NHN-10, but bind to NHN-2 and NHN-3 were shown from CBA studies to recognise the same antigenic site A. We therefore place MAbs 4D6, NHN-8 and NHN-10 in epitope group A1 and MAbs NHN-2 and NHN-3 in epitope groups A2 and A3, respectively. Moreover, it is only MAbs which belong to group A1 which continue to bind to reduced HN protein (Fig. 1b).

TABLE 3

Cross-reactivity tests [a]

Selecting MAb	Epitope specificity	Mutant	MAb used									
			None	4D6	NHN-8	NHN-10	NHN-2	NHN-3	8C11	445	NHN-6	NHN-1
4D6	A1	7	+	+	+	+	–	–	–	–	–	–
NHN-8	A1	Z5	+	+	+	+	–	–	–	–	–	–
NHN-8	A1	Z6	+	+	+	+	–	–	–	–	–	–
NHN-10	A1	Z1	+	+	+	+	–	–	–	–	–	–
NHN-10	A1	Z3	+	+	+	+	–	–	–	–	–	–
NHN-2	A2	Z13	+	–	–	–	+	–	–	–	–	–
NHN-2	A2	Z15	+	–	–	–	+	–	–	–	–	–
NHN-2	A2	Z16	+	–	–	–	+	–	–	–	–	–
NHN-3	A3	F1	+	–	–	–	–	+	–	–	–	–
NHN-3	A3	F3	+	–	–	–	–	+	–	–	–	–
8C11	B1	U2	+	–	–	–	–	–	+	–	–	–
8C11	B1	MN2	+	–	–	–	–	–	+	–	–	–
445	B2	V3	+	–	–	–	–	–	+	+	–	–
445	B2	E2	+	–	–	–	–	–	–	+	–	–
445	B2	Q1	+	–	–	–	–	–	–	+	+	–
NHN-6	B3	F5	+	–	–	–	–	–	–	–	+	–
NHN-6	B3	F6	+	–	–	–	–	–	–	–	+	–
NHN-1	C1	Z17	+	–	–	–	–	–	–	–	–	+
NHN-1	C1	Z18	+	–	–	–	–	–	–	–	–	+
NHN-1	C1	Z20	+	–	–	–	–	–	–	–	–	+

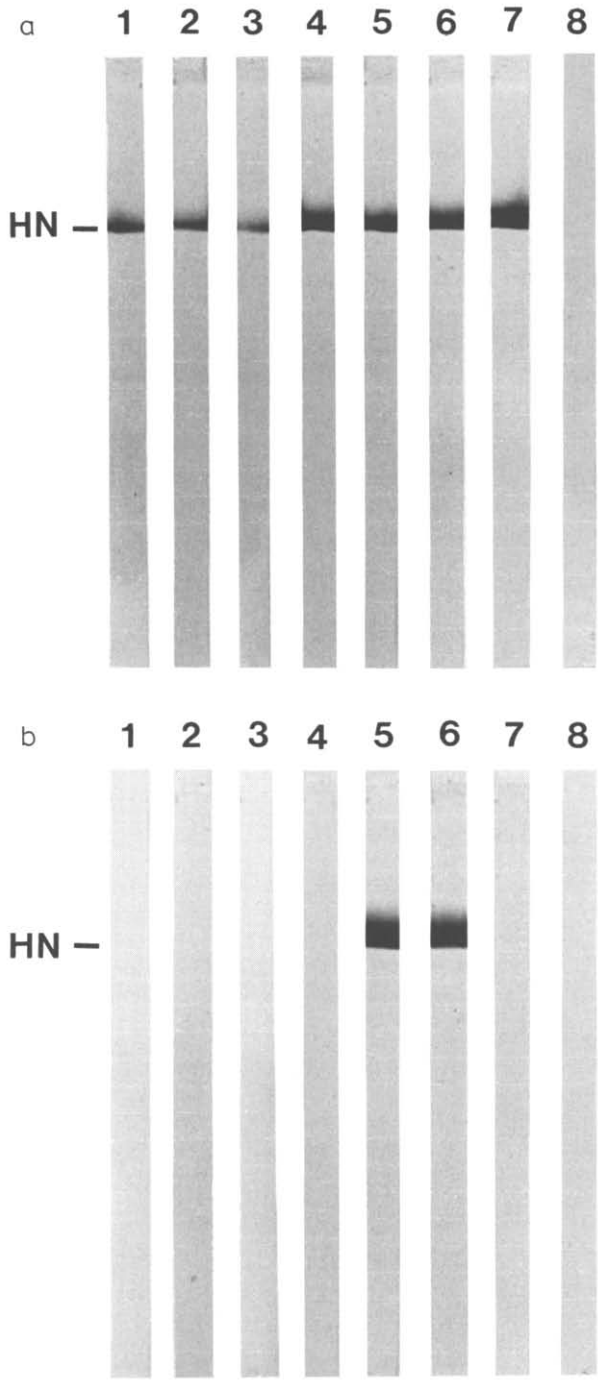
[a] Determined from both plaque assays and Western blotting in the presence and absence of each MAb. The methods used have been described previously (Samson et al., 1988).

+: Normal plaque titers/no binding.

–: No plaques/binding.

Table 3 shows that all MAb 8C11 resistant mutants bind MAb 445. The reciprocal, however, is not necessarily the case; one MAb 445 resistant mutant (V3) does not bind MAb 8C11. Similarly, all the MAb NHN-6-resistant mutants bind MAb 445. However, the MAb 445 resistant mutant (Q1) does not bind MAb NHN-6. These results are consistent with the partial competition observed between MAbs 8C11 and NHN-6 (Table 1). Thus the epitope for MAb 8C11 (B1) appears to overlap the epitope for MAb 445 (B2). The epitope for MAb NHN-6 (B3) overlaps B2 and is perhaps sufficiently close to B1 for there to be steric hindrance when both MAbs NHN-6 and 8C11 bind to the HN protein. MAb NHN-1 resistant mutants

Fig. 1. Sensitivity of wild-type HN protein to reducing agent. Purified wild type virions were boiled for 2 min in 1% SDS sample buffer (a) without or (b) with 2.5% 2-mercaptoethanol as reducing agent prior to separation by 10% SDS-PAGE (Laemmli, 1970). The gel was blotted onto nitrocellulose paper, blocked with bovine serum albumin and cut into 0.5 cm wide vertical strips and antigen was detected on blots using peroxidase conjugated antibody (Samson, 1986) after incubation with the following MAbs: lane 1, NHN-1; lane 2, NHN-2; lane 3, NHN-3; lane 4, NHN-6; lane 5, NHN-8; lane 6, NHN-10; lane 7, 445; lane 8, no MAb (control). The blot analyses for MAbs 4D6 and 8C11 have been shown previously (Samson et al., 1988).


a
1
2
3
4
5
6
7
8
HN
b
1
2
3
4
5
6
7
8
HN

only fail to bind to the selecting MAb and therefore form a separate epitope and defines a third antigenic site C.

Sequence analysis of MAb-resistant mutants

Several workers have shown that single amino acid changes in the primary structures of paramyxovirus HN proteins are involved in the loss of recognition by the neutralising MAb used to select resistant mutants (Coelingh et al., 1987; Thompson and Portner, 1987; Gotoh et al., 1988). To locate the epitope for a neutralising MAb within the amino acid sequence of NDV HN, the entire HN genes of the wild type and the MAb resistant mutants were sequenced directly from the genomic RNA using reverse transcriptase. Occasionally, we found difficulty in unequivocally identifying the nucleotides at certain locations due to the presence of 'cross-bands' located at the same positions in the mutant and wild type HN gene sequences. Only one nucleotide change from the wild type sequence was found for each mutant. The nucleotide sequence of the wild type HN gene obtained from our sequencing studies was identical to the sequence published by Millar et al. (1986) except for an A to G mutation changing Thr(502)(ACG) to Ala(GCG). This change is also observed in all the MAb resistant mutants in this study. Thr(502) is part of a

TABLE 4

Predicted amino acid changes in MAb-resistant mutants

MAb used for selection	Mutants	Mutation	Amino acid residue	Amino acid change	Epitope specificity
14 [a]	P6	GAG → AAG	347	Glu → Lys	A1
14	V4	GAG → AAG	347	Glu → Lys	A1
4D6	7	GAC → TAC	349	Asp → Tyr	A1
NHN-8	Z5	GAG → GGG	347	Glu → Gly	A1
NHN-8	Z6	GAC → TAC	349	Asp → Tyr	A1
NHN-10	Z1	GAG → AAG	347	Glu → Lys	A1
NHN-10	Z3	GAG → AAG	347	Glu → Lys	A1
NHN-2	Z13	GCT → GAT	457	Ala → Asp	A2
NHN-2	Z15	CCT → CTT	454	Pro → Leu	A2
NHN-2	Z16	CAG → CAT	456	Gln → His	A2
NHN-3	F1	AGA → GGA	460	Arg → Gly	A3
NHN-3	F3	AGA → GGA	460	Arg → Gly	A3
8C11	U2	AAG → GAG	284	Lys → Glu	B1
8C11	MN2	AAG → AAT	284	Lys → Asn	B1
445	V3	GAC → TAC	287	Asp → Tyr	B2
445	E2	ACA → AAA	290	Thr → Lys	B2
445	Q1	GAG → AAG	293	Glu → Lys	B2
NHN-6	F5	CCC → TCC	325	Pro → Ser	B3
NHN-6	F6	CCC → CTC	325	Pro → Leu	B3
NHN-1	Z17	AAC → GAC	481	Asn → Asp	C1
NHN-1	Z18	AAC → GAC	481	Asn → Asp	C1
NHN-1	Z20	AAC → GAC	481	Asn → Asp	C1

[a] From Chambers et al. (1988).

potential glycosylation site which was considered not to be favourable for glycosylation due the presence of a proline at position 501 (Millar et al., 1986). In addition, this potential glycosylation site is not conserved in any of the other NDV strains which have been sequenced so far, although there is an analogous site in a similar position in SV5 and Sendai virus (Millar et al., 1986).

The nucleotide and deduced amino acid changes in each of the mutants are shown in Table 4. The deduced amino acid substitutions were all nonconservative. All the mutants which are resistant to MAbs 4D6, NHN-8 and NHN-10 (epitope A1) have one of the following changes: Glu(347) to Lys or Gly; or Asp(349) to Tyr. These results agree with our earlier results with MAb 14-resistant mutant (P6) which had a change from Glu(347) to Lys (Chambers et al., 1988). An independent MAb 14 resistant mutant (V4) had the same mutation. All these MAbs recognise the same epitope (A1).

The amino acid changes for epitopes A2 and A3 were located close to each other. The A2 epitope had mutations at Pro(454) to Leu; Gln(456) to His; and Ala(457) to Asp. The A3 epitope had a change at Arg(460) to Gly.

The mutations in the B1 epitope were all at Lys(284) to Glu or Asn. The mutations in the B2 epitope were at Glu(293) to Lys; Thr(290) to Lys; and Asp(287) to Tyr. One mutation in the B3 epitope associated with the NHN-6 resistant mutant F5 had a change from Pro(325) to Ser which could create an additional potential glycosylation site in the HN protein, whilst F6 (another NHN-6 resistant mutant) has a mutation at Pro(325) to Leu.

All the MAb NHN-1 resistant mutants had an amino acid change Asn(481) to Asp. This is similar to the mutation at antigenic site IV reported by Gotoh et al. (1988), which removed a potential glycosylation site.

Discussion

In this study we have located seven epitopes (A1, A2, A3, B1, B2, B3 and C1) in the HN protein of NDV strain Beaudette C by direct sequencing of the genomic RNA of antibody resistant mutants. We previously located a continuous epitope in the HN protein by Western blotting of subgenomic HN fragments expressed in *E. coli* and RNA sequencing (Chambers et al., 1988). That epitope corresponds to the epitope A1 described here. In the recent study by Gotoh et al. (1988) four antigenic binding sites were identified in NDV strain D26 (designated I, II, III and IV), three of which were located by RNA sequencing. The amino acid residue position of sites I and IV from strain D26 correspond to epitopes A1 and C1 in Beaudette C.

The epitope A1 in Beaudette C is continuous as shown by resistance to the reducing agent 2-mercaptoethanol (Samson et al., 1988; Chambers et al., 1988; Fig. 1) but differs in this respect from antigenic site I in the D26 strain (Nishikawa et al., 1986). The amino acid, Cys-123 has been shown to be conserved only in NDV strains in which the HN glycoprotein exists as dimers (Sheehan et al., 1987). Strain D26 possesses Cys-123 (Sato et al., 1987) and the site I epitope is sensitive to reduction, whereas Beaudette C does not possess this cysteine residue (Millar et al.,

1986), and the A1 epitope is resistant to reduction. Perhaps the integrity of the D26 site I epitope requires Cys-123 in disulphide bridge formation but the Beaudette C A1 epitope does not. The frequent isolation of MAbs which recognise the above site(s) suggests that it is the most immunodominant site on HN in Beaudette C.

MAbs to site I of Gotoh et al. (1988) inhibited NA activity in NDV (with neuraminlactose as substrate) whereas none of the MAbs which bind to site A1 in our study showed NI. It is possible that antibodies to site I in strain D26 recognise a conformational epitope which may rely on amino acids involved in neuraminidase function. However, antibodies to site A1 in strain Beaudette C recognise a linear epitope, not involved in neuraminidase function. Only MAbs to epitopes A2 and A3 showed NI. Sequence analysis of antigenic variants of the influenza virus selected with MAbs which inhibited NA activity showed a series of amino acid substitutions within the enzyme active site (Air et al., 1985) and is in agreement with the three-dimensional structure of influenza virus NA (Varghese et al., 1983). Similar observations have been made with parainfluenza type 3 virus (Coelingh et al., 1987) and Sendai virus (Thompson and Portner, 1987). We believe that amino acids 454, 456 and 457 (epitope A2), and 460 (epitope A3) are topologically very close to the NA active site of HN because MAbs to these epitopes inhibit to a varying degree the NA activity of the NH protein. This region, however, is not located at the sialic acid receptor-binding site (amino acids 212–303) proposed by Jorgensen et al. (1987).

The complex antigenic site B consists of three partially overlapping epitopes. An additional potential glycosylation site Asn-Ser-Ser(325) is created in the MAb NHN-6 resistant mutant F5 (epitope B3) at Pro(325) to Ser. The electrophoretic mobility of the HN protein in this mutant is significantly lower than that of any of the other mutants or of the wild type (it changes from an apparent molecular weight of 72 to 75 kDa, data not shown). Preliminary experiments using tunicamycin indicate that the electrophoretic mobilities of F5 and wild type unglycosylated HN proteins are similar. This finding is consistent with an additional glycosylation event having occurred in this mutant. The other MAb NHN-6 resistant mutant, F6, has Pro(325) changed to Leu and neither creates an extra potential glycosylation site nor exhibits any change in HN protein migration. It is not yet possible, however, to say whether addition of an extra carbohydrate chain in the F5 mutant prevents MAb NHN-6 binding to HN. A similar observation was made earlier by Air et al. (1985) using X-7(F1) influenza N2 neuraminidase mutants, in which Asp(329) changed to Asn to form a potential glycosylation site. Although MAb NHN-6 showed complete competition with MAb 445 and partially competed with MAb 8C11 (Table 1), there is distinction in their biological functions within the site. MAbs 8C11 and 445 inhibit hA function and have a high neutralising titer, whereas MAb NHN-6 does not inhibit hA and has a low neutralising titer. MAbs 445 and 14 were raised against NDV strain Ulster 2C (Russell et al., 1983b) and it was shown that MAb 14 bound to cells infected with NDV strains Beaudette C, Ulster, B1 and Texas GB whereas MAb 445 bound to cells infected with strains Beaudette C, Ulster 2C and Texas GB but not B1 (Russell and Alexander, 1983). We propose, on the basis of our studies and the published HN sequences, that this is because residue Glu(293), implicated

in the binding of MAb 445 (epitope B2), is a Gly in this position in the B1 strain (Jorgensen et al., 1987). All the amino acid residues proposed here to be involved in the binding of MAbs 445 and 14 are common to strains B1, Beaudette C, Ulster and Texas GB (Millar et al., 1986, 1988; Jorgensen et al., 1987; Schaper et al., 1988).

In this study only one epitope was found in site C. All of the MAb NHN-1 resistant mutants which map to this site have lost a potential glycosylation site at amino acid positions 481-483 on the HN protein. This mutation, from Asn(481) to Asp, was also observed by Gotoh et al. (1988) for site IV in strain D26. Unlike their observation, the HN protein in our mutants migrated with the same mobility as that of the wild type virus, suggesting that this site may not be glycosylated in Beaudette C.

We are currently testing the binding of MAbs to cells infected with different (sequenced) NDV strains in order to ascertain whether the presence or absence of amino acid residues implicated in the binding of MAbs to Beaudette C is reflected in the binding of MAbs to other NDV strains.

Acknowledgements

We thank Dr. N.S. Millar for preparing some of the oligonucleotides, Dr P.H. Russell for generously providing MAbs 14 and 445, and Dr. G. Meulemans for generously providing MAbs 4D6 and 8C11. This work was supported by the Agriculture and Food Research Council. M. Nesbit was supported by a Science and Engineering Research Council postgraduate training award.

References

Air, G.M., Els, M.C., Brown, L.E., Laver, W.G. and Webster, R.G. (1985) Location of antigenic sites on the three dimensional structure of the influenza N2 virus neuraminidase. Virology 145, 237–248.

Aminoff, D. (1961) Methods for the quantitative estimation of N-acetylneuraminic acid and their application to hydrolysates of sialomucoids. Biochem. J. 81, 384–392.

Both, G.W. and Air, G.M. (1979) Nucleotide sequencing coding for the N-terminal region of the matrix protein of influenza virus. Eur. J. Biochem. 96, 363–372.

Cavanagh, D. and Davis, P.J. (1988) Evolution of avian coronavirus IBV: sequence of the matrix glycoprotein gene and intergenic region of several serotypes. J. Gen. Virol. 69, 621–629.

Chambers, P. and Samson, A.C.R. (1980) A new structural protein for Newcastle disease virus. J. Gen. Virol. 50, 155–166.

Chambers, P., Nesbit, M., Yusoff, K., Millar, N.S., Samson, A.C.R. and Emmerson, P.T. (1988) Location of a neutralizing epitope for the haemagglutinin-neuraminidase glycoprotein of Newcastle disease virus. J. Gen. Virol., 69, 2115–2122.

Clavell, L.A. and Bratt, M.A. (1972) Hemolytic interaction of Newcastle disease virus and chicken erythrocytes. II. Determining factors. Appl. Microbiol. 23, 461–470.

Coelingh, K.L., Winter, C.C., Jorgensen, E.D. and Murphy, B.R. (1987) Antigenic and structural properties of the HN glycoprotein of the human parainfluenza type 3 virus: sequence analysis of variants selected with monoclonal antibodies which inhibit infectivity, hemagglutination, and neuraminidase activities. J. Virol. 61, 1473–1477.

Coelingh, K.L., Winter, C.C., Murphy, B.R., Rice, J.M., Kimball, P.C., Olmsted, R.A. and Collins, P.L. (1986) Conserved epitopes on the hemagglutinin-neuraminidase proteins of human and bovine parainfluenza type 3 viruses: nucleotide sequence analysis of variants selected with monoclonal antibodies. J. Virol. 60, 90–96.

Engvall, E. and Perlmann, P. (1972) Enzyme-linked immunosorbent assay, ELISA III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. J. Immunol. 109, 129–135.

Gotoh, B., Sakaguchi, T., Nishikawa, K., Inocencio, N.M., Hamaguchi, M., Toyoda, T. and Nagai, Y. (1988) Structural features unique to each of the three antigenic sites on the hemagglutinin-neuraminidase protein of Newcastle disease virus. Virology 163, 174–182.

Hsu, M.C., Scheid, A. and Choppin, P.W. (1979) Reconstitution of membranes with individual paramyxovirus glycoproteins and phospholipid in cholate solution. Virology 95, 476–491.

Iorio, R.M. and Bratt, M.A. (1983) Monoclonal antibodies to Newcastle disease virus: delineation of four epitopes on the HN glycoprotein. J. Virol. 48, 440–450.

Iorio, R.M. and Bratt, M.A. (1984) Monoclonal antibodies as functional probes on the HN glycoprotein of Newcastle disease virus: antigenic separation of the hemagglutinating and neuraminidase sites. J. Immunol. 133, 2215–2219.

Iorio, R.M., Lawton, K.A., Nicholson, P.M. and Bratt, M.A. (1984) Monoclonal antibodies identify a strain-specific epitope on the HN glycoprotein of Newcastle disease virus strain Australia-Victoria. Virus Research 1, 513–525.

Iorio, R.M., Borgman, J.B., Glickman, R.L. and Bratt, M. (1986) Genetic variation within a neutralizing domain on the haemagglutinin neuraminidase glycoprotein of Newcastle disease virus. J. Gen. Virol. 67, 1391–1403.

Jorgensen, E.D., Collins, P.L. and Lomedico, P.T. (1987) Cloning and neucleotide sequence of Newcastle disease virus hemagglutinin-neuraminidase mRNA: identification of a putative sialic acid binding site. Virology 156, 12–24.

Kohama, T., Garten, W. and Klenk, H.-D. (1981) Changes in conformation and charge paralleling proteolytic activation of Newcastle disease virus glycoproteins. Virology 111, 364–376.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227, 680–685.

Le Long, Portetelle, D., Ghysdael, J., Gonze, M., Burny, A. and Meulemans G. (1986) Monoclonal antibodies to hemagglutinin-neuraminidase and fusion glycoproteins of Newcastle disease virus: relationship between glycosylation and reactivity. J. Virol. 57, 1198–1202.

Meulemans, G., Gonze, M., Carlier, M.C., Petit, P., Burny, A. and Le Long. (1987) Evaluation of the use of monoclonal antibodies to hemagglutinin and fusion glycoproteins of Newcastle disease virus for virus identification and strain differentiation purposes. Arch. Virol. 92, 55–62.

Millar, N.S., Chambers, P. and Emmerson, P.T. (1986) Nucleotide sequence analysis of the haemagglutinin-neuraminidase gene of Newcastle disease virus. J. Gen. Virol. 67, 1917–1927.

Millar, N.S., Chambers, P. and Emmerson, P.T. (1988) Nucleotide sequence of the fusion and haemagglutinin-neuraminidase glycoprotein genes of Newcastle disease virus, strain Ulster: molecular basis for variations in pathogenicity between strains. J. Gen. Virol. 69, 613–620.

Nishikawa, K., Isomura, S., Suzuki, S., Watanabe, E., Hamaguchi, M., Yoshida, T. and Nagai, Y. (1983) Monoclonal antibodies to the HN glycoprotein of Newcastle disease virus. Biological characterization and use for strain comparisons. Virology, 130, 318–330.

Nishikawa, K., Morishima, T., Toyoda, T., Miyadai, T., Yokochi, T., Yoshida, T. and Nagai, Y. (1986) Topological and operational delineation of antigenic sites on the HN glycoprotein of Newcastle disease virus and their structural requirements. J. Virol. 60, 987–993.

Ozawa, M., Asano, A. and Okada, Y. (1979) Biological activities of glycoproteins of HJV (Sendai virus) studied by reconstitution of hybrid envelope and by concanavalin A-mediated binding: a new function of HANA proteins and structural requirements for F protein in hemolysis. Virology 99, 197–202.

O'Sullivan, M.J. and Marks, V. (1981) Methods for the preparation of enzyme-antibody conjugates for use in enzyme immunoassay. In: J.J. Langone and H.V. Vunakis (Eds.), Methods in Enzymology, Vol. 73, pp. 147–166. Academic Press, Inc., London.

Portner, A., Scroggs, R.A. and Metzger, D.W. (1987) Distinct functions of antigenic sites of the HN glycoprotein of Sendai virus. Virology 158, 61–68.

Russell, P.H. and Alexander, D.J. (1983) Antigenic variation of Newcastle disease virus strains detected by monoclonal antibodies. Arch. Virol. 75, 243–253.

Russell, P.H., Griffiths, P.C. and Cannon, M.J. (1983a) A microwell immunoperoxidase test for screening hybridomas and for diagnosing Newcastle disease virus and Sendai virus. J. Immunol. Methods 61, 165–170.

Russell, P.H., Griffiths, P.C., Goswami, K.K.A., Alexander, D.J., Cannon, M.J. and Russell, W.C. (1983b) The characterization of monoclonal antibodies to Newcastle disease virus. J. Gen. Virol. 64, 2069–2072.

Sato, H., Hattori, S., Ishida, N., Imamura, Y. and Kawakita, M. (1987) Nucleotide sequence of the hemagglutinin-neuraminidase gene of Newcastle disease virus avirulent strain D26: evidence for a longer coding region with a carboxyl terminal extension as compared to virulent strains. Virus Res. 8, 217–232.

Samson, A.C.R. (1986) Anomalous behaviour of Newcastle disease virus haemagglutinin-neuraminidase protein in Western blotting analysis of monoclonal antibody binding sites. J. Gen. Virol. 67, 1199–1203.

Samson, A.C.R., Nesbit, M., Lyon, A.M. and Meulemans, G. (1988) Identification of haemagglutinin-neuraminidase antibody binding sites by western blot analysis of antibody-resistant mutants and partial digest fragments of Newcastle disease virus. J. Gen. Virol. 69, 473–480.

Schaper, U.M., Fuller, F.J., Ward, M.D.W., Mehrotra, Y., Stone, H.O., Stripp, B.R. and De Buysscher, E.V. (1988) Nucleotide sequence of the envelope protein genes of a highly virulent, neurotropic strain of Newcastle disease virus. Virology 165, 291–295.

Scheid, A. and Choppin, P.W. (1973) Isolation and purification of envelope proteins of Newcastle disease virus. J. Virol. 11, 263–271.

Scheid, A. and Choppin, P.W. (1974) Identification of biological activities of paramyxovirus glycoproteins. Activation of cell fusion, hemolysis, and infectivity by proteolytic cleavage of an inactive precursor protein of Sendai virus. Virology 57, 475–490.

Sheehan, J.P., Iorio, R.M., Syddall, R.J., Glickman, R.L. and Bratt, M.A. (1987) Reducing agent-sensitive dimerization of the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus correlates with the presence of cysteine at residue 123. Virology 161, 603–606.

Thompson, S.D. and Portner, A. (1987) Localization of functional sites on the hemagglutinin-neuraminidase glycoprotein of Sendai virus by sequence analysis of antigenic and temperature-sensitive mutants. Virology 160, 1–8.

Umino, Y., Kohama, T., Kohase, M., Sugaira, A., Klenk, H.-D. and Rott, R. (1984) Biological functions of monospecific antibodies to envelope glycoproteins of Newcastle disease virus. Arch. Virol. 81, 53–65.

Varghese, J.N., Laver, W.G. and Colman, P.M. (1983) Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 A resolution. Nature (London) 303, 35–40.

(Received 28 July 1988; revision received 26 August 1988)