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## Identification of Haemagglutinin–Neuraminidase Antibody Binding Sites by Western Blot Analysis of Antibody-resistant Mutants and Partial Digest Fragments of Newcastle Disease Virus

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### SUMMARY

A collection of monoclonal antibodies (MAbs) which react with the haemagglutinin–neuraminidase (HN) protein of Newcastle disease virus (NDV) has been used to isolate MAb-resistant mutants of the Beaudette C strain of NDV. The patterns of cross-reactivity of the HN proteins of these mutants against the collection of MAbs determined by Western blotting allowed the MAbs to be sorted into different groups. Protease V8 partial digest fragments of purified wild-type virions and subsequent reaction against the collection of MAbs using Western blotting provided an alternative method of grouping MAbs which broadly agreed with the former method. Chemical cleavage of the HN protein at aspartate–proline bonds followed by Western blotting of the fragments allowed the approximate position of certain MAb binding sites to be determined.

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The paramyxovirus Newcastle disease virus (NDV) contains two glycoproteins, one required for virus–cell fusion [the fusion (F) glycoprotein], the other for virus–cell adsorption [the haemagglutinin–neuraminidase (HN) glycoprotein] (Choppin & Scheid, 1980). Monoclonal antibodies (MAbs) against either glycoprotein can neutralize the virus and be used to select MAb-resistant mutants (Russell *et al.*, 1983; Iorio & Bratt, 1985; Samson *et al.*, 1985; Meulemans *et al.*, 1986).

Monoclonal antibodies can be used to identify separate antigenic sites within a protein and epitopes within a given site using a variety of techniques such as competition antibody binding and cross-reaction of antibody-resistant mutants and virus strains (Russell *et al.*, 1983; Russell & Alexander, 1983; Iorio *et al.*, 1986; Iorio & Bratt, 1984; Nishikawa *et al.*, 1983, 1986). The positions of epitopes with a given protein sequence can be determined from genome sequence analysis of antibody-resistant mutants, a technique pioneered with influenza virus (Wiley *et al.*, 1981) and now being used with some paramyxoviruses (Portner *et al.*, 1987; Coelingh *et al.*, 1986).

A total of nine MAbs was used in this study each of which react with the HN protein of NDV. MAbs 8C11, 4D6, AC3, JB6, 2B6 and 8E8 have been described elsewhere (Le Long *et al.*, 1986). MAbs 14 and 445 were kindly provided by Dr P. H. Russell (Royal Veterinary College, London, U.K.) and have also been described elsewhere (Russell *et al.*, 1983). The isolation and characterization of MAb C2E9 together with other MAbs isolated in Newcastle will be the subject of a separate communication. (MAb C2E9 is the only one which does not neutralize the Beaudette C strain of NDV.)

The results in Fig. 1(a) show that each of the nine MAbs bound to the HN protein of the Beaudette C strain (wild-type) of NDV even when virions had been boiled for 2 min in the presence of 1% SDS. However, when virions were boiled for 2 min in the presence of

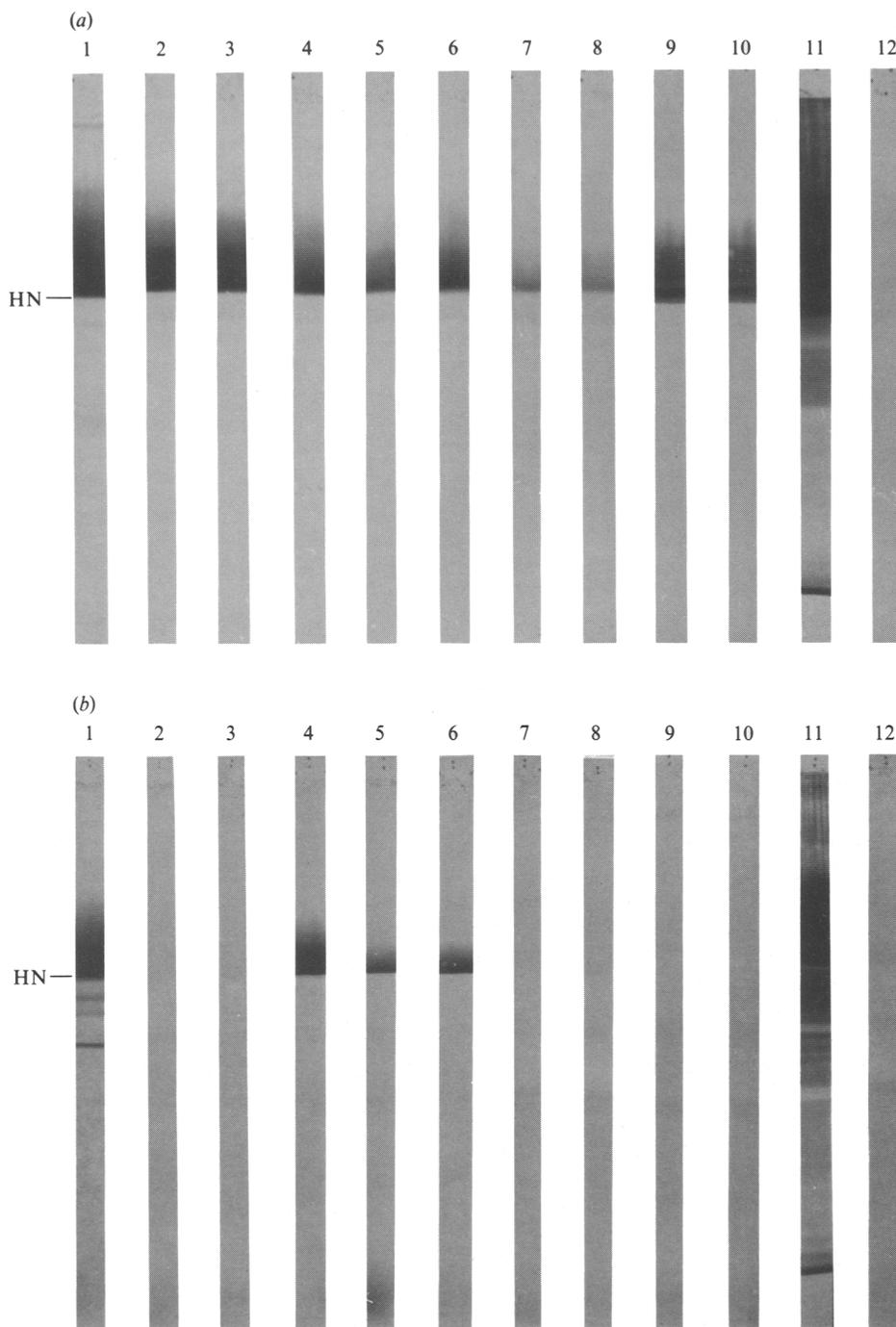


Fig. 1. Sensitivity of wild-type HN protein to reducing agent. Purified wild-type virions were boiled for 2 min in 1% SDS sample buffer without (a) or with (b) 2.5% 2-mercaptoethanol as reducing agent prior to separation by 10% SDS-PAGE (Laemmli, 1970). The gel was blotted on to nitrocellulose paper, 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, 14; lane 2, 445; lane 3, 8C11; lane 4, AC3; lane 5, JB6; lane 6, 4D6; lanes 7 and 9, 2B6; lanes 8 and 10, 8E8; lane 11, C2E9; lane 12, no MAb (control). Lanes 9 and 10 were from a gel loaded with five times the concentration of virus used for the other lanes.

Table 1. Cross-reactivity Western blot tests between various NDV mutants and the nine MAbs

Source of NDV antigen	Western blot response to MAb								
	14	JB6	2B6*	4D6	AC3	8E8*	C2E9	445	8C11
No virus	-†	-	-	-	-	-	-	-	-
Wild-type	+	+	+/-	+	+	+/-	+	+	+
8C11-R1‡	+	+/-	+/-	+	+/-	-	+/-	+	-
8C11-R2	+	+	+/-	+	+	+/-	+	+	-
8C11-R3	+	+	+/-	+	+	+/-	+	+	-
8C11-R4	+	+	+/-	+	+	+/-	+	+	-
8C11-R5	+	+	+/-	+	+	+/-	+	+	-
4D6-R1	-	-	-	-	-	-	+	+	+
4D6-R2	-	-	-	-	-	-	+	+	+
4D6-R3	-	-	-	-	-	-	+	+	+
AC3-R1	-	-	-	-	-	-	+	+	+
AC3-R2	-	-	-	-	-	-	+	+	+
AC3-R3	-	-	-	-	-	-	+	+	+
AC3-R4	-	-	-	-	-	-	+	+	+
AC3-R5	-	-	-	-	-	-	+	+	+
JB6-R1	-	-	-	-	-	-	+	+	+
JB6-R2	-	-	-	-	-	-	+	+	+
JB6-R3	-	-	-	-	-	-	+	+	+
JB6-R4	-	-	-	-	-	-	+	+	+
JB6-R5	-	-	-	-	-	-	+	+	+

\* MAbs 2B6 and 8E8 gave considerably weaker reactions than other MAbs (see Fig. 1a).

† +, Positive reaction; -, no reaction; +/-, weak positive reaction.

‡ Mutants are denoted by the MAb to which they are resistant.

2-mercaptoethanol as reducing agent with 1% SDS then only MAbs 14, AC3, JB6, 4D6 and C2E9 continued to detect any HN antigen (Fig. 1b). These data are consistent with the finding of Le Long *et al.* (1986) for Western blotting against the Italian strain of NDV using MAbs AC3, JB6 and 4D6 and that of Samson (1986) for MAb 14 against Beaudette C. MAb C2E9 although reacting strongly against the HN protein also reacted non-specifically with other proteins present in purified ND virions. MAbs that continue to react with reduced HN protein are likely to recognize linear rather than conformational epitopes within HN.

Neutralizing MAbs 8C11, 4D6, AC3 and JB6 were initially used to select antibody-resistant mutants of the Beaudette C strain of NDV. The procedure involved plating dilutions of plaque-purified wild-type virus and overlaying with HEPES-buffered Medium 199 containing 5% calf serum and 1% agar to which was added unheated ascites fluid at 1/500 or 1/1000 dilution as previously described (Samson *et al.*, 1985). Wild-type plaque-purified antibody-resistant mutants were grown *in ovo*; chorioallantoic fluid was collected, clarified by centrifugation at 3000 g for 5 min and prepared for SDS-PAGE and Western blotting as previously described (Samson, 1986). No further purification was necessary for cross-reactivity blotting tests (Table 1), however wild-type virions were purified on sucrose/potassium tartrate density gradients (Chambers & Samson, 1980) prior to testing the sensitivity of HN protein to 2-mercaptoethanol as reducing agent (Fig. 1), for the preparation of protease digest fragments of virus proteins (Fig. 2) and for the chemical cleavage of virus proteins (Fig. 4).

Spontaneous mutants resistant to 8C11, 4D6, AC3 and JB6 were isolated and clarified chorioallantoic fluid from infected eggs was used directly for SDS-PAGE and Western blotting to determine the cross-reactivity pattern of these mutants to these and other MAbs rapidly. This procedure is quicker and easier to perform than cross-neutralization tests though the latter have been conducted using these mutants and fully corroborate the blotting studies (not shown).

Table 1 shows the results of the cross-reactivity blot test between the various mutants and the nine MAbs. As expected, all MAb-resistant mutants failed to bind the respective selecting antibody. Furthermore all 4D6-, AC3- and JB6-resistant mutants also failed to bind any of the following MAbs: 14, JB6, 2B6, 4D6, AC3 or 8E8. The 8C11-resistant mutants failed to bind only

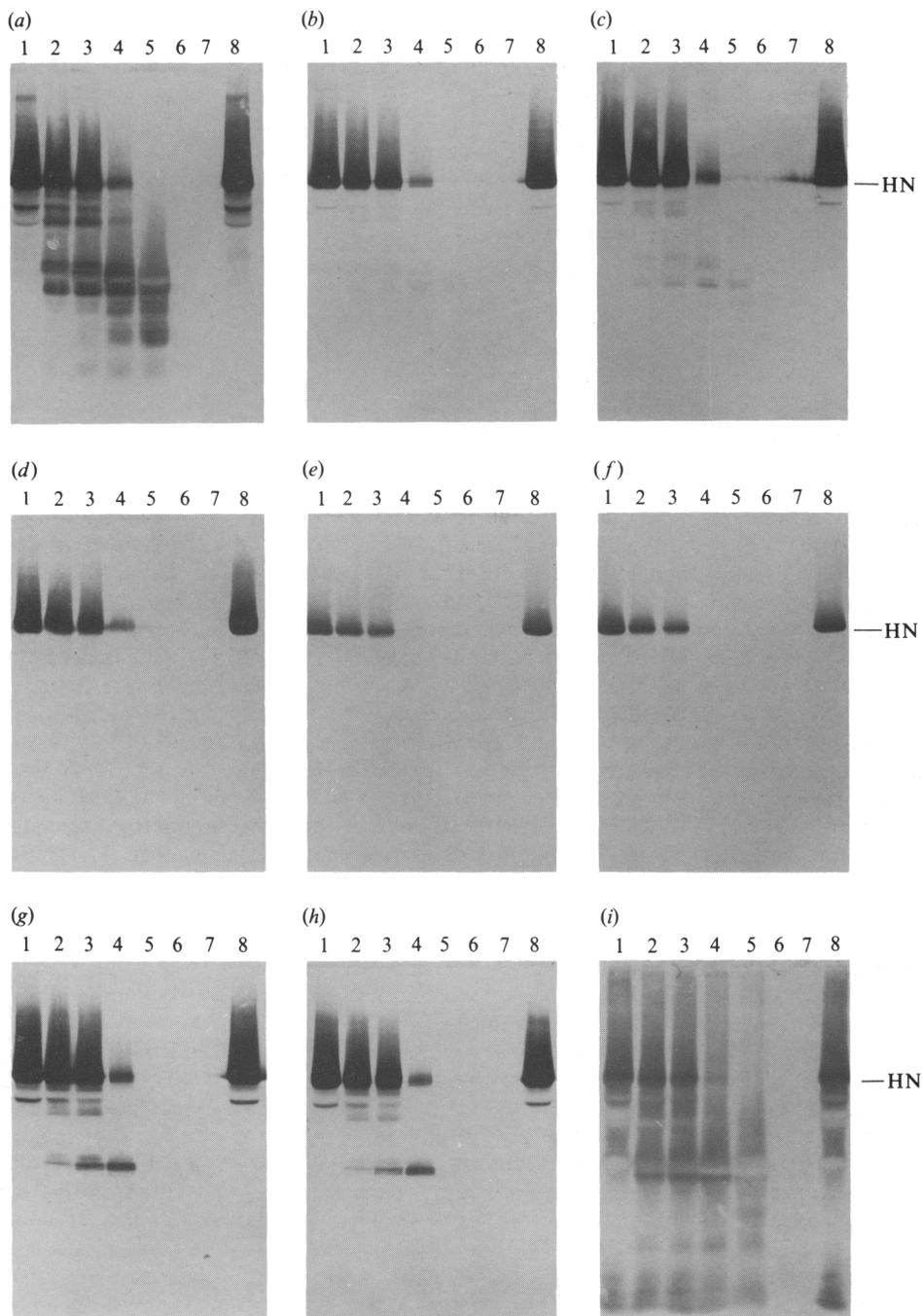


Fig. 2. Protease partial digest blot test. Purified wild-type virions were boiled for 2 min in 0.1% SDS sample buffer (Laemmli, 1970) without reducing agent and dispensed into five tubes containing various amounts of *S. aureus* V8 protease, a sixth tube contained V8 protease but no virus. The tubes were incubated at 37°C for 30 min and frozen at -20°C. Samples were boiled again for 2 min prior to loading onto 10% SDS-polyacrylamide gels and detection of antigen binding as described for Fig. 1. Lanes 1 and 8, virus, no V8; lane 2, virus plus 5 µg V8; lane 3, virus plus 15 µg V8; lane 4, virus plus 50 µg V8; lane 5, virus plus 150 µg V8; lane 6, 150 µg V8, no virus; lane 7, sample buffer. (a) MAb 14, (b) MAb AC3, (c) MAb 4D6, (d) MAb 4B6, (e) MAb 2B6, (f) MAb 8E8, (g) MAb 8C11, (h) MAb 445 and (i) MAb C2E9.

MAB 8C11. On the basis of this test alone we can provisionally group together 14, JB6, 2B6, 4D6, AC3 and 8E8 which are definitely distinct from MAB 8C11. At this stage 445 and C2E9 may be placed in another group(s).

The grouping of the nine MABs was then examined using an independent criterion, the rationale for which is as follows. If two MABs react with a common linear sequence (epitope), or indeed overlapping or closely linked sequences within a polypeptide, then if the polypeptide is digested with a variety of proteases to give a ladder of partial digests which are then Western blotted against MABs, identical and often overlapping or closely linked epitopes should reveal similar peptide blotting fingerprints. Epitopes which are separated by protease cleavage should reveal different fingerprints.

Fig. 2 shows the result of such an analysis using the SDS-tolerant protease V8 from *Staphylococcus aureus*. We have also successfully used diphenylcarbonyl chloride-treated trypsin and chymotrypsin to generate different digest patterns (not shown). The blotted gels are purposely overloaded to allow detection of minor cleavage bands and this accounts for the very broad undigested HN bands in certain wells. Two of the MABs, 2B6 and 8E8, which bind comparatively weakly to HN protein detect no cleavage products of the HN protein; the binding sites recognized by these MABs are also sensitive to reducing agent (Fig. 1). These data suggest that the epitope(s) recognized by 2B6 and 8E8 is (are) complex and not merely sensitive to destruction of cysteine-cysteine disulphide bridges but by any disruption of the integrity of the HN protein, such as that brought about by protease treatment. In contrast MABs 8C11 and 445, which also only bind to unreduced HN protein, both react with partial digest products of HN and show the same partial digest fragment blotting patterns. This implies that the epitope(s) recognized by 8C11 and 445 are made up of linked sequences probably held in juxtaposition by disulphide bridge(s). Apart from C2E9 which showed a very ill-defined complex pattern, the remaining MABs (14, JB6, 4D6 and AC3) appeared to be related by their pattern of digest reactions. MAB 14 was extremely avid and showed a very clear and distinct pattern, JB6, 4D6 and AC3 were less avid and the digest fragments did not show up as well; however, examination of the original blots revealed several common partial digest bands among this group which are distinct from the other (445/8C11 and C2E9) patterns. The above digest patterns taken together with the cross-reactivity data (Fig. 2) and sensitivity to reducing agent (Fig. 1) suggest the following groupings: group 1a, MABs 14, 4D6, AC3 and JB6; group 1b, MABs 2B6, 8E8 (sensitive to reducing agent); group 2a, MAB 445 (sensitive to reducing agent); group 2b, MAB 8C11 (sensitive to reducing agent); group 3, MAB C2E9. Russell *et al.* (1983) and Russell & Alexander (1983) earlier made a similar distinction between MAB 14 (their group HN-1) and MAB 445 (their group HN-2) on the basis of competition binding, haemagglutination inhibition and strain variation. This distinction is fully supported by our findings.

In an attempt to determine the location of the group 1a epitope within the HN polypeptides (likely to be a linear epitope because of its resistance to boiling in SDS under reducing conditions), we decided to use a variety of chemical cleavage procedures which should cleave the HN protein at known positions (the HN gene sequence for this strain being known; Millar *et al.*, 1986). The formic acid cleavage at aspartate-proline linkages (Piszkiwicz *et al.*, 1970) proved the most successful. The published HN sequence predicts that there should be three aspartate-proline sites at proline amino acid positions 130, 386 and 473 within the 577 residue protein for the Beaudette C strain of NDV (see Fig. 3).

Within the four complete digest fragments (1 to 4, Fig. 3) predicted from the gene sequence there are respectively zero, seven, three and four cysteine residues which could be involved in disulphide linkages. Fragments 1, 2 and 3 each have one and fragment 4 has two potential (and conserved) glycosylation sites (Millar *et al.*, 1986; Jorgensen *et al.*, 1987 and N. S. Millar, personal communication). Taking into consideration the predicted sizes of these four fragments and partial digest fragments, together with the Western blot pattern obtained following a two-dimensional non-reduced/reduced SDS-PAGE separation of formic acid-cleaved purified virions, the following spot assignments could be made (compare Fig. 3 and 4).

Fragments which have mobilities *a*, *b*, *c* and *d* in the non-reduced first dimension are made up of linear subsets of fragments 1 to 4 which are partially cleaved, or cleaved and held together by

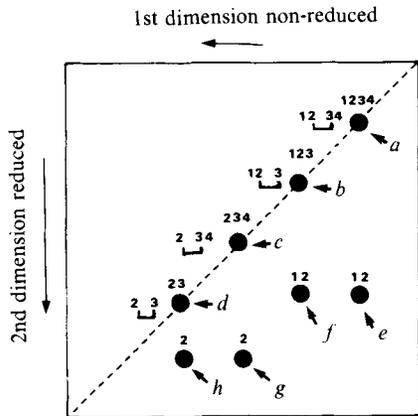
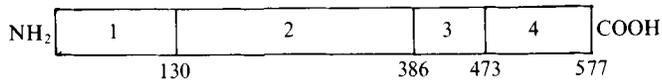


Fig. 3

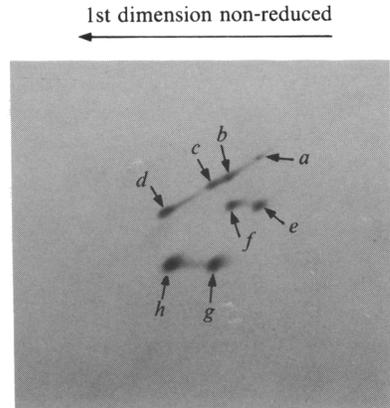


Fig. 4

Fig. 3. Origin of aspartate-proline cleavage products of HN protein. Complete digest fragments are numbered 1 to 4 within the HN molecule. The proline residues are numbered at the three *Asp-Pro* locations. The non-reduced/reduced gel blot sketch indicates the positions of the eight spots that react with MAb 4D6 (see Fig. 4) together with their derivation from the partially cleaved HN molecule. Fragments 2 and 3 are linked by disulphide(s) indicated by  $\curvearrowright$ . These fragments dissociate in the reduced second dimension and take up positions *e*, *f*, *g* and *h* beneath spots *a*, *b*, *c* and *d* respectively.

Fig. 4. Two-dimensional separation of aspartate-proline cleavage products of HN protein. Purified wild-type virions were suspended in 70% (v/v) formic acid and incubated at 40 °C for 24 h (Piszkiwicz *et al.*, 1970). The suspension was dialysed overnight against Tris-saline buffer (25 mM-Tris-HCl, 137 mM-NaCl, 0.7 mM-Na<sub>2</sub>HPO<sub>4</sub> pH 7.2) and boiled for 2 min in 1% SDS sample buffer without reducing agent prior to separation by 10% SDS-PAGE (Laemmli, 1970). A vertical 0.5 cm wide strip of this first dimension gel was cut out and equilibrated for 30 min in sample buffer containing 2.5% 2-mercaptoethanol prior to the second dimension separation also by 10% SDS-PAGE. The gel was blotted onto nitrocellulose paper and antigen detected as in Fig. 1 using MAb 4D6.

disulphide bridge(s) between fragments 2 and 3. In the second dimension the disulphides are reduced and cleaved fragments now fall below the diagonal. It must be remembered that in this Western blot analysis only polypeptides that contain the epitope-bearing fragment will be detected by the MAb. For Fig. 4, MAb.4D6 was used and the same spot pattern was obtained with the remaining MAbs in group 1a, namely 14, AC3 and JB6 (not shown).

The only common region for all spots that bound the group 1a MAbs was region 2 (Fig. 3), i.e. amino acid residues 130 to 385, which we predict contains this linear epitope. The data also predict that region 2 is disulphide-linked to region 3 (residues 386 to 473).

The cross-reactivity blot tests alone using antibody-resistant mutants (Table 1) suggested that MAbs 14, JB6, 2B6, 4D6, AC3 and 8E8 recognized a common epitope or portion of an epitope. Further support for this has been obtained from a set of ten independent MAb 14-resistant mutants which all failed to bind MAbs 14, 2B6 and AC3 but continued to bind MAb 445 (other MAbs were not tested). The common region recognized by these MAbs was thought to be linear in the case of 14, JB6, 4D6 and AC3 but to involve another region(s) of the HN polypeptide for the binding of MAbs 2B6 and 8E8 which were both sensitive to reducing agent and to limited protease cleavage.

MAbs 8C11 and 445 on the other hand are in different groups to the above MAbs and on the basis of the cross-reactivity blot tests alone appear to be in two different groups. Their identical protease digest data however suggest that their epitopes are contained on a common polypeptide

(see Fig. 2). Although so far none of our 8C11-resistant mutants failed to bind MAb 445, recently we found that approximately 50% of our 445-resistant mutants failed to bind both MAb 445 and 8C11. Further support for grouping MAb 8C11 and 445 together comes from ELISAs which showed that individual MABs from group 1a or 1b showed additive binding to HN antigen when mixed with individual MABs from group 2a or 2b but that 445 (2a) was not additive against MAb 8C11 (2b) (not shown).

The non-reduced/reduced two-dimensional SDS-PAGE blot provided information concerning the location of the 1a linear epitope recognized by MABs 14, JB6, 4D6 and AC3. The analysis predicted that the epitope was between residues 130 and 385. Recent cloning/expression work using synthetic oligonucleotides has now localized this epitope to within eight amino acid residues in the above region (P. Chambers *et al.*, unpublished data). Furthermore, preliminary RNA sequencing of a MAB 14-resistant mutant has shown a significant codon change within this eight amino acid region (K. Yusoff *et al.*, unpublished data). Current work in this laboratory is aimed at pinpointing nucleotide base changes in a variety of antibody-resistant mutants in order to delineate neutralizing epitopes within NDV surface glycoproteins.

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#### REFERENCES

- CHAMBERS, P. & SAMSON, A. C. R. (1980). A new structural protein for Newcastle disease virus. *Journal of General Virology* **50**, 155-166.
- CHOPPIN, P. W. & SCHEID, A. (1980). The role of viral glycoproteins in adsorption, penetration and pathogenicity of viruses. *Reviews of Infectious Diseases* **2**, 40-61.
- COELINGH, K. J., WINTER, C. C., MURPHY, B. R., RICE, J. M., KIMBALL, P. C., OLMSTED, R. A. & 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. *Journal of Virology* **60**, 90-96.
- IORIO, R. M. & BRATT, M. A. (1984). Monoclonal antibodies as functional probes of the HN glycoprotein of Newcastle disease virus: antigenic separation of the hemagglutinating and neuraminidase sites. *Journal of Immunology* **133**, 2215-2219.
- IORIO, R. H. & BRATT, M. A. (1985). Selection of unique antigenic variants of Newcastle disease virus with neutralizing monoclonal antibodies and anti-immunoglobulin. *Proceedings of the National Academy of Sciences, U.S.A.* **82**, 7106-7110.
- IORIO, R. M., BORGMAN, J. B., GLICKMAN, R. L. & BRATT, M. A. (1986). Genetic variation within a neutralizing domain on the haemagglutinin-neuraminidase glycoprotein of Newcastle disease virus. *Journal of General Virology* **67**, 1393-1403.
- JORGENSEN, E. D., COLLINS, P. L. & LOMEDICO, P. T. (1987). Cloning and nucleotide sequence of Newcastle disease virus haemagglutinin-neuraminidase mRNA: identification of a putative sialic acid binding site. *Virology* **156**, 12-24.
- 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. & MEULEMANS, G. (1986). Monoclonal antibodies to hemagglutinin-neuraminidase and fusion glycoproteins of Newcastle disease virus: relationship between glycosylation and reactivity. *Journal of Virology* **57**, 1198-1202.
- MEULEMANS, G., GONZE, M., CARLIER, M. C., PETIT, P., BURNY, A. & LE LONG (1986). Protective effects of HN and F glycoprotein-specific monoclonal antibodies on experimental Newcastle disease. *Avian Pathology* **15**, 761-768.
- MILLAR, N. S., CHAMBERS, P. & EMMERSON, P. T. (1986). Nucleotide sequence analysis of the haemagglutinin-neuraminidase gene of Newcastle disease virus. *Journal of General Virology* **67**, 1917-1927.
- NISHIKAWA, K., ISOMURA, S., SUZUKI, S., WATANABE, E., HAMAGUCHI, M., YOSHIDA, T. & 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. & NAGAI, Y. (1986). Topological and operational delineation of antigenic sites on the HN glycoprotein of Newcastle disease virus and their structural requirements. *Journal of Virology* **60**, 987-993.
- PISZKIEWICZ, D., LANDON, M. & SMITH, E. L. (1970). Anomalous cleavage of aspartyl-proline peptide bonds during amino acid sequence determinations. *Biochemical and Biophysical Research Communications* **40**, 1173-1178.
- PORTNER, A., SCROGGS, R. A. & NAEVE, C. W. (1987). The fusion glycoprotein of Sendai virus: sequence analysis of an epitope involved in fusion and virus neutralization. *Virology* **157**, 556-559.

- RUSSELL, P. H. & ALEXANDER, D. J. (1983). Antigenic variation of Newcastle disease virus strains detected by monoclonal antibodies. *Archives of Virology* **75**, 243-252.
- RUSSELL, P. H., GRIFFITHS, P. C., GOSWAMI, K. K. A., ALEXANDER, D. J., CANNON, M. J. & RUSSELL, W. C. (1983). The characterization of monoclonal antibodies to Newcastle disease virus. *Journal of General Virology* **64**, 2069-2072.
- SAMSON, A. C. R. (1986). Anomalous behaviour of Newcastle disease virus haemagglutinin-neuraminidase protein in Western blotting analysis of monoclonal antibody binding sites. *Journal of General Virology* **67**, 1199-1203.
- SAMSON, A. C. R., RUSSELL, P. H. & HALLAM, S. E. (1985). Isolation and characterization of monoclonal antibody-resistant mutants of Newcastle disease virus. *Journal of General Virology* **66**, 357-361.
- WILEY, D. C., WILSON, I. A. & SKEHEL, J. J. (1981). Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. *Nature, London* **289**, 373-378.

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