Inhibition of fusion by neutralizing monoclonal antibodies to the haemagglutinin–neuraminidase glycoprotein of Newcastle disease virus

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The majority of neutralizing monoclonal antibodies (MAbs) to the haemagglutinin–neuraminidase (HN) glycoprotein of Newcastle disease virus prevent attachment of the virus to cellular receptors and inhibits virion-induced fusion from without (FFWO) and fusion from within (FFWI) mediated by the virus glycoprotein-laden infected cell surface. For these antibodies, the inhibition of fusion is presumed to be the result of the prevention of HN-mediated bridging of potential fusion partners. MAbs against antigenic sites 3 and 4 neutralize virus infectivity, but by a mechanism other than the prevention of attachment, the exact nature of which remains to be established. Antibodies to both of these sites effectively inhibit virion-induced FFWO, even when the inducing virus is not infectious. This is consistent with the mechanism of neutralization of these MAbs involving the inhibition of an early, post-attachment step in infection. MAbs to site 3 also inhibit FFWI, but those to site 4 do not, even when added at high concentrations. This suggests that the requirement for HN may be different in the two modes of fusion. The epitopes recognized by MAbs to sites 3 and 4 have been delineated by the identification of individual nucleotide substitutions in the HN genes of neutralization escape variants. Some of the deduced amino acid substitutions result in additional N-linked glycosylation sites in HN, which are utilized and presumably account for the escape from neutralization.

Introduction

The haemagglutinin–neuraminidase (HN) glycoprotein of paramyxoviruses serves two related but antagonistic functions: attachment to sialic acid-containing receptors, for which haemagglutination (HA) of chicken erythrocytes serves as a model, and the cleavage of sialic acid catalysed by the neuraminidase (NA) activity (Scheid & Choppin, 1973). The HN of Newcastle disease virus (NDV) is a 74K glycoprotein which is inserted into the virion envelope near its N terminus (Schuy et al., 1984) and has six potential N-linked glycosylation sites, five of which are clustered in the C-terminal half of the molecule (Sakaguchi et al., 1989).

Neutralizing monoclonal antibodies (MAbs) to the Australia-Victoria (AV) (1932) isolate of the virus define seven overlapping antigenic sites on HN (Iorio & Bratt, 1983; Iorio et al., 1986, 1989a). MAbs to the seven sites can be divided into three classes on the basis of functional inhibition studies: those which inhibit both HA and NA (MAbs to sites 12, 2 and 23), those which inhibit only HA (MAbs to sites 14 and 1) and those which inhibit neither function (MAbs to sites 3 and 4). However, MAbs belonging to each of the three classes inhibit virus-induced haemolysis of chicken erythrocytes (Iorio & Bratt, 1984).

All antibodies capable of inhibiting HA neutralize predominantly by preventing virion attachment to cellular receptors (Iorio et al., 1989a). The epitopes recognized by these MAbs map to three widely separated domains in the molecule as deduced from sequencing studies of the HN gene of antibody-selected variants (Iorio et al., 1991). However, the exact mechanism of neutralization of infectivity, as well as the location of the epitopes recognized by antibodies to sites 3 and 4, remain to be determined.

Another characteristic of NDV, as well as other members of the Paramyxoviridae, is its ability to induce cell–cell fusion, mediated by the second virion surface glycoprotein, the fusion (F) protein. The possible role of HN in this process has not been elucidated. Bratt & Gallaher (1969, 1972) defined two sets of conditions under which NDV may induce fusion. One is early fusion, mediated by high levels of input virus, and is called fusion from without (FFWO). Fusion that occurs later in infection, mediated by newly synthesized viral glycoproteins deposited on the infected cell surface, is called fusion from within (FFWI). Unlike FFWI,
FFWO does not require the inducing virus to be infectious. The ability of members of the NDV serotype to induce the two types of fusion is isolate-specific. The AV isolate is a comparatively good inducer of FFWI, but not of FFWO.

In this study, using heterologous isolates that are good inducers of FFWO, MAbs to all seven sites have been shown to inhibit this process, even when the inducing virus has been rendered non-infectious by u.v. irradiation. Given that MAbs to sites 3 and 4 do not prevent attachment, it is likely that the site of their neutralizing activity is a post-attachment step early in the interaction of the virion with the host cell. However, antibodies to the two sites have different effects on FFWI. MAbs to site 3 inhibit this process, but those to site 4 do not. The implications of this finding for the role of HN in the two modes of fusion are discussed.

We have also mapped the epitopes recognized by MAbs to sites 3 and 4 through the identification of individual amino acid substitutions in the HN glycoproteins of several neutralization escape variants. Some of the substitutions result in the addition of N-linked glycosylation sites to HN which are utilized and presumably responsible for the escape from neutralizing antibody.

**Methods**

**Virus and cells.** The AV, B (B1-Hitchner, 1947), E (England F, 1949), F (NJ-Roakin, 1946), HP (Israel-HP, 1953), IS (Iowa-Saltsbury, 1949), RO (California-RO, 1944) and W (Wisconsin-Appleton, 1950) isolates of NDV, as well as variants derived from the AV isolate, were grown in the allantoic sac of 10-day-old embryonated hen eggs at 37 °C from a stock of virus one egg passage after cloning (Bratt & Gallaher, 1969). After the death of the majority of the embryos, allantoic fluid was harvested and virus was purified as described previously (Clavell & Bratt, 1972; Weiss & Bratt, 1974).

Primary and secondary chicken embryo cells were prepared and maintained as described by Bratt & Gallaher (1969) and Weiss & Bratt (1974).

**Hybridomas and MAbs.** The preparation of hybridomas and initial characterization of MAbs have been described previously (Iorio & Bratt, 1983; Iorio et al., 1986, 1989a).

**Assays of FFWO and FFWI inhibition.** Chicken embryo fibroblast monolayers at 60% to 70% confluence in 35 mm tissue culture plates were used for fusion assays. For FFWO, the plates were pre-chilled and infected at a multiplicity of 500 with virus previously inactivated by u.v. irradiation (Iorio & Bratt, 1983). After adsorption for 30 min at 4 °C, secondary medium containing MAbs at a concentration of 200 μg/ml was added and the plates were incubated for another 15 min at 4 °C. After the addition of 1 ml of warm medium, the plates were incubated in 5% CO₂ at 37-5 °C for 3 h.

For FFWI, a multiplicity of 10 was used. Secondary medium containing 2% sodium bicarbonate and 5 mM-HEPES (pH 8.2) was added and the plates were incubated at 37 °C. After 3 h, the medium was removed and replaced with fresh MAb-containing medium, and the plates were incubated for an additional 4 h. The medium was again removed and indicator chicken embryo fibroblasts (5 × 10⁵/ml) were added for 1 h.

After washing with PBS, the cells from both assays were fixed with methanol and stained with Giemsa Accustain (Sigma) prior to microscopic examination for syncytium formation.

**Selection of variants.** Variants were selected from cloned passaged stocks of the AV isolate by neutralization escape using rabbit antiserum (Iorio & Bratt, 1985). Each variant originated from a different passaged stock, ensuring that they were independent isolates.

**Primer extension and dideoxynucleotide sequencing.** Nucleotide sequencing was performed using 17-mer oligonucleotides complementary to the HN gene of the AV isolate (McGinnes et al., 1987) to prime dideoxynucleotide chain termination sequencing reactions. Details of the sequencing protocol, including the primers and their purification, as well as the purification of the virion RNA template, the primer extension reactions and the sequencing gel protocols have all been described previously (Iorio et al., 1989b; Sheehan et al., 1987).

**Infected cell lysates.** Chicken embryo fibroblasts infected with virus at a multiplicity of 10 were labelled with [³⁵S]methionine (Amersham) (Hightower et al., 1975; Peeples & Bratt, 1984). For inhibition of glycosylation, tunicamycin (Calbiochem) was added at a concentration of 1 μg/ml. Lysates were analysed by SDS-PAGE (Laemmli, 1970) in the presence of 2-mercaptoethanol.

**Endoglycosidase F (endo F) digestion of [³⁵S]methionine-labelled virions and immunoprecipitation.** Virions labelled with [³⁵S]methionine were prepared as described by Peeples & Bratt (1984), except that labelling medium was left on the monolayer overnight. Labelled virions were concentrated in an Amicon Microconcentrator 30 and washed with 250 mM-sodium acetate (pH 7.5) containing 20 mM-EDTA, prior to treatment with endo F (Boehringer Mannheim) for 18 h at 37 °C in the same buffer and subsequent analysis by SDS-PAGE.

**Results**

**MAb inhibition of the induction of FFWO by non-infectious virus**

FFWO requires only that input virus particles bridge adjacent cells and promote the fusion of their membranes, leading to syncytium formation; virus replication is not required. The induction of FFWO by non-infectious virus serves as a model system to assay the effect of neutralizing antibody on the early steps in the infection process.

Fig. 1 shows the effect of treatment with several MAbs to the inducition of FFWO by non-infectious virus. Since the AV isolate of NDV does not induce FFWO, two other isolates, B and HP, which are good inducers of this mode of fusion, were used for these experiments. The induction of fusion by virions of the HP isolate, relative to uninfected cells (Fig. 1a), is shown in the HP-infected control (Fig. 1b). A neutralizing MAb to the F glycoprotein completely prevented syncytium formation (Fig. 1c). Three MAbs to HN that prevent attachment of the virus to host cell receptors, i.e. those to sites 1, 2 and 14 (Iorio et al., 1989a), also effectively prevented
syncytium formation (Fig. 1d to f). This is expected because these MAbs probably block the HN-mediated bridging of potential fusion partners.

An antibody to site 4 which does not prevent attachment (Iorio et al., 1989a) is still capable of preventing syncytium formation by HP virions (Fig. 1g). However, MAbs to site 3 cannot be assayed with the HP isolate because they do not recognize this virus (data not shown). When these MAbs were used in the same assay with virions of the B isolate, which is also a good inducer of this mode of fusion (Fig. 1h), they inhibited FFWO very effectively (Fig. 1i).

**Effect of anti-HN MAbs on FFWI**

The effect of antibodies to the seven HN antigenic sites on the ability of the virus to induce FFWI was determined using the homologous AV isolate, which is an excellent inducer of this mode of fusion (Bratt & Gallaher, 1972) (Fig. 2b). As expected, a neutralizing antibody to the F protein completely blocked syncytium formation (Fig. 2c). All antibodies that neutralize by preventing attachment also prevented syncytium formation in this assay, as shown for MAbs to sites 2 (Fig. 2d) and 14 (Fig. 2e). Moreover, MAbs to site 3, which do not prevent attachment, also inhibited FFWI (Fig. 2f).

However, MAbs to HN antigenic site 4, at a concentration of 200 μg/ml, did not block syncytium formation in this assay, as shown for antibodies 4a (Fig. 2g) and 4c (Fig. 2h). Even the addition of a fivefold greater amount of the latter antibody, at a concentration of 1 mg/ml, failed to inhibit syncytium formation (Fig. 1i).

**Isolation and sequence analysis of variants**

Variants were selected with three MAbs, 3a, 3b, and 3c, specific for overlapping epitopes in site 3, and MAb 4b, which is specific for an epitope in site 4. To map the epitopes recognized by these MAbs in the linear amino acid sequence of HN, the entire HN gene of each variant was sequenced. The single deduced amino acid substitution in each variant is shown in Table 1. Variants 3a-1 and 3a-2, selected with MAb 3a, both have a substitution...
Fig. 2. Effect of MAbs on FFWI. The effect of MAb treatment on the induction of FFWI in chicken embryo cells by the AV isolate.
(a) Uninfected cells; (b) no MAb; (c) MAb to the F glycoprotein; (d) MAb 2a; (e) MAb 14a; (f) MAb 3a; (g) MAb 4a; (h and i) MAb 4c.
The concentration of the MAbs was 200 μg/ml, except in (i) in which it was 1 mg/ml.

Table 1. Nucleotide and deduced amino acid substitutions in site 3 and 4 variants

<table>
<thead>
<tr>
<th>Variant</th>
<th>Nucleotide substitution*</th>
<th>Amino acid substitution*</th>
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<tbody>
<tr>
<td>3a-1:3a-2</td>
<td>878, A→G</td>
<td>263, Asn→Asp</td>
</tr>
<tr>
<td>3a-5:3a-6</td>
<td>950, G→A</td>
<td>287, Asp→Asn†</td>
</tr>
<tr>
<td>3a-8</td>
<td>1053, A→G</td>
<td>321, Lys→Arg</td>
</tr>
<tr>
<td>3a-2</td>
<td>1053, A→C</td>
<td>321, Lys→Thr</td>
</tr>
<tr>
<td>3a-5</td>
<td>1054, A→C</td>
<td>321, Lys→Asn†</td>
</tr>
<tr>
<td>3c-11</td>
<td>1086, G→A</td>
<td>332, Gly→Glu</td>
</tr>
<tr>
<td>3c-8</td>
<td>1088, A→G</td>
<td>333, Arg→Gly</td>
</tr>
<tr>
<td>3c-7</td>
<td>1090, A→T</td>
<td>333, Arg→Ser</td>
</tr>
<tr>
<td>4c-4:4c-6</td>
<td>1159, G→T</td>
<td>356, Lys→Asn†</td>
</tr>
</tbody>
</table>

* The numbering systems for nucleotide and amino acid residues are +1 and +3, respectively, relative to the published sequences (McGinnes et al., 1987). This is necessary to account for the presence of a tyrosine at residue 185 which had originally been reported as absent in the HN of this isolate. This had necessitated the introduction of a deletion at this position to afford maximal alignment of the sequence of its HN with those of other NDV isolates.
† Amino acid substitutions resulting in the addition of new potential glycosylation sites.

of aspartic acid for asparagine at residue 263 of the wild-type. Two variants, selected with MAbs 3a and 3c, both have asparagine for aspartic acid at position 287 of the wild-type. Three other variants selected by escape from neutralization by MAbs 3a and 3c have a substitution of one of three different residues for the lysine at position 321 of the wild-type. The fact that antibody 3a selected variants with substitutions quite removed from those of variants selected with the other site 3 MAbs is consistent with earlier data, suggesting the existence of distinct, yet overlapping, epitopes within site 3 (Iorio et al., 1986).

MAb 3a recognizes an isolate-specific epitope on the HN of the homologous virus (Iorio et al., 1984). This antibody fails to, or only weakly neutralize the vast majority of the NDV isolates. Thus, one would expect the 3a epitope to be generally non-conserved among the members of the NDV serotype. The nucleotide sequence was determined in this region of the molecule for several isolates shown to escape neutralization by MAb 3a. The deduced amino acid sequences are shown in Table 2 for the limited domain defined by residues 261 to 270.
occupied by several different amino acids in this group of viruses. Similar sequence heterogeneity in this region of these isolates have been tested and are also not recognized by MAb 3a (Iorio et al., 1984). The isolate specificity of MAb 3a is probably related to the comparative uniqueness of the HN sequence of the AV isolate in this region.

Although residue 263 itself is conserved in these heterologous isolates, several residues nearby are different in the AV isolate relative to the other isolates: residues 265 and 266 are different in the AV isolate relative to all the other isolates shown; position 269 is occupied by several different amino acids in this group of viruses. Similar sequence heterogeneity in this region of HN, including some divergence at residue 263, is evident upon comparison with the published sequences of HN from several other isolates (Sakaguchi et al., 1989). Many of these isolates have been tested and are also not recognized by MAb 3a (Iorio et al., 1984). The isolate specificity of MAb 3a is probably related to the comparative uniqueness of the HN sequence of the AV isolate in this region.

Antibody 4b selects variants with substitutions of glutamic acid for the glycine at residue 332, glycine or serine for the arginine at residue 333 or asparagine for the lysine at residue 356 (Table 1).

<table>
<thead>
<tr>
<th>NDV isolate</th>
<th>Amino acid sequence*</th>
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<tbody>
<tr>
<td>AV</td>
<td>261-DYNSVIPTSM-270</td>
</tr>
<tr>
<td>IS</td>
<td>AV L</td>
</tr>
<tr>
<td>RO</td>
<td>AV L</td>
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<tr>
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<td>GB</td>
<td>AV L</td>
</tr>
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* Amino acid (single-letter code) differences only are shown.
† The position (residue 263) of the amino acid substitution in two of the variants selected with MAb 3a.

The amino acid substitutions in some of the site 3 and site 4 variants introduce new N-linked glycosylation sites

Some of the amino acid substitutions in the variants shown in Table 1 create new potential glycosylation sites: NVT at residues 287 to 289 (variants 3c-5 and 3c-6); NPS at residues 321 to 323 (variant 3c-5); NSS at residues 356 to 358 (variants 4c-4 and 4c-6).

To determine whether the new glycosylation sites are utilized, [35S]methionine-labelled variant-infected chicken embryo cell lysates were analysed by SDS-PAGE under reducing conditions. Fig. 3(a) shows that four of the variants with additional potential glycosylation sites (lanes 2, 3, 5 and 6) all have larger HN proteins than those of either the wild-type (lane 1), variant 3c-5 (lane 4) or variant 4c-8 (lane 7). The additional site in the HN variant 3c-5 is not glycosylated. This is not surprising because sites having proline as the middle residue are usually not utilized (Kornfeld & Kornfeld, 1985). Fig. 3(b) shows that when virus is produced in the presence of the glycosylation inhibitor tunicamycin, all the variants have unglycosylated HN proteins of the same size, confirming that the slower migration of the HN of some of the variants is due to the utilization of the additional glycosylation site.

Differential sensitivity of glycosylation variants to endo F

The HN proteins of the glycosylation variants may not be recognized by neutralizing antibody because the sugar moiety either directly masks the antibody-binding epitope or alters it conformationally. [35S]Methionine-labelled wild-type virions and some of the site 3 and 4 variants were deglycosylated by treatment with endo F and tested for MAb recognition. Since the antibodies recognize only native HN, and the sugar moieties, being proximal to antigenic sites, are likely to be in an exposed region of the molecule, virions were treated intact rather than after SDS denaturation.

Treatment of wild-type virions of the AV isolate with endo F at a concentration of 2 units/ml produced three distinct, faster migrating bands (Fig. 4, lanes 2 and 4) relative to the untreated HN band (Fig. 4, lanes 1 and 3), all of which are recognized by MAbs 3b and 14a. Presumably these bands result from sequential deglycosylation of the molecule at different glycosylation sites. The migration of the maximally deglycosylated form of HN resulting from this treatment was the same as that of the single band resulting from endo F treatment of SDS-denatured HN (data not shown). Endo F treatment of the glycosylation variant 3b-5 produces four faster migrating bands (Fig. 4, lane 8) relative to the untreated control (Fig. 4, lane 7), all of which are recognized by the MAb to antigenic site 14. However, neither untreated HN from the glycosylation variant 3b-5 (lane 5) nor any of the deglycosylated forms derived from it by endo F digestion (lane 6) were immunoprecipitated by MAb 3b. Thus, endo F treatment does not enable antibody to recognize the HN protein of this variant.

Similar studies of site 4 glycosylation variants revealed them to be much more sensitive to endo F than their site 3 counterparts. When one of the site 4 glycosylation variants, 4b-6; was treated with endo F at a concentration similar to that used in the experiment shown in Fig. 4, a protein the size of the maximally deglycosylated HN was the predominant species identified on gels (Fig. 5, lane 11). The use of five- to tenfold lower concentrations of endo F was necessary to demonstrate the partially deglycosylated intermediate forms of HN from virions of this variant (Fig. 5, lanes 8 and 9). Treatment with this concentration of endo F had comparatively little effect on the HN of the wild-type (Fig. 5, lanes 2
Fig. 3. SDS-PAGE of site 3 and 4 variant-infected chicken embryo fibroblast cell lysates. Cells were labelled at 7 h post-infection for 1 h with [[35S]methionine and lysed in reducing gel buffer prior to analysis on gels in the absence (a) or presence (b) of 1 μg/ml tunicamycin. Lanes 1, wild-type AV isolate; lanes 2, variant 3b-5; lanes 3, variant 3¢-6; lanes 4, variant 3¢-5; lanes 5, variant 4b-4; lanes 6, variant 4b-6; lanes 7, variant 4¢-9. HNg and Fg, and HNag and Fag are the glycosylated and unglycosylated forms, respectively, of the two glycoproteins. NP, M and P are the nucleocapsid, membrane and phosphoproteins, respectively.

Fig. 4. Immunoprecipitation of endo F-treated HN from intact virions of a site 3 glycosylation variant. Labelled virions of the wild-type (lanes 1 to 4) and variant 3¢-5 (lanes 5 to 8), were untreated (odd-numbered lanes) or treated with 2 μg/ml endo F (even-numbered lanes), immunoprecipitated with MAb 3¢ (lanes 1, 2, 5 and 6) or 14¢ (lanes 3, 4, 7 and 8) and analysed by SDS–PAGE.

Discussion

Antibodies to HN of NDV can be separated into two groups with respect to their mechanism of neutralization: those which prevent receptor recognition (MAbs to sites 14, 1, 12, 2 and 23) and those which do not (MAbs to sites 3 and 4) (Iorio et al., 1989a). The exact mechanism by which members of the latter group of antibodies neutralize virus infectivity is still unknown.
Fig. 5. Endo F treatment of a site 4 glycosylation variant. [35S]Methionine-labelled virions of the wild-type (lanes 1 to 6), glycosylation variant 4a-6 (lanes 7 to 12) and variant 4a-7 (lanes 13 to 18) were treated with various concentrations of endo F for 18 h at 37 °C. The endo F concentrations were 0 units/ml (lanes 1, 7 and 13), 0.2 units/ml (lanes, 2, 8 and 14), 0.4 units/ml (lanes 3, 9 and 15), 0.86 units/ml (lanes 4, 10 and 16), 1.6 units/ml (lanes 5, 11 and 17), and 3.3 units/ml (lanes 6, 12 and 18).

We have determined the effect of antibodies to all seven sites on the induction of FFWO by non-infectious virus as a means of understanding both the role of HN in NDV-induced fusion and the mechanism of neutralization by the site 3 and 4 antibodies. All of the antibodies are inhibitory in this assay, suggesting that (i) the HN glycoprotein is required for virion-induced fusion and (ii) neutralizing antibodies that do not prevent virus attachment still act at an early step in the interaction of the virion with the host cell.

Evidence from a number of recent studies suggests that HN, in addition to F protein, is required for the induction of fusion by paramyxoviruses (Sakai & Shibuta, 1989; Ebata et al., 1991; Morrison et al., 1991; Moscona & Peluso, 1991), although there is also evidence to the contrary (Markwell et al., 1985; Paterson et al., 1985; Olmstead et al., 1986; Alkhatib et al., 1990). The reasons for the discrepancy in the findings of these groups is unclear, but our data are consistent with HN being required for the induction of cell fusion and apparently for more than merely bringing the two membranes into close proximity to one another.

The inhibition of FFWO by neutralizing MAbs by preventing virion attachment is in agreement with the prevailing evidence that HN is required to bridge adjacent cells in fusion induction. All of these antibodies also prevent FFWI, indicating that receptor recognition is also a requirement for this mode of fusion. However, an argument can be made that there is an additional role for HN in cellular fusion. Antibodies to sites 3 and 4, because they inhibit neither virion attachment to host cells (Iorio et al., 1989a) nor NA activity on neuraminidase (Iorio & Bratt, 1984), appear to bind to domains not involved in any function commonly attributed to HN. Yet these MAbs inhibit input virus-mediated syncytium formation. Similar results have been obtained with MAbs to the attachment proteins of Sendai (Miura et al., 1982), measles (De Vries et al., 1987) and mumps viruses (Tsurudome et al., 1986).

HN possibly contributes a second, undefined function required for FFWO. A similar conclusion is indicated from the results of studies with vesicles reconstituted using the Sendai virus glycoproteins (Gitman & Loyter, 1984) and with cells persistently infected with human parainfluenza virus type 3 (Moscona & Peluso, 1991). The inhibition of such a secondary function of HN could account for the inhibition of FFWO and possibly the neutralization of virus infectivity by the anti-site 3 and 4 MAbs.

An alternative explanation for the effect of the anti-site 3 and 4 antibodies on fusion invokes the existence of an interaction between the HN and F glycoprotein spikes on the virion or on the surface of the infected cell. Evidence has been presented for such an interaction. The glycoproteins of Sendai virus have been shown to have a critical ratio in vesicles used for the promotion of fusion.
and the same membrane (Citovsky et al., 1986). This conformational change correlates with fusogenic activity. Thus, one possibility not ruled out by our data is that anti-HN MAbs block fusion and neutralize virus infectivity by interfering with a necessary interaction between the HN and F glycoprotein spikes.

The inhibition of FFWO, but not FFWI, by anti-HN antibodies, as shown for the anti-site 4 MAbs, has also been demonstrated for MAbs to the HN of mumps virus (Tsurudome et al., 1986). This finding strongly argues that virion–cell and cell–cell membrane fusion are not equivalent processes. The failure of MAbs to site 4 to inhibit FFWI is not inconsistent with their neutralizing activity. Inhibition of FFWO, but not inhibition of FFWI, is analogous to inhibition of the infection of a cell by a virion. An antibody that neutralizes by preventing entry of the virus into the monolayer need not also prevent spread of the virus through the monolayer by cell–cell fusion. This suggests that the relationship between the two types of glycoprotein spikes on the surface of the virion and on the surface of the infected cell may be quite different. In fact, the inhibition of FFWI by the anti-site 3, but not the anti-site 4 MAbs indicates that the effect of MAbs to these two sites on the proposed secondary function of HN in fusion or on the putative interaction between the HN and F glycoprotein spikes is not equivalent.

To begin to map the epitopes recognized by MAbs that do not prevent attachment in the linear amino acid sequence of HN, the entire nucleotide sequence of the HN gene of variants selected by escape from antibodies to sites 3 and 4 was determined. The identification of a single nucleotide substitution in each identified a single amino acid substitution in the HN protein of each variant. Substitutions are at residues 263, 287 or 321 for site 3 variants and residues 332, 333 or 356 for site 4 variants.

Although the anti-site 3 and 4 MAbs bind to domains on HN that are distinct from its receptor recognition and NA sites, it is important to note that the amino acid substitutions in the site 4 variants are very close in the linear amino acid sequence to those in variants selected with anti-site 1 (residue 345) and anti-site 14 (residues 347, 350 and 353) MAbs, which block receptor recognition (Iorio et al., 1989a). Indeed, site 4 topologically overlaps site 14 (Iorio et al., 1986). The proximity of site 4 to sites recognized by MAbs that prevent attachment suggests the possibility that the putative secondary function proposed for HN may reside very close to the receptor recognition site. Thus the secondary function of HN may be very closely linked to its attachment function. Analysis of the fusogenic properties of the variants described here and those selected with attachment-inhibiting antibodies (Iorio et al., 1991) may help to elucidate the relationship between the two functional domains.

In some of the variants, the single amino acid substitution results in the introduction of a new potential glycosylation site, which is utilized and responsible for escape from neutralizing antibody. The phenomenon of masking neutralizing antibody binding sites through the addition and use of a new glycosylation site has been described for the haemagglutinin of influenza virus (Skehel et al., 1984), the E2 glycoprotein of Sindbis virus (Davis et al., 1987), the gp120 of human immunodeficiency virus (Davis et al., 1990) and HN of the Beaudette isolate of NDV (Yusoff et al., 1988). In the latter instance, the selecting MAb only weakly inhibits HA and does not inhibit NA activity on neuraminidase. The additional glycosylation site results from a substitution of serine for the proline residue at position 325 of wild-type HN. Mutants that escape neutralization by loss of the glycosylation site at residues 481 to 483 in the HN of the D26 (Gotoh et al., 1988) and Beaudette (Yusoff et al., 1988) isolates of NDV have also been described.

Endo F treatment of native HN has made possible the demonstration of intermediate deglycosylated forms of HN not normally seen with the denatured protein. Since the maximally deglycosylated form of HN obtained by endo F digestion of native HN comigrates with that resulting from treatment of the SDS-denatured protein, the intermediate forms probably result from deglycosylation at different sites. The simplest explanation for this is that three of the six potential glycosylation sites are utilized in wild-type HN. The finding that the endo F-sensitive glycosylation sites in the site 4 glycosylation variants all have increased sensitivity to the glycosidase suggests that the substitution in these variants (4-4 and 4-6) has somehow altered the molecule such that the original sites in the non-denatured molecule are more accessible to the glycosidase.

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