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Fusion Mutants of Newcastle Disease Virus Selected with Monoclonal Antibodies to the Hemagglutinin-Neuraminidase

RONALD M. IORIO* AND RHONA L. GLICKMAN

Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, 55 Lake Avenue, North, Worcester, Massachusetts 01655

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The Australia-Victoria (AV) isolate of Newcastle disease virus (NDV) induces fusion from within but not fusion from without. L1, a neuraminidase (NA)-deficient virus derived from AV, has the opposite fusion phenotype from the wild-type virus. It fails to induce the former mode of fusion, but has gained a limited ability to promote the latter. Monoclonal antibodies to antigenic site 23 on the hemagglutinin-neuraminidase (HN) glycoprotein have previously been shown to select variants of the AV isolate that have altered NA activity or receptor-binding affinity. By using an antibody to this site, variants of L1 have been selected. Three of the variants have gained an increased affinity for sialic acid-containing receptors, as evidenced by the resistance of their hemagglutinating activity to the presence of reduced amounts of sialic acid on the surface of chicken erythrocytes. All four variants still have very low levels of NA activity, comparable to that of the parent virus, L1. The alteration in receptor-binding affinity results in a decreased potential for elution from cellular receptors and correlates with an increased ability to promote both modes of fusion. A single amino acid substitution in the HN protein of each variant, responsible for its escape from neutralization, has been identified. These studies identify two HN residues, 193 and 203, at which monoclonal antibody-selected substitution influences the receptor recognition properties of NDV and may influence its ability to promote syncytium formation.

One of the hallmark cytopathic effects of the infection of cells by paramyxoviruses is the formation of syncytia as a result of the induction of cell-cell fusion by the virus (4). Two sets of conditions under which a member of this group of viruses, Newcastle disease virus (NDV), can promote cellular fusion have been defined (2). Early fusion is mediated by input virus particles, which need not be infectious, at high multiplicities and is called fusion from without (FFWO). The fusion that occurs late after infection at low multiplicity and requires infectious virus is mediated by the newly synthesized viral glycoproteins deposited on the surface of the infected cell. It is thus called fusion from within (FFWI).

The requirement for a proteolytically cleaved form of the fusion (F) glycoprotein in the induction of both modes of fusion is well established (24). However, the contribution(s) of the hemagglutinin-neuraminidase (HN) glycoprotein in the promotion of fusion is far less well understood. Even the expression of the two glycoproteins in mammalian cells by recombinant DNA technology has yielded conflicting results with respect to the requirement for HN in paramyxovirus-induced fusion (1, 6, 9, 19, 20, 22, 32), although a requirement for HN in the induction of fusion by NDV has been demonstrated (8, 17).

Bratt and Gallaher (2, 3) presented evidence consistent with a cause-and-effect relationship between properties commonly attributed to the HN glycoprotein of NDV and its fusogenic potential. The ability to cause FFWO correlated with the strength of the initial interactions of the virus with the cell membrane, a property commonly attributed to the HN protein spike. They suggested that a good inducer of FFWO can be characterized as spending a relatively longer time in association with the cell surface. This is consistent with HN, through the nature of its interaction with cellular receptors, being a major determinant of the fusogenic activity of NDV. Members of the NDV serotype differ in their ability to induce the two modes of fusion, and for many isolates, the two types are mutually exclusive. For example, the Australia-Victoria (AV) isolate is a particularly strong inducer of FFWI, but FFWO has not been detected with this isolate.

There is also strong evidence to suggest that the neuraminidase (NA) of paramyxoviruses profoundly affects their fusing activities. Naturally occurring strains of mumps virus (16) and bovine parainfluenza virus type 3 (27, 28) were shown to exhibit an inverse relationship between NA and the ability to induce FFWI. Those viruses with low levels of NA were the strongest inducers of this mode of fusion. Subsequently, a fusing, NA-deficient variant was selected from a nonfusing strain, again indicating that the NA activity of mumps virus HN modulates cell fusion (36).

We have previously shown that monoclonal antibodies (MAbs) to one of seven sites on the HN glycoprotein of NDV (site 23) bind to a domain that is apparently very close to both the NA and receptor recognition sites. These MAbs inhibit the NA activity of the molecule on neuraminlactose (12). A competitive inhibitor of NA which binds at the active site of the molecule inhibits the binding of site 23 MAbs to virions by more than 90% (13). Three of four variants selected by escape from the neutralizing activity of these MAbs have altered NA activity presumably due to a single amino acid substitution at either residue 194 or 201 (13). A fourth site 23 variant has a substitution of leucine for the phenylalanine at residue 193 of the wild type (wt) with no measurable effect on NA activity. The hemagglutinating (HA) activity of this virus is nearly threefold more resistant to periodate treatment of erythrocytes than that of the wt (14). The periodate sensitivity test (33) has been used previously to identify substitutions in the hemagglutinin of influenza virus which influence its affinity for sialic acid-containing receptors (34). Thus, a substitution of leucine for the phenylalanine at residue 193 influences the affinity with

* Corresponding author.

which the virus binds to sialic acid-containing receptors on the cell surface.

The temperature-sensitive (*ts*) mutant C1 and two sequentially selected revertants, L1 and N1, originally isolated and characterized by Smith and Hightower (30, 31) have also proved very useful in mapping the NA active site in the linear amino acid sequence of HN. L1 was isolated as a spontaneously arising, second-step revertant of C1 which formed large plaques at the nonpermissive temperature. It was subsequently found to have a severe deficiency in NA activity. A revertant, N1, with partially restored activity was isolated from L1 by using a chromogenic substrate. All three viruses fail to induce FFWI in chicken embryo cells.

The HN genes of these viruses have been sequenced completely (13). C1 has a single amino acid substitution of asparagine for the aspartic acid at HN residue 129. L1 has two substitutions, the one at residue 129 and an additional one at residue 175 (isoleucine to methionine). Residue 175 of the HN of NDV corresponds to residue 181 in the mumps virus protein, the nature of which affects the integrity of its NA site (35), and to residue 193 in bovine parainfluenza virus type 3 HN, substitution at which also affects both HN functions (29) and the ability of the virus to promote syncytium formation (22). However, we have recently shown that the introduction of the substitution at residue 175 of the HN of NDV results in a protein that is no longer capable of adsorbing chicken erythrocytes, indicating that the nature of residue 175 influences receptor recognition as well as NA (25).

N1 has the two substitutions (D-129→N and I-175→M) found in L1, plus a third (phenylalanine to leucine) at residue 193, and is not recognized by the site 23 MAbs (12). This is not surprising, since the F-193→L substitution in N1 is identical to the single substitution in the site 23 variant that has increased affinity for cellular receptors (14).

We have now shown that both revertants, L1 and N1, have a limited capacity to induce FFWO, even though induction of this mode of fusion has not been demonstrated for either the wt AV isolate or the *ts* mutant, C1, from which the revertants were derived. Using escape from neutralization by an MAb to HN antigenic site 23, we have isolated variants of L1, all of which retain its phenotype of drastically reduced NA activity and some of which have increased affinity for cellular receptors, decreased elution potential, and markedly enhanced fusogenic activity in assays of both FFWO and FFWI.

MATERIALS AND METHODS

Virus. The AV (1932) isolate of NDV and mutants and variants derived from it were grown in the allantoic sac of embryonated hen eggs, and virions were purified as described previously (14).

Cells. Primary and secondary chicken embryo cells were prepared and grown in a 5% CO_2 atmosphere at 39°C in standard medium (Eagle minimal essential medium supplemented with 2.5% calf serum, 2.5% tryptone phosphate broth, 0.07% $NaHCO_3$, 0.1% amphotericin B [Fungizone], 10 U of penicillin per ml, and 10 μg of streptomycin per ml). All medium components were obtained from GIBCO Laboratories (Grand Island, N.Y.).

Hybridomas, MAbs, and selection of variants. The preparation of hybridomas and initial characterization of MAbs have been described previously (12).

Variants were selected from cloned, passaged stocks of mutant L1 by escape from neutralization by MAb 23_f using rabbit anti-mouse immunoglobulin (11).

Neutralization and NA assays and HN content of virions. The plaque neutralization assay and the determination of virion NA activity and HN content have been described previously (13).

Primer extension and dideoxy sequencing. Nucleotide sequencing was performed with 17-mer oligonucleotides complementary to the HN gene of the AV isolate (15) to prime dideoxynucleotide chain termination sequencing reactions. Details of the sequencing protocol, including the primers and their purification, as well as that of the virion RNA template, the primer extension reactions, and the sequencing gel protocols, have all been described previously (26).

Fusion assays. Chicken embryo cells at 60 to 70% confluency in 35-mm-diameter tissue culture plates were used for the fusion assays. For FFWO, the cells were infected at a multiplicity of 500 for 30 min at room temperature. Fresh standard medium was added, and the plates were incubated in 5% CO_2 at 37.5°C for 3 h.

For FFWI, a multiplicity of 10 was used. Standard medium containing 2% sodium bicarbonate and 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 8.0) was added, and the plates were incubated at 37°C for 7 h. The medium was withdrawn, and indicator chicken embryo fibroblasts (5×10^5/ml) were added for 1 h.

After being washed with phosphate-buffered saline (PBS) (10 mM Na_2PO_4, 3 mM KCl, 1.8 mM KH_2PO_4, 8.5% NaCl), the cells in both assays were fixed with methanol and stained with Giemsa Accustain (Sigma Chemical Co., St. Louis, Mo.) prior to microscopic examination for syncytium formation. The extent of fusion (fusion index) is expressed as the percentage of nuclei that were found in syncytia involving three or more nuclei.

Neuraminidase sensitivity tests. The resistance of the HA activity of virions to the presence of reduced amounts of sialic acid receptors on erythrocytes used in the HA assay was determined. Chicken erythrocytes were washed twice in PBS containing 1% $CaCl_2$ and 1% $MgCl_2$ and treated with 1/10 volume of *Vibrio cholerae* neuraminidase [NA(VC)] (Calbiochem, La Jolla, Calif.) for 1 h at 37°C. The erythrocytes were then pelleted by centrifugation and resuspended in the same buffer for use in HA assays of the variants (12). The concentration of NA(VC) that decreased the HA activity of each virus by 4 HA units was determined and expressed relative to the wt level.

Elution. Chicken erythrocytes (2×10^8/ml), washed as described above, were incubated at 4°C for 1 h with an equal volume of virions (10^7 PFU/ml). After an initial wash to remove unbound virions, the erythrocytes were resuspended, aliquoted into several tubes, and incubated at 37°C. At various times, the erythrocytes were pelleted, and the titer of infectious virus in the supernatants on chicken embryo cells was determined. The total amount of virus bound is that associated with the erythrocyte pellet before the shift to the higher temperature.

RESULTS

Fusogenic activities of the site 23 variants. Three variants selected with a site 23 MAb have either decreased NA (variant 23_a-1), increased NA (variant 23_a-2) (13), or increased affinity for cellular receptors (variant 23_a-3) (14). However, all have wt levels of HN in virions. To determine the effect of each of these properties on the fusogenic

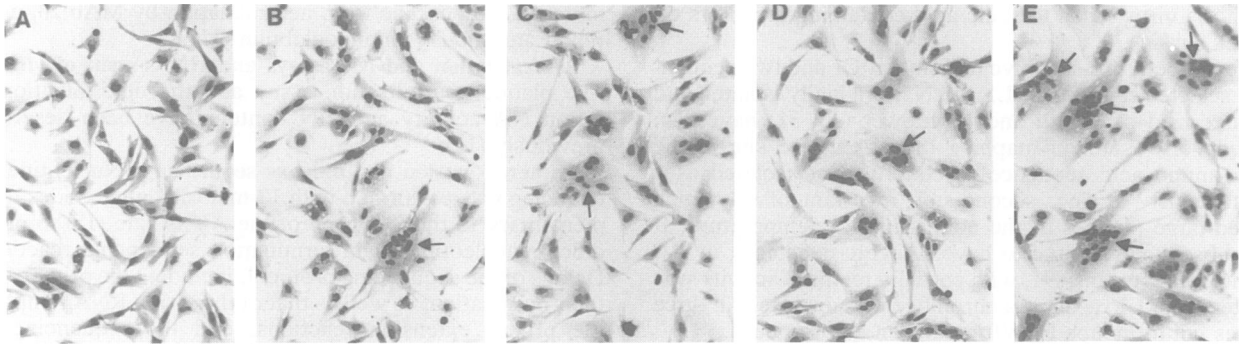

FIG. 1. Induction of FFWI by site 23 variants. Chicken embryo cells were infected and assayed for syncytium formation in the FFWI assay. (A) Uninfected cells. Other panels show cells infected with AV (B), 23_a-1 (C), 23_a-2 (D), and 23_a-3 (E). Syncytia are indicated by arrows.

activity of the virus, the ability of each of these variants to induce FFWI (Fig. 1) and FFWO (Fig. 2) in chicken embryo cells was assayed. The fusion indices, or percentages of nuclei involved in syncytia, are listed in Table 1. The uninfected monolayers show a total absence of multinucleate cells in both assays (Fig. 1A and 2A). As originally described by Bratt and Gallaher (3), the AV isolate of the virus is a very effective inducer of FFWI (Fig. 1B) (fusion index of 22.6) but does not induce FFWO in chicken embryo cells (Fig. 2B) (fusion index of 1.4).

Variant 23_a-1, a virus with only 17% of the NA activity of the wt (13) shows no measurable change in its ability to induce FFWI (Fig. 1C) (fusion index of 22.4), indicating that loss of NA activity to this extent has no effect on the ability of the virus to induce this mode of fusion. However, variant 23_a-1 is more effective (fusion index of 6.4) than the wt virus in inducing FFWO (Fig. 2C).

Variant 23_a-2 has a 79% increase in NA activity relative to the wt (11), exhibits a decreased ability to induce FFWI (Fig. 1D) relative to the wt virus (Fig. 1B) (its fusion index is 73%

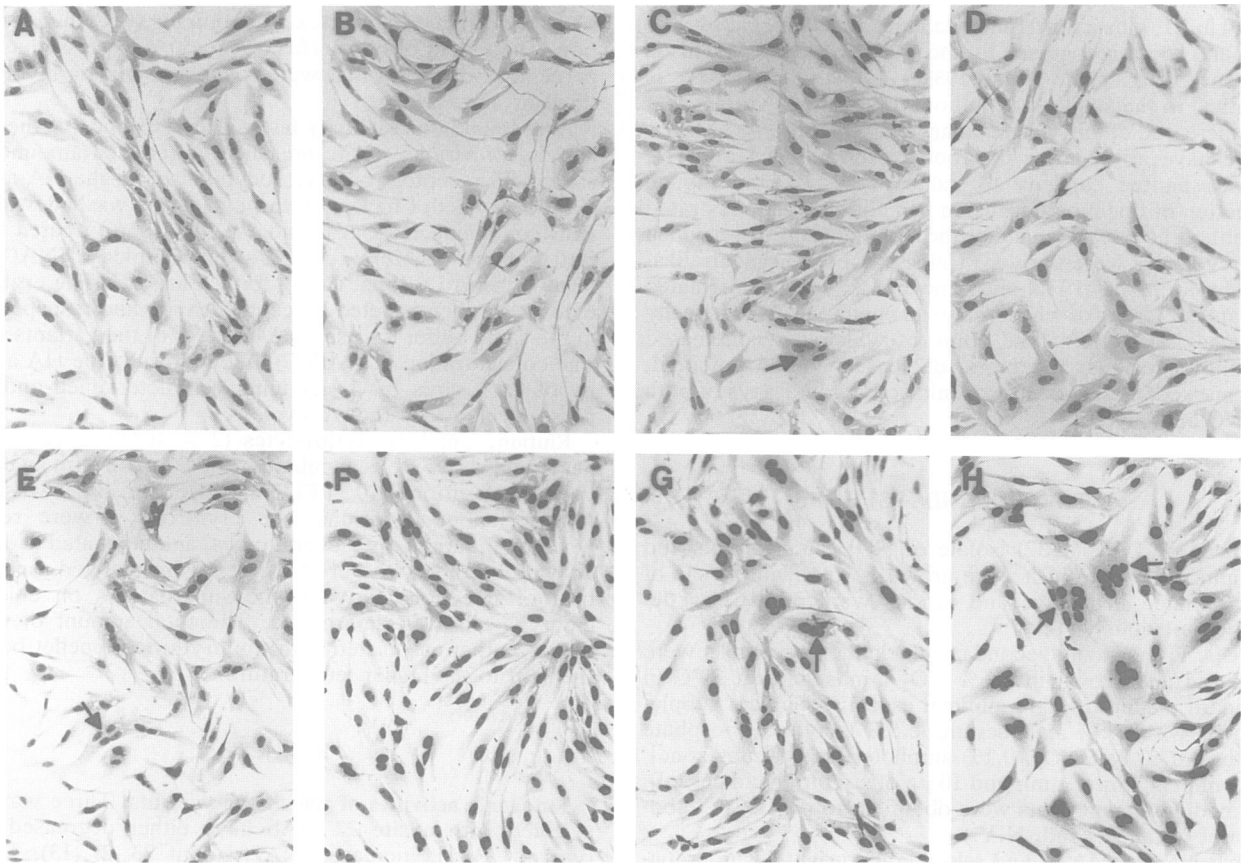

FIG. 2. Induction of FFWO by site 23 variants and by C1, L1, and N1. Chicken embryo cells were infected and assayed for syncytium formation in the FFWO assay. (A) uninfected cells. Other panels show cells infected with AV (B), 23_a-1 (C), 23_a-2 (D), 23_a-3 (E), C1 (F), L1 (G), and N1 (H). Syncytia are indicated by arrows.

TABLE 1. Fusion indices of site 23 variants and the C1-L1-N1 series of viruses

Viruses	Fusion index[a]	
	FFWI	FFWO
None[b]	<1	<1
AV	22.6	1.4
23_a-1	22.4	6.4
23_a-2	16.4	<1
23_a-3	43.9	8.2
C1	<1	1.6
L1	<1	7.4
N1	<1	12.3

[a] Percentage of nuclei in cells having three or more nuclei.
[b] Cells were uninfected.

TABLE 2. Properties of and nucleotide and amino acid substitutions in site 23 variants selected from L1

Virus	% PFU[a]	NA[b]	HN[c]	NA/HN	Additional substitution	
					Nucleotide[d]	Amino acid[e]
L1[f]	18 (+)	0.02	0.89	0.02	None	None
N1[f]	80 (+)	0.11	0.85	0.13	T-668→C	F-193→L
L1-23_f-1	110 (−)	0.02	0.76	0.03	A-680→G	R-197→G
L1-23_f-2	104 (−)	0.06	0.95	0.07	T-668→A	F-193→I
L1-23_f-3	83 (+)	0.03	0.62	0.05	T-700→A	H-203→Q
L1-23_f-4	73 (+)	0.10	0.67	0.15	C-698→T	H-203→Y

[a] Percent persistent fraction (percentage of virus PFU surviving treatment with MAb 23_f). Treatment of the wt virus with the MAb results in a persistent fraction of only 6.5% (11). A + or − means that the MAb does or does not, respectively, bind to the virus, as determined by the ability of added anti-immunoglobulin to effect neutralization (11).
[b] NA content of the variant relative to that of the wt.
[c] HN content of the variant relative to that of the wt.
[d] All of the viruses have the two substitutions, G-476→A and A-616→G, present in L1. Only the additional substitution in each is shown.
[e] All of the viruses have the two substitutions, D-129→N and I-175→M, present in L1. Only the additional substitution in each is shown.
[f] The data for L1 and N1 were published previously (12) and are included for comparative purposes.

of the wt fusion index [Table 1]) and does not induce FFWO (Fig. 2D).

Variant 23_a-3, which has wt NA activity but an increased affinity for cellular receptors (13, 14), is more effective than the wt virus in the induction of both FFWI (fusion index of 43.9) (Fig. 1E; Table 1) and FFWO (Fig. 2E; Table 1) (fusion index of 8.2). In fact, of all those tested, this virus produces the highest fusion index in assays of FFWI. It is also important to note the correlation between the size and the number of syncytia. As an example, variant 23_a-1, a comparatively weak promoter of FFWO, induces syncytia having only three or four nuclei, while variant 23_a-3, a strong inducer of this mode of fusion, promotes the formation of giant multinucleate cells having as many as seven nuclei.

Ability of the C1-L1-N1 series of viruses to cause FFWO. The two revertants L1 and N1, isolated from the *ts* mutant C1, have some properties in common with the group of site 23 variants. L1 has only 3%, and N1 has 16%, of the NA activity of the wt virus (30). N1 also has the F-193→L amino acid substitution found in variant 23_a-3 (12); this substitution is responsible for its binding with increased affinity (13).

All three of these viruses were previously shown to be unable to induce FFWI, although they were isolated from AV (30), which is a good inducer of this mode of fusion (3). We have confirmed these findings (Table 1). However, C1, L1, and N1 had not been tested for the ability to cause FFWO. Figure 2 shows that, like AV (Fig. 2B), the *ts* mutant C1 (Fig. 2F) fails to induce FFWO. However, both revertants, L1 (Fig. 2G) and N1 (Fig. 2H), have gained the ability to promote FFWO, exhibiting fusion indices progressively greater than that of the wt (Table 1).

Site 23 variants selected from L1. Since MAbs to antigenic site 23 have been shown to select variants with altered receptor recognition properties (14), an MAb specific for this site was used to select variants of the NA-deficient L1 virus with the goal of isolating one or more viruses which combine the two properties of increased affinity for cellular receptors and diminished NA activity. Such viruses might be expected to spend a significantly longer time in association with cell surface receptors than the wt virus, which may result in an enhanced ability to promote syncytium formation.

Four viruses were isolated from L1 by escape from neutralization by MAb 23_f and were named L1-23_f-1, L1-23_f-2, L1-23_f-3, and L1-23_f-4. The high level of infectious virus (persistent fraction) remaining after treatment of all four viruses, relative to that with the wt virus (Table 2), confirms that they escape neutralization by the selecting MAb, even though two of the four are capable of binding that MAb. We have described the latter phenomenon previously for MAbs to this antigenic site (13).

The complete nucleotide sequence of the HN gene of each site 23 variant of L1 was determined. From this, the amino acid sequence of the HN glycoprotein of each was deduced. Each variant retains the original nucleotide substitutions present in L1; i.e., the HN proteins of all four have the D-129→N and I-175→M amino acid substitutions present in L1. Each variant also has one additional nonconservative nucleotide substitution in the HN gene resulting in a third amino acid substitution in the gene product. These substitutions, F-193→I, R-197→G, H-203→Q, and H-203→Y, are presumably responsible for the escape from neutralization by MAb 23_f and map close to a domain in HN previously identified as part of antigenic site 23 (13).

NA and receptor recognition properties of site 23 variants of L1. As shown in Table 2, each of the variants still has drastically reduced NA activity, with only that of L1-23_f-4 markedly different from that of the parent virus, L1. Each of the viruses also packages significant amounts of HN into virions, as does L1 (13). This ensures that the diminished NA activity in virions is due in each case to a direct effect on NA rather than to diminished HN content in virions.

In order to determine the influence, if any, of the site 23 amino acid substitutions on the receptor recognition properties of the virus, each of the variants was tested for its ability to agglutinate chicken erythrocytes having reduced amounts of sialic acid-containing receptors as a result of pretreatment with NA(VC). This approach was originally used to demonstrate that mutants of influenza virus having a selective growth advantage in the presence of a defined mixture of MAbs specific for all four sites on the hemagglutinin were actually adsorptive mutants with increased avidity for cellular receptors (37). The results are shown (Fig. 3) as the concentration of exogenous enzyme that abolishes agglutination of chicken erythrocytes by 4 HA units of each virus. The HA activity of virions of the AV isolate is resistant to treatment with only about 2.5 mU of enzyme. The levels of resistance of the *ts* mutant, C1, and its two revertants, L1 and N1, are comparable to that of the wt. Variant 23_a-3, which was previously shown to be more resistant to perio-

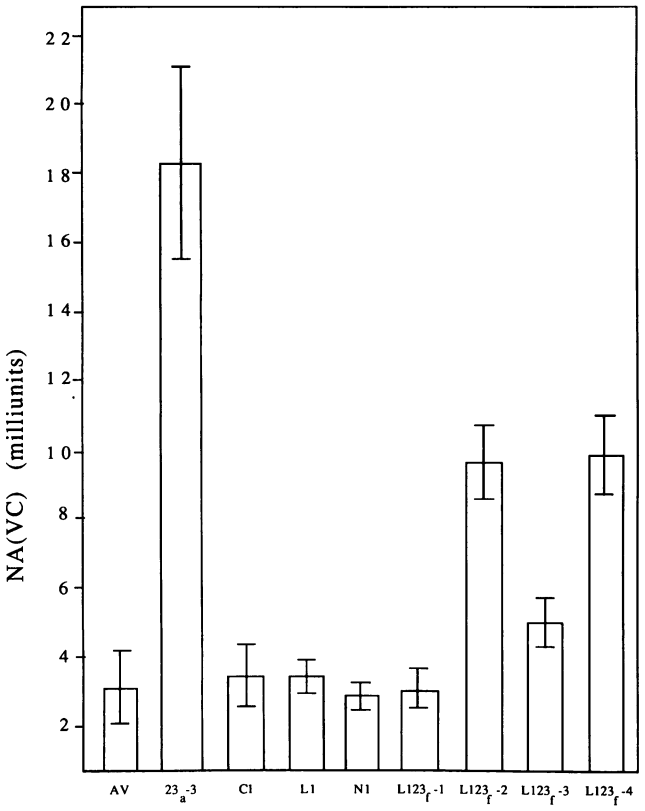

FIG. 3. Effect of NA depletion of sialic acid receptors on erythrocytes on the HA activity of virions. Chicken erythrocytes were treated for 1 h at 37°C with various amounts of NA(VC) before use in hemagglutination assays. The bar graph shows the amount of enzyme that decreased the HA activity of each virus by 4 HA units. Error bars indicate standard deviations.

date-induced alteration in sialic acid receptors (14), is also more resistant than the wt to this treatment. Its HA activity survives treatment with concentrations of NA(VC) that are more than sevenfold higher than that needed to abolish the HA activity of AV.

One of the variants, L1-23_f-1, shows a level of resistance to the NA(VC) treatment similar to that of the parent virus, L1. However, the HA activity of the remaining three variants shows increased resistance to NA(VC) treatment of erythrocytes. L1-23_f-2 and L1-23_f-4 are both more than threefold more resistant to the treatment than the parent virus, L1 (Fig. 3). These findings indicate that the substitutions in these variants, F-193→I and H-203→Y, render the HA activity of the virus significantly more resistant to the presence of reduced amounts of sialic acid-containing receptors on the erythrocyte surface. The HA activity of L1-23_f-3 also shows increased resistance to the enzyme, but less than twice that of L1.

Fusogenic properties of L1-23_f variants correlate with their receptor recognition properties. In contrast to the wt AV isolate from which it was derived, L1 no longer induces FFWI (30) but has gained the ability to induce FFWO to a limited extent (Fig. 2G). Since three of the four site 23 variants selected from L1 have also gained the ability to bind to cellular receptors with various degrees of increased affinity (Fig. 3), the fusogenic potential of each virus was determined by assaying each of the variants for their ability to induce both FFWO and FFWI (Fig. 4). The fusion indices of each variant in the two modes of fusion are indicated in Fig. 5.

While L1, as well as L1-23_f-1 and L1-23_f-3 (Fig. 4B and D), is capable of inducing FFWO, the site 23 variants of L1 having the most significantly increased receptor-binding affinities (L1-23_f-2 and L1-23_f-4) have the ability to induce much more extensive syncytium formation via virion-mediated FFWO (Fig. 4C and E) than the parent virus, L1, can induce (Fig. 5).

With the exception of L1-23_f-1, the site 23 variants selected from L1 have also reverted to different extents with respect to the ability to induce FFWI (Fig. 4). Moreover, the relative efficiencies of these viruses in the induction of this mode of fusion correlate with their abilities to promote FFWO and the resistance of their HA activities to reduced amounts of sialic acid receptors (Fig. 3); i.e., L1-23_f-2 > L1-23_f-4 > L1-23_f-3.

Elution properties of L1-23_f variants. Smith and Hightower (31) have previously shown that L1 is impaired in elution from chicken erythrocytes relative to the wt virus. L1-23_f-2 and L1-23_f-4, on the basis of their minimal NA activity and increased affinity for cellular receptors, would be expected to elute even more slowly. Thus, the elution rates of two of the L1-23_f variants were compared to that of the parent L1 virus (Fig. 6). L1-23_f-2, a strong inducer of both types of fusion, elutes extremely slowly, with less than 5% of the virus eluting in 60 min. L1 and L1-23_f-1, whose HA activities are very sensitive to a reduction in the number of sialic acid receptors on the cell surface (Fig. 3) and have similarly weak fusion-inducing properties (Fig. 4 and 5), also elute much faster from erythrocytes (Fig. 6). The apparent difference in the elution rates of L1 and L1-23_f-1 is not reproducible. In other experiments, the variant eluted faster than the parent virus (data not shown). On the other hand, the extremely low elution rate of L1-23_f-2, relative to the others, was consistent over several experiments (data not shown).

DISCUSSION

The AV isolate of NDV is an effective inducer of FFWI, but the induction of FFWO by its virions has not been demonstrated. L1 is an NA-deficient virus derived from this isolate which has lost the ability to induce the former but has become a moderate inducer of the latter mode of fusion. An MAb to antigenic site 23 was used to select variants of the L1 virus that escaped neutralization by the selecting antibody. Two of these variants were also proficient inducers of both modes of fusion. This phenotype correlates with an increased affinity for sialic acid-containing receptors. A requirement for the attachment function of HN was also indicated from studies of the fusion properties of CV-1 cells persistently infected with human parainfluenza virus type 3 (18), which fuse only with cells having the cell surface receptor for the virus, sialic acid. Our findings suggest that the attachment function of HN not only is required for the induction of fusion but also may be a major determinant of the fusogenic potential of the virus, specifically through the nature of its interaction with cellular receptors.

These findings add to the previous evidence (14) that substitutions in antigenic site 23 influence the receptor recognition properties of the HN spike. Indeed, of eight site 23 variants isolated from either AV or L1, four have demonstrated increased affinity for sialic acid-containing receptors (summarized in Table 3). These viruses had a single amino acid substitution at either residue 193 or 203, suggesting that these two residues strongly influence the attachment function of HN and, in turn, the fusogenic potential of the

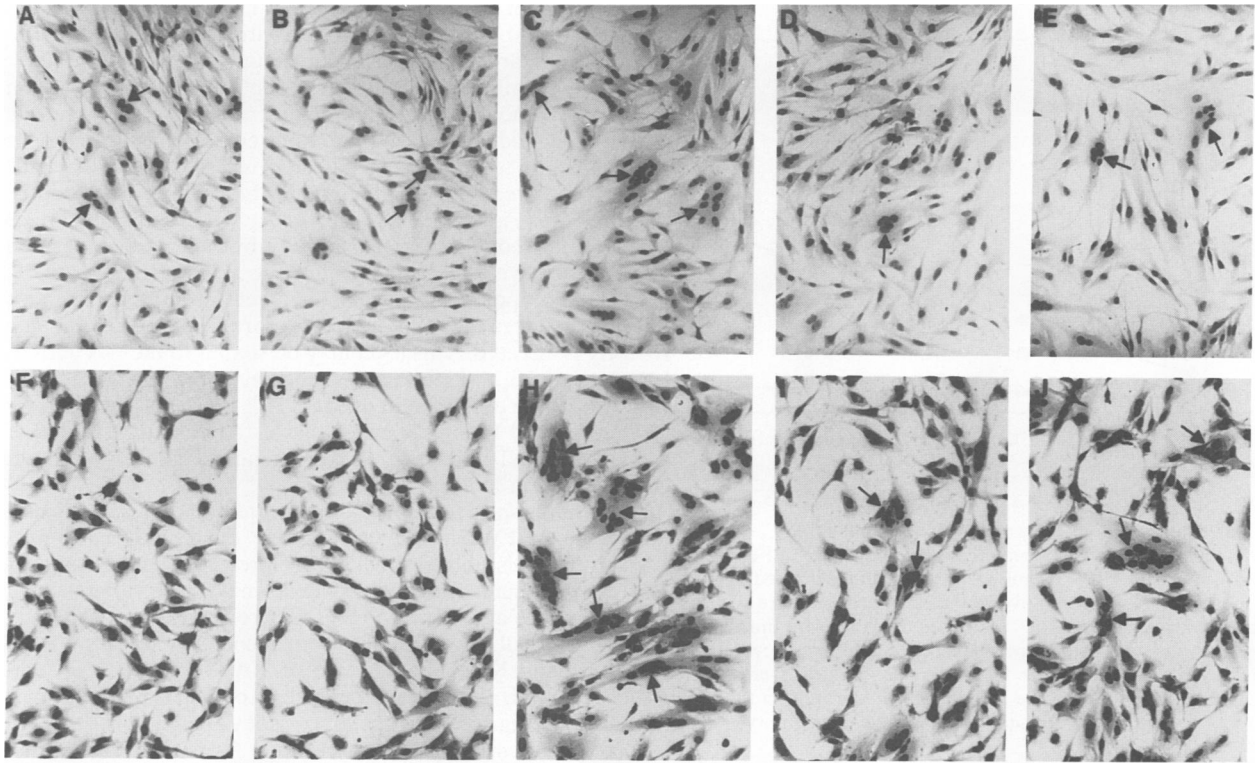

FIG. 4. Induction of FFWO and FFWI by site 23 variants selected from L1. Chicken embryo cells were infected and assayed for syncytium formation in the FFWO (A to E) and FFWI (F to J) assays. The cells were infected with L1 (A and F), L1-23$_f$-1 (B and G), L1-23$_f$-2 (C and H), L1-23$_f$-3 (D and I), or L1-23$_f$-4 (E and J). Syncytia are indicated by arrows.

virus. Moreover, three of the remaining substitutions in site 23 alter the NA activity of HN (Table 3). This means that this region includes a relatively high density of functionally important amino acids: two residues that influence receptor recognition (193 and 203) and two that influence NA activity (194 and 201) within a span of 11 amino acids (193 to 203) in the linear sequence of the protein. Both Chou-Fasman (5) and Garnier (7) analyses predict that, in the HN of the AV isolate, residues 193 and 203 constitute the ends of a long turn connecting two regions of extended coil in a long beta stretch (data not shown). They are also coincident with a peak of hydrophilicity (14). Thus, each residue is exposed, and one can speculate that these residues are part of a receptor-binding pocket lined by the ends of the beta sheet.

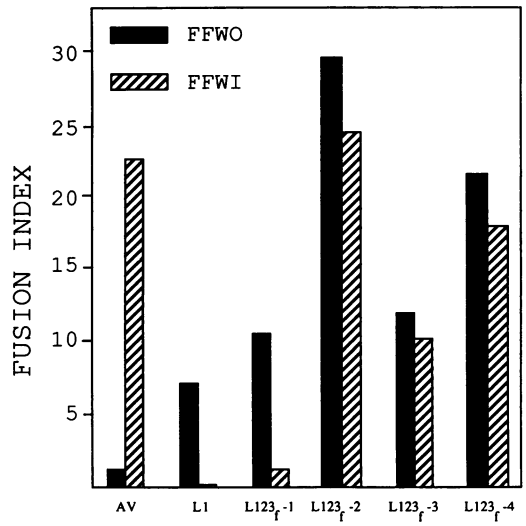

FIG. 5. Comparison of the abilities of site 23 variants selected from L1 to induce both modes of fusion. The bar graph shows the fusion indices (percentages of nuclei involved in syncytia with three or more nuclei) of AV, L1, and the site 23 variants of L1 in assays of FFWO and FFWI.

The proximity of residues that influence the attachment and NA activities of NDV's HN raises anew the long-debated question of the separability of the two sites. Functional inhibition studies with MAbs and analyses of mutants selected with antibodies and inhibitors (summarized in reference 10) have made a strong case for the two sites being separate. However, the domain recognized by site 23 MAbs includes several residues at which substitution influences either attachment or NA. Thus, site 23 may be a region of the molecule involved in both activities. However, the separability of the two sites can still be rationalized with this finding if one assumes a requirement for a conformational change in the molecule in order to bring the NA active site to the bound receptor. It may be that the site 23 MAbs recognize a flexible domain through which the proposed conformational change is transmitted. Clearly, only the determination of the three-dimensional structure of HN will resolve this question.

Bratt and Gallaher (2) postulated that viruses that are good inducers of FFWO spend a prolonged period of time on the cell surface and can be characterized as having a low requirement for sialic acid-containing receptors and a low potential for elution from those receptors. Clearly, the fusion mutants isolated from L1 that have increased receptor-

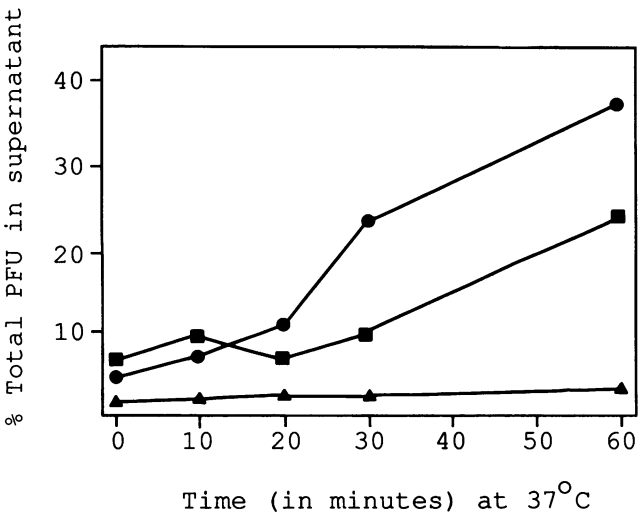

FIG. 6. Elution of L1 (●), L1-23_f-1 (■), and L1-23_f-2 (▲) from chicken erythrocytes. Equal volumes of virions (10^7 PFU/ml) and chicken erythrocytes (2×10^8/ml) were mixed and incubated at 4°C for 1 h. Unbound virus was removed by washing the mixture with cold PBS containing Ca^{2+} and Mg^{2+}. After removal of a zero time sample, the erythrocytes with bound virions were aliquoted into several tubes and shifted to 37°C. At various time intervals, the cells were pelleted from different samples, and the amount of virus present in the supernatant was determined by plaque titration on chicken embryo cells.

binding affinity and decreased NA activity, most notably L1-23_f-2 and L1-23_f-4, support this theory. They have a low requirement for sialic acid receptors (Fig. 3), carry a low potential for elution (Fig. 6), and are strong inducers of FFWO (Fig. 4C and E). However, direct confirmation of this theory will require determination of the effect of the mutations in HN identified here on the fusion properties of eucaryotic cells expressing the HN and F proteins.

We have examined the other NDV isolates whose HN genes have been sequenced (summarized in reference 21) for the presence of isoleucine at residue 193 or tyrosine at residue 203. None is predicted to have an isoleucine at residue 193 of its HN protein. However, two, the LaSota (1946) (21) and Texas-GB (1948) (23) isolates, have a tyrosine residue at position 203, like variant L1-23_f-4, and both are strong inducers of FFWO (3). Again, this is consistent with residue 203 being a determinant of the virus's ability to induce FFWO. Also, preliminary sequence determination of the site 23 region of another virus, the Italy-Milano (1945) isolate, which was also previously shown to be a good inducer of FFWO (3), predicts a histidine at position 203 of its HN protein, a rather conservative change from tyrosine.

TABLE 3. Summary of the effect of amino acid substitutions in site 23 on HN function

Amino acid sequence	Effect of substitution
190 200 210	
NVIFSGCRDHSHSHQYLALGV	
I	Increased affinity for receptors
L	Increased affinity for receptors
F	Increased NA activity
P	Decreased NA activity
P	Increased NA activity
Q	Increased affinity for receptors
Y	Increased affinity for receptors

What are the relative contributions of low NA and increased affinity for cellular receptors to the acquisition of FFWO-inducing activity by the noninducing AV isolate? These studies suggest that either correlates with a moderate capacity to induce FFWO. Variant 23_a-3, which had increased receptor-binding affinity but wild-type NA activity, and L1 (Fig. 2G) and L1-23_f-1 (Fig. 4B), which had at least a 50-fold reduction in NA activity but no increase in binding affinity, were moderately effective inducers of FFWO. Also, there is apparently a threshold level to which the NA activity of the virus must be reduced before FFWO-inducing capacity is affected. This level may be similar to the NA activity of variant 23_a-1 (17% of the wt activity), since this virus induces FFWO to only a limited extent (Fig. 2C). Only when a virion combined both properties, as in the case of L1-23_f-2 (Fig. 4C) and L1-23_f-4 (Fig. 4E), did it induce extensive syncytium formation in assays of FFWO.

Smith and Hightower (30) reported that L1 and N1 did not induce FFWI; those results were confirmed in the present study. It is unlikely that the failure of L1 and N1 to induce FFWI results from their NA deficiency, since variants L1-23_f-2 (Fig. 4H) and L1-23_f-4 (Fig. 4J) are both extremely effective inducers of this mode of fusion, despite having only 6 and 8%, respectively, of the wt level of NA (Table 1). Also, variant 23_a-1, with only 17% of the wt level of NA, is perfectly capable of inducing FFWI. Thus, our studies are not consistent with a requirement for NA for syncytium formation. Indeed, diminished NA activity may have the opposite effect on fusion. It may act synergistically with increased receptor-binding affinity, resulting in a further enhancement of fusogenic activity, as has been suggested for other viruses (16, 27, 28).

We feel that the FFWI-inducing deficiency of L1 is a function of the nature of its attachment to cellular receptors rather than its NA deficiency. When the I-175→M substitution present in L1 is introduced into HN by site-directed mutagenesis and the mutated protein is expressed in COS cells, the gene product not only has minimal NA activity but, more importantly, also fails to adsorb chicken erythrocytes (25). Thus, the nature of HN residue 175 apparently exerts a strong influence on the interaction of HN with cellular receptors, an effect that is not manifested in altered resistance to sialic acid depletion of erythrocyte surfaces (Fig. 3).

The F-193→L substitution in N1 can correct for the inability of the I-175→M mutated protein to bind erythrocytes (25) but not its inability to promote FFWI. However, the F-193→I and H-203→Y substitutions in L1-23_f-2 and L1-23_f-4, respectively, can correct for whatever problem L1 has in promoting FFWI. Moreover, these substitutions overcome the mutual exclusivity of FFWO and FFWI in AV (and L1). It may be that the quality of the interaction between HN and cellular receptors has a strong influence on fusogenicity. Also, another possibility that cannot yet be ruled out is that the mutants recognize a different sialic acid-containing receptor that is resistant to the exogenous NA. However, the fact that AV can compete for the induction of FFWO by L1-23_f-2 (data not shown) argues against this possibility.

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