JOURNAL OF VIROLOGY, Feb. 1989, p. 952–954
0022-538X/89/020952-03$02.00/0

Vol. 63, No. 2

Mutations Located on Both F1 and F2 Subunits of the Newcastle Disease Virus Fusion Protein Confer Resistance to Neutralization with Monoclonal Antibodies

C. NEYT,[1]* J. GELIEBTER,[2] M. SLAOUI,[3] D. MORALES,[4] G. MEULEMANS,[4] AND A. BURNY[1]

Laboratory of Biological Chemistry[1] and Laboratory of Immunology,[3] Free University of Brussels, 67, rue des Chevaux, 1640 Rhode-St-Genèse, and National Institute for Veterinary Research, 1180 Brussels,[4] Belgium, and Rockefeller University, New York, New York 10021[2]

Received 20 June 1988/Accepted 17 October 1988

The fusion gene sequence of six Newcastle disease virus escape mutants revealed that residues important for the integrity of antigenic site 1 and antigenic site 2 were located, respectively, on the F2 subunit and within the cysteine-rich domain of the F1 subunit. We further report the antibody-binding capacity of these mutants.

Newcastle disease virus (NDV) is an avian paramyxovirus the strains of which differ widely in pathogenicity but demonstrate little antigenic variability (19, 27). The fusion protein plays a key role in viral virulence and is a major target for the immune response.

On the one hand, the fusion protein mediates fusion, a process involved in virus penetration and syncytium formation (23). Activation of these functions depends on the specific cleavage of an inactive precursor by a host protease to yield two disulfide-linked polypeptides, F1 and F2 (15, 16, 24). The presence of pairs of basic residues in the cleavage peptide and the accessibility of this region are essential features required for the efficient spread of infection through a wide variety of tissues (8, 26, 28). The cleavage generates, on the F1 polypeptide, a new NH_2 terminus which has been suggested to play an active role in the membrane fusion reaction (1, 20, 21). Computer modeling studies further revealed that the NH_2-terminal peptide adopts an oblique insertion with respect to the lipid acyl chains of artificial membranes. This oblique insertion is expected to destabilize the lipid bilayer (3).

On the other hand, the immunity raised against the fusion protein is effective in the neutralization of NDV infectivity. Chickens were successfully immunized with a recombinant vaccinia virus expressing the fusion protein of NDV strain Italian (13a).

We previously obtained five hybridoma lines producing neutralizing monoclonal antibodies (MAbs) against the fusion protein of NDV (10). One neutralization escape mutant was selected in the presence of each MAb (variants I1C3r, I2C1r, I8B1r, I10F2r, and I12C4r). Cross-neutralization tests (12) suggested that our MAbs were raised against epitopes clustered into two nonoverlapping, operationally defined (30) antigenic sites in the fusion protein: mutants I1C3r, I2C1r, I8B1r, and I10F2r defined antigenic site 1, and mutant I12C4r defined antigenic site 2.

In this study, the results of the operational analysis were substantiated by sequencing the mRNA coding for the fusion protein of six antigenic variants. These were also examined for their antibody-binding capacity.

Sequence analysis. Using a dideoxynucleotide primer extension method (7) (reverse transcriptase; Boerhinger GmbH, Mannheim, Federal Republic of Germany), we sequenced the fusion gene of the I1C3r, I2C1r, I8B1r, I10F2r, and I12C4r variants as well as the fusion gene of a multisite mutant.

(i) Antigenic site 1. The I1C3r variant shows an A-to-G transition (GAC to GGC) leading to an Asp-72-to-Gly substitution. The I2C1r variant shows a GAG-to-AAG codon change resulting in a Glu-74-to-Lys substitution. The I8B1r and I10F2r variants undergo the same nucleotide transversion, C to A (GCG to GAG), and amino acid substitution, Ala-75 to Glu (Fig. 1). Three conclusions arose from this and previous studies (10). The F2 subunit plays a significant role in the structure of site 1; indeed substitutions cluster in the vicinity of the cysteine implicated in the interchain disulfide bond. Moreover, its folding depends on adequate glycosylation (10) and is destroyed by disruption of disulfide bridges (data not shown). We suggest that antigenic site 1 is made up of clusters of residues on F1 and F2, which are brought close to each other by the interchain disulfide bond. Another possibility is that glycosylation, disulfide bridges, and the F2 fragment are required for the proper conformation of this antigenic site. A similar situation has been reported for antigenic site C on the hemagglutinin of influenza virus strain Hong Kong (29).

(ii) Antigenic site 2. The I12C4r mutant undergoes a T-to-C nucleotide transition (TTG to TCG) resulting in a Leu-343-to-Ser substitution. The multisite escape mutant selected from I8B1r with MAbs 1C3, 10F2, and 12C4 showed, besides the expected Ala-75-to-Glu change, an Ala-378-to-Val substitution (GCA to GTA). This change is suspected to be driven by the selection pressure imposed on I8B1r by MAb 12C4, which is the only MAb to neutralize the parental I8B1r mutant population. An alteration in position 343 or 378 may conformationally disturb a distant (12C4) epitope. It should be noted, however, that critical substitutions are located in the cysteine-rich domain of F1 (Fig. 1). Antigenic site 2 is sensitive to denaturation by mercaptoethanol and 1% sodium dodecyl sulfate (10).

Antibody-binding capacity. An enzyme-linked immunosorbent assay (ELISA) and a radioimmunoprecipitation (RIP) assay (10, 13) were performed to characterize the antibody-binding capacity of the variants. A comparison of these data with the previously published (12) neutralizing properties of the mutants is shown in Table 1. The Asp-72-to-Gly substitution did not affect the antibody-binding and neutralizing properties of MAb 12C4 but induced a variable pattern of

* Corresponding author.

FIG. 1. Consensus protein sequence (without the signal peptide) of seven NDV fusion proteins generated from the comparison of four velogenic, one mesogenic, and two lentogenic NDV strains (strains Italian [6], Miyadera [26], Australia Victoria [11], Ulster [14], Beaudette [4], La Sota [9], and D26 [22]). A dash indicates positions at which amino acid changes occurred among the seven NDV fusion proteins. mRNA sequencing of the fusion protein of NDV strain Italian revealed, at position 51, an asparagine (AAC) and not the previously reported aspartic acid (GAC). Potential glycosylation sites are underlined; cysteines are indicated by asterisks; amino acid substitutions detected in antigenic sites 1 and 2 in neutralization escape mutants are indicated by circles and plus symbols, respectively.

reactivities of the MAbs to site 1. Binding detected in either the ELISA or the RIP assay (MAb 2C1 and MAbs 1C3, 8B1, and 10F2, respectively) did not necessarily correlate with efficient neutralization (Table 1). The amino acid substitution was especially efficient in discriminating between MAbs 8B1 and 10F2 whose reactivities were otherwise identical (compare results for various mutants in Table 1). The Ala-75-to-Gly substitution (mutants I8B1r and I10F2r) drastically decreased the neutralizing and antibody-binding (in both the ELISA and the RIP assay) capacities of all MAbs to site 1 (Table 1). The nearby Glu-74-to-Lys alteration (mutant I2C1r) also induced a drastic effect, but one MAb to site 1 (MAb 1C3) bound sufficiently to induce partial neutralization (Table 1). Substitutions at positions 74 and 75 (to site 1) enhanced the neutralization efficiency of MAb 12C4 (to site 2), although its binding was poorly detected by the ELISA (Table 1). This discrepancy may have been related to an altered structure or a poor accessibility of the 12C4 epitope on viruses bonded to the solid substrate (ELISA). The relationship between antigenic sites 1 and 2 was further examined by analysis of mutant I12C4r, which, as compared with the wild-type virus, was neutralized more by some MAbs to site 1 (MAbs 8B1 and 10F2). MAb 2C1 bound extensively in the ELISA, but this feature did not correlate with an increased neutralization capacity.

In conclusion, residues important for the integrity of antigenic site 1 are located on the F2 subunit. Substitutions within the cysteine-rich region of F1 alter antigenic site 2. From cross-neutralization and antibody-binding assays, it is reasonable to assume that antigenic sites 1 and 2 are interrelated. Moreover, the mechanism of neutralization implies antibody binding in a precise structural environment (2, 5, 17, 18, 25). A slight modification of this environment could drastically lower the antibody efficiency in terms of neutralization.

TABLE 1. Reactivity of MAbs with various mutants

Mutant	Assay	Reactivity of the following MAb:				
		1C3	2C1	8B1	10F2	12C4
I1C3r	Neutralization[a]	−	−	+	−	+
	RIP[b]	+	ND	+	+	+
	ELISA[c]	−	+	−	−	+
I2C1r	Neutralization	±	−	−	−	++
	RIP	+	−	−	−	+
	ELISA	−	−	−	−	−
I8B1r and I10F2r	Neutralization	−	−	−	−	++
	RIP	−	ND	−	−	+
	ELISA	−	−	−	−	±
I12C4r	Neutralization	±	±	++	++	−
	RIP	+	ND	+	+	+
	ELISA	−	++	−	−	−

[a] −, Neutralization index (12) of <0.51; +, neutralization to the same extent as for parental strain Italian; ±, poorer neutralization relative to strain Italian; ++, better neutralization relative to strain Italian.

[b] ND, Not done; + and −, immunoprecipitation and no immunoprecipitation of the fusion protein, respectively.

[c] −, No binding; +, binding to the same extent as for parental strain Italian; ±, poorer binding relative to strain Italian; ++, better binding relative to strain Italian.

We thank Le Long for helpful discussions on experimental procedures. We are grateful to C. Wemers for powerful and stimulating discussions, to D. Espion for help, and to Y. Cleuter for valuable technical advice.

Part of this work was supported by a contract from Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture-Smith Kline Animal Health. C.N. is a recipient of an Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture fellowship.

LITERATURE CITED

1. **Barett, T., D. K. Clarke, S. A. Evans, and B. K. Rima.** 1987. The nucleotide sequence of the gene encoding the F protein of canine distemper virus: a comparison of the deduced amino acid sequence with other paramyxoviruses. Virus Res. **8**:373–386.
2. **Blondel, B., R. Crainic, O. Fichot, G. Dufraisse, A. Candrea, D. Diamond, M. Girard, and F. Horaud.** 1986. Mutations conferring resistance to neutralization with monoclonal antibodies in type 1 poliovirus can be located outside or inside the antibody-binding site. J. Virol. **57**:81–90.
3. **Brasseur, R., P. Lorge, E. Goormaghtigh, J.-M. Ruysschaert, D. Espion, and A. Burny.** 1988. The mode of insertion of the paramyxovirus F1 N-terminus into lipid matrix, an initial step in host cell/virus fusion. Virus Genes **1**:325.
4. **Chambers, P., N. Millar, and P. Emmerson.** 1986. Nucleotide sequence of the gene encoding the fusion glycoprotein of Newcastle disease virus. J. Gen. Virol. **67**:2685–2694.
5. **Dimmock, N. J.** 1987. Multiple mechanisms of neutralization of animal viruses. Trends Biochem. Sci. **12**:70–74.
6. **Espion, D., S. de Henau, C. Letellier, C. D. Wemers, R. Brasseur, J. F. Young, M. Gross, M. Rosenberg, G. Meulemans, and A. Burny.** 1987. Expression at the cell surface of native fusion protein of the Newcastle disease virus (NDV) strain Italian from cloned cDNA. Arch. Virol. **95**:79–95.
7. **Geliebter, J.** 1987. Dideoxynucleotide sequencing of RNA and

uncloned cDNA. Focus **9**:5–8.

8. **Kawaoka, Y., C. Naeve, and R. Webster.** 1984. Is virulence of H5N2 influenza viruses in chickens associated with loss of carbohydrate from the hemagglutinin? Virology **139**:303–316.
9. **Le Long, R. Brasseur, C. Wemers, G. Meulemans, and A. Burny.** 1988. Fusion (F) protein gene of Newcastle disease virus: sequence and hydrophobicity comparative analysis between virulent and avirulent strains. Virus Genes **1**:333.
10. **Le Long, D. Portetelle, J. Ghysdael, M. Gonze, A. Burny, and G. Meulemans.** 1986. Monoclonal antibodies to hemagglutinin-neuraminidase and fusion glycoproteins of Newcastle disease virus: relationship between glycosylation and reactivity. J. Virol. **57**:1198–1202.
11. **Mc Ginnes, L. W., and T. Morrison.** 1986. Nucleotide sequence of the gene encoding the Newcastle disease virus fusion protein and comparisons of paramyxovirus fusion protein sequences. Virus Res. **5**:343–356.
12. **Meulemans, G., M. Gonze, M. C. Carlier, P. Petit, A. Burny, and L. Long.** 1987. Pathogenicity of antigenic variants of Newcastle disease virus Italian strain selected with monoclonal antibodies. Ann. Rech. Vet. **18**:371–374.
13. **Meulemans, G., and P. Halen.** 1982. Enzyme-linked immunosorbent assay (ELISA) for detecting infectious laryngotracheitis viral antibodies in chicken serum. Avian Pathol. **11**:361–369.
14. **Millar, N., P. Chambers, and P. Emmerson.** 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.
15. **Morrison, T., L. J. Ward, and A. Semerjian.** 1985. Intracellular processing of the Newcastle disease virus fusion glycoprotein. J. Virol. **53**:851–857.
16. **Nagai, Y., H. D. Klenk, and R. Rott.** 1976. Proteolytic cleavage of the viral glycoproteins and its significance for virulence of Newcastle disease virus. Virology **72**:494–508.
17. **Nicholaisen-Strouss, K., H. Kumar, T. Fitting, C. Grant, and J. Elder.** 1987. Natural feline leukemia virus variant escapes nautralization by monoclonal antibody via an amino acid change outside the antibody-binding epitope. J. Virol. **61**:3410–3415.
18. **Pellett, P. E., K. Kousoulas, L. Pereira, and B. Roizman.** 1985. Anatomy of herpes simplex virus 1 strain F glycoprotein B gene: primary sequence and predicted protein structure of the wild type and of monoclonal antibody-resistant mutants. J. Virol. **53**:243–253.
19. **Reeve, P., and G. Poste.** 1971. Studies on the cytopathogenicity of Newcastle disease virus: relation between virulence, polykaryocytosis and plaque size. J. Gen. Virol. **11**:17–24.
20. **Richardson, C., and P. Choppin.** 1983. Oligopeptides that specifically inhibit membrane fusion by paramyxoviruses: studies on the site of action. Virology **131**:518–532.
21. **Richardson, C., A. Scheid, and P. Choppin.** 1980. Specific inhibition of paramyxovirus and myxovirus replication by oligopeptides with amino acid sequences similar to those at the N-termini of F1 or HA2 viral polypeptides. Virology **105**:205–222.
22. **Sato, H., M. Oh-hira, N. Ishida, Y. Imamura, S. Hattori, and M. Kawakita.** 1987. Molecular cloning and nucleotide sequence of P, M and F genes of Newcastle disease virus avirulent strain D26. Virus Res. **7**:241–255.
23. **Scheid, A., and P. Choppin.** 1974. Identification of the 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**:470–490.
24. **Scheid, A., and P. W. Choppin.** 1977. Two disulfide-linked polypeptide chains constitute active F protein of paramyxoviruses. Virology **80**:54–66.
25. **Stave, J., J. Card, D. O. Morgan, and V. N. Vakharia.** 1988. Neutralization sites of type O1 foot-and-mouth disease defined by monoclonal antibodies and neutralization-escape virus variants. Virology **162**:21–29.
26. **Toyoda, T., T. Sakaguchi, K. Imai, N. Inocencio, B. Gotoh, M. Hamaguchi, and Y. Nagai.** 1987. Structural comparison of the cleavage-activation site of the fusion glycoprotein between virulent and avirulent strains of Newcastle disease virus. Virology **158**:242–247.
27. **Waterson, A. P., T. M. Pennington, and W. M. Allan.** 1967. Virulence in Newcastle disease virus. A preliminary study. Br. Med. Bull. **23**:138–143.
28. **Webster, R., and R. Rott.** 1987. Influenza virus A pathogenicity: the pivotal role of hemagglutinin. Cell **50**:665–666.
29. **Wiley, D. C., and I. A. Wilson.** 1981. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature (London) **289**:373.
30. **Yewdell, J. W., and W. Gerhard.** 1981. Antigenic characterization of viruses by monoclonal antibodies. Annu. Rev. Microbiol. **35**:185–206.