JOURNAL OF VIROLOGY, May 1988, p. 1845–1848
0022-538X/88/051845-04$02.00/0

Vol. 62, No. 5

Evidence for a Complex Structure of Neutralization Antigenic Site I of Poliovirus Type 1 Mahoney

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Received 29 October 1987/Accepted 8 February 1988

We have selected neutralization escape mutants by using a monoclonal antibody (nt-MAb) against a sequential epitope between amino acids 93 through 104 (neutralization antigenic site I) of poliovirus type 1 Mahoney. The majority of mutants were also resistant against five strain-specific nt-MAbs which recognized conformation-dependent epitopes, suggesting that the neutralization antigenic site I must be involved in the formation of such epitopes. An analysis of all mutants by the binding of nt-MAbs and by isoelectric focusing of VP1 allowed discrimination of five classes of mutants. Sequence analysis of mutant RNAs revealed point mutations and deletions in the antibody-binding site.

Poliovirus neutralization epitopes of all three serotypes have been mapped by sequencing neutralization escape mutants to neutralizing monoclonal antibodies (nt-MAbs) (2, 5, 11–13). In the course of these studies it became evident that for poliovirus types 2 and 3, mutations were clustered between amino acids 89 through 100 of VP1, a site which has been defined as neutralization antigenic site I (N-AgI) (22). These results were in contrast to those obtained with poliovirus type 1 Mahoney in which the mutations were distributed over the three major coat proteins and were defined as neutralization antigenic sites II and III (N-AgII and N-AgIII, respectively) (5, 22). It was concluded that the BALB/c mouse is unable to respond to N-AgI of poliovirus type 1 despite the close structural relationship of the serotypes at this site (9). However, all three sites have been mapped on the virion surface by high-resolution X-ray crystallography (8).

Recently, we have described nt-MAbs by using a combined in vivo-in vitro immunization procedure, i.e., in vivo priming with purified polypeptide and in vitro stimulation of splenocytes with infectious virus (21). These antibodies (no. 67, 87, and 95) recognized a sequential epitope within N-AgI between amino acid residues 93 through 104 of VP1 of poliovirus type 1 Mahoney (20). Another nt-MAb (C3) that recognized a sequential epitope within this site of VP1 of poliovirus type 1 was obtained after immunization of BALB/c mice with heat-inactivated virus (1, 18). These were the only reports in which N-AgI was involved in the neutralization of poliovirus type 1 Mahoney. Neutralization escape mutants selected by C3 antibody were still sensitive to other nt-MAbs, demonstrating the independence of this epitope (2).

In the present report, we describe the neutralization escape mutants selected by one of our nt-MAbs (no. 95), which are also resistant to a whole group of nt-MAbs that recognize conformation-dependent epitopes. To our knowledge, this is the first demonstration that N-AgI of wild-type poliovirus type 1 can function as a conformation-dependent neutralization epitope.

To address the question of whether mutations at the binding site of antibody 95 could influence the binding and neutralization of other nt-MAbs prepared against poliovirus type 1, we have isolated 14 neutralization-resistant clones of poliovirus type 1 Mahoney and propagated them according to standard procedures (2, 16). Each clone was assayed for neutralization and antibody binding by 13 nt-MAbs specific for poliovirus type 1 that recognize conformation-dependent epitopes (Fig. 1).

The antibodies were obtained by immunizing BALB/c mice with infectious virus. Antibodies 70, 71, 72, 73, 77, 197, 387, 581, B1, B2, and UC3 were obtained by priming mice with 25 μg of virus subcutaneously in complete Freund adjuvant. Six weeks later they were given booster injections three times intraperitoneally with 50 μg of virus without adjuvant (standard immunization protocol). The splenocytes were fused on day 4 with NS-1 myeloma cells (17; H. Uhlig, Ph.D. Thesis, 1986, University of Hamburg, Hamburg, Federal Republic of Germany). Antibodies 19.2, 19.9, 46.5, and 46.7 were obtained after in vitro stimulation of spleen cells primed in vivo with infectious virus (21). In the case of antibodies 46.5 and 46.7, Ag8.653 myeloma cells were used as fusion partners. According to cross-neutralization and isoelectric focusing (IEF) of escape mutants, antibodies 197 and 46.7 presumably map to N-AgII, and antibodies 387, 581, and 46.5 map to N-AgIII, respectively (data not shown).

Virus neutralization (Fig. 1A) was carried out in a microneutralization test (17). Hybridoma supernatants (100 μl) or a 1/50 dilution of ascites fluid (antibody 95) was incubated with 50 μl of virus (1,000 PFU per ml) for 2 h at 37°C. Then, 100 μl of trypsinized HeLa cells (5×10^5 per ml) was added. After 3 days, cells were stained with crystal violet (0.1% in phosphate-buffered saline [PBS; pH 7.2], 20% ethanol). Four of 14 clones (no. 1 to 4) were still sensitive to all 13 antibodies tested. However, 10 mutant virus clones (no. 5 to 14) were also resistant against five nt-MAbs (no. 70, 71, 72, 73, and 77). According to their reaction patterns against virus and virus-related antigens, these antibodies had already been shown to belong to one group (17), which in addition had a strong specificity for the Mahoney strain of poliovirus type 1 (17).

Antibody binding (Fig. 1B) was measured in an enzyme-linked immunosorbent assay (ELISA) (4). Rabbit anti-poliovirus immunoglobulins were bound to microdilution plates for 3 h at 37°C in 50 mM Tris hydrochloride (pH 9.3). After being washed with PBS-Tween (0.05%), mutant virus-infected cell culture supernatant was added (50 μl) and incu-

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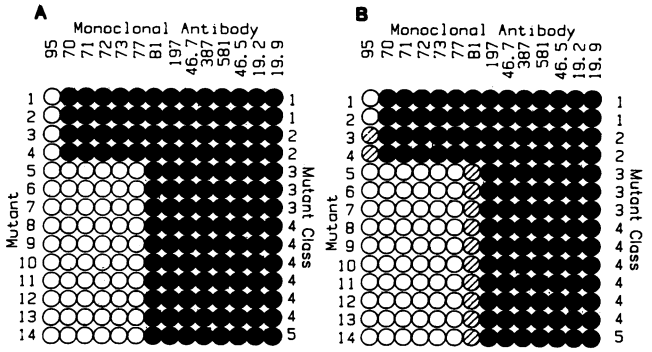

FIG. 1. Microneutralization and antibody-binding patterns of poliovirus type 1 mutants. Poliovirus type 1 mutants selected by antibody 95 were assayed for neutralization (A) and antibody binding (B) by nt-MAbs. Virus neutralization was determined in a microneutralization assay. Antibody binding was assayed in a sandwich ELISA. Symbols: ●, Neutralization and antibody binding (absorbance values of <50% compared with wild type); ○, neutralization resistance and lack of antibody binding (values of <20%); ◌, partial antibody binding (values between 20 to 50%).

bated overnight at 4°C. Plates were washed once with PBS-Tween and incubated with hybridoma supernatant diluted 1/10 in minimum essential medium–2% fetal calf serum–20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.2) for 2 h at 37°C. The plates were washed twice as described above and incubated for another 2 h at 37°C with peroxidase-labeled rabbit anti-mouse immunoglobulins (Dako, Copenhagen, Denmark) diluted 1:500 in PBS–10% newborn calf serum. Results were measured in a multiscan ELISA reader (Flow Laboratories, Meckenheim, Federal Republic of Germany). In the ELISA (Fig. 1B), roughly the same pattern as that in the neutralization test was observed; however, mutant clones 3 and 4 could be distinguished from clones 1 and 2 by still significant binding to the antibody used for the selection. In addition, clones 1 to 4 and 5 to 14 are distinguished by antibody B1, which showed a reduced binding to clones 5 to 14, although they were still neutralized (Fig. 1A). Preliminary analysis of mutant clones by IEF in urea revealed charge shifts of VP1 of 1 unit charge for clones 5, 6, and 7 and of 2 unit charges for clones 8 to 13. From these data, the mutants could be divided into five classes, as indicated in Fig. 1. The evidence for a distinction of class 4 and 5 mutants is given below.

For further analysis, wild-type virus and one member of each mutant class were labeled with [^{35}S]methionine and partially purified by CsCl density gradient centrifugation (1). Labeled virus was assayed after an at least 20-fold dilution in PBS in a protein A-aided microimmunoprecipitation test, which was carried out in microdilution plates (19). Virus (10 μl) was incubated with 100 μl of serial dilutions of hybridoma supernatant of antibody or a 1/10 dilution of ascites fluid (antibody 95) for 1 h at room temperature. Then Formalin-fixed Cowan 1 strain of *Staphylococcus aureus* was added. After 30 min at 4°C, the plates were centrifuged for 15 min at 260 g and 100 μl supernatant was removed for determination of radioactivity.

The results are shown in Fig. 2. In the control (Fig. 2a) in which each mutant was titrated with nt-MAb 19.2, which is not affected by a mutation at this site (Fig. 1), the titration curves obtained with mutant and wild-type virus are almost superimposed, demonstrating the independence of this epitope from N-AgI. The reduced binding of the selecting antibody to class 2 mutants is also reflected in this assay (Fig. 2b). However, this time class 1 mutants also showed a residual binding, although slightly less so.

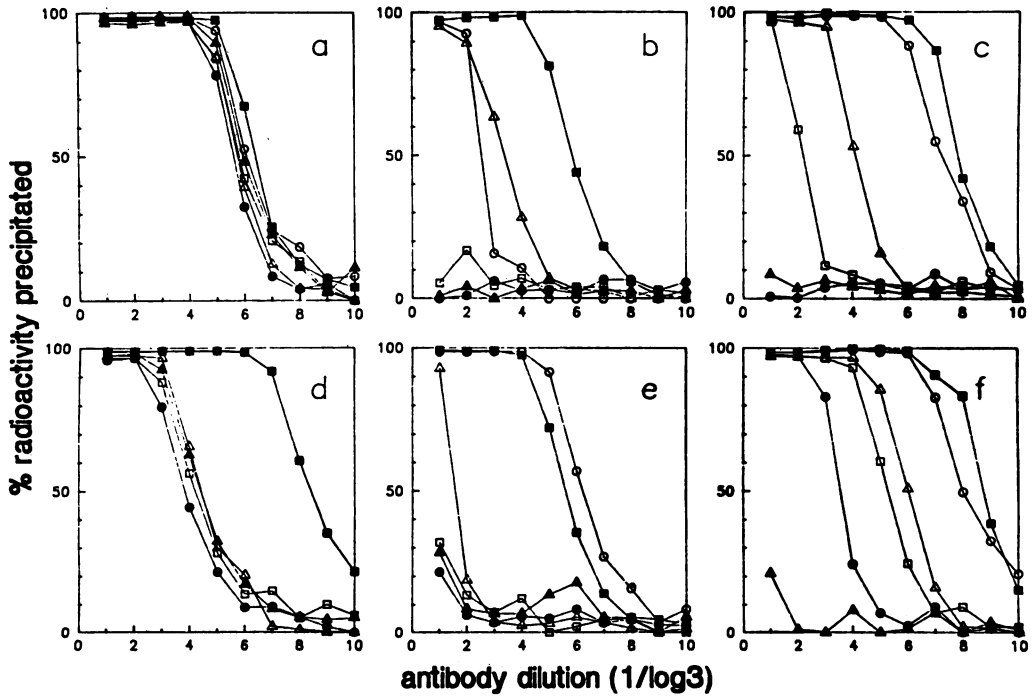

FIG. 2. Immunoprecipitation of wild-type and mutant viruses with nt-MAbs. Protein A-aided immunoprecipitation of [^{35}S]methionine-labeled wild-type (■) and class 1 (○), 2 (△), 3 (□), 4 (●), and 5 (▲) mutants was done with serial dilutions of hybridoma supernatants of antibodies 19.2 (a), 70 (c), B1 (d), B2 (e), and UC3 (f) and with a 1/10 dilution of antibody 95 (b) from ascites fluid.

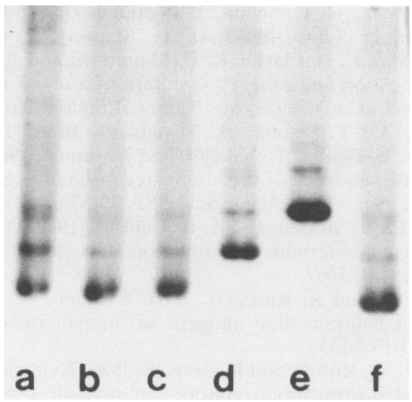

FIG. 3. IEF of wild-type and mutant viruses. [^{35}S]methionine-labeled wild-type virus (lane a) and class 1 (lane b), 2 (lane c), 3 (lane d), 4 (lane e), and 5 (lane f) mutants were analyzed. Only VP1 is shown.

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  2780                2800                Virus/Mutant
    *         *         *
GGAUAACCCAGCUUCCACCACGAAUAAGGAUAAGG   Mahoney
 AspAsnProAlaSerThrThrAsnLysAspLys
        95                100

GGAUAACCCAGCUUUCACCACGAAUAAGGAUAAGC   Class 1  (2)
            Phe

GGAUAACCCAGCUUCCACCACGAGUAAGGAUAAGC   Class 2  (3)
                      Ser

GGAUAACCCAGCUUCCACCACGAAUAAUGAUAAGC   Class 3  (5)
                         Asn

GGAUAACCCAGCUUCCACCACGAAUGAGGAUAAGC   Class 4 (13)
                         Glu

GGAUAACCCAGCUUCCAC···GAAUAAGGAUAAGC   Class 5 (14)
 AspAsnProAlaSerThr   AsnLysAspLys
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FIG. 4. Nucleotide and predicted amino acid sequences of neutralization escape mutants. Poliovirus type 1 mutants selected by antibody 95 were sequenced by the dideoxy-chain termination method (15). Nucleotides were numbered according to Nomoto et al. (14). Base exchanges are underlined. The nomenclature of mutants is as shown in Fig. 1.

The results obtained with antibody 70, a representative of antibodies 70 to 77, reveal a differential binding pattern of the mutants (Fig. 2c), with class 1 mutants being indistinguishable from the wild-type, whereas mutants of classes 4 and 5 were no longer precipitated by this antibody. Class 2 and 3 mutants showed an intermediate binding. In the neutralization test, both class 1 and 2 mutants were sensitive to neutralization to this antibody (Fig. 1A). The lower binding of class 3, 4, and 5 versus class 1 and 2 mutants with antibody B1 (Fig. 1B) was not observed in this assay (Fig. 2d). However, the class 1 mutant behaved like the wild type; the curves are identical. It has to be mentioned that this antibody, in contrast to antibodies 70 to 77, reacted with both the Mahoney and Sabin strains of poliovirus type 1. In Fig. 2e and f, the titers of mutants were determined with two additional Mahoney-specific nt-MAbs against N-AgI; these nt-MAbs were obtained from a different fusion, depicting nicely the proposed hierarchy of the mutants (Fig. 2f).

The analysis of the mutants by IEF is shown in Fig. 3. IEF in urea was done as described previously (7). [^{35}S]methionine-labeled wild-type virus and mutants were dissociated in 9 M urea in the presence of pancreatic ribonuclease for 1 h at 25°C. Samples were reduced with 10 mM dithiotreitol and loaded onto a vertical slab gel which had been prerun for 30 min at 200 V and for 1 h at 400 V. Focusing was done for 1 h at 200 V and 5 h at 400 V. After being fixed, the gel was soaked with Amplify (Amersham Corp., Little Chalfont, United Kingdom) for 30 min and subjected to autoradiography. The mutations of class 1, 2, and 5 mutants did not involve charge shifts of VP1. However, class 3 and 4 mutants revealed charge shifts of -1 and -2 unit charges, respectively.

All mutants of both the Mahoney and Sabin strains selected by C3 antibody had amino acid substitutions at position 100 of VP1, which would result in a charge shift of -1 unit charge (2). Amino acid substitutions due to single point mutations and leading to a charge shift of -2 can only occur at positions of basic amino acids. This would involve substitutions of a lysine residue at position 101 or 103 of VP1.

Recently, we have sequenced the RNA of one member of each mutant class through this region of VP1 by the dideoxy-chain termination method (15), using reverse transcriptase (Promega, Madison, Wis.) and an appropriate oligonucleotide primer. The results are summarized (Fig. 4). Classes 1 and 2 had mutations in positions 2789 (C $\rightarrow$ U) and 2798 (A $\rightarrow$ G), resulting in an amino acid exchange at positions 97 (Ser $\rightarrow$ Phe) and 100 (Asn $\rightarrow$ Ser), respectively. Class 5 mutants had a deletion of three nucleotides, resulting in the loss of one Thr at position 98 or 99. In classes 3 and 4, nucleotide exchanges at positions 2802 (G $\rightarrow$ U) and 2800 (U $\rightarrow$ G) resulted in the exchange of Lys at position 101 to Asn (class 3) or Glu (class 4), which is in accordance with the charge alterations found by IEF. The point mutations (class 3 and 4) and the deletions (class 5) demonstrate sequence changes affecting both a sequential and a conformation-dependent epitope. Furthermore, we have also selected escape mutants with nt-MAb 70. The majority of mutants obtained were also resistant to antibody 95, supporting the close dependence of the epitopes. Obviously, we also have to sequence these mutants to obtain an insight into the structure of these epitopes.

The mutations affecting the epitope of one monoclonal antibody (Fig. 2c), as revealed by a successive loss of affinity and neutralization, offer excellent tools to study the mechanism of poliovirus neutralization and the concomitant structural changes of the virus particle as indicated, for example, by the low pI of neutralized virus (3, 6, 10).

The question of why we found nt-MAbs against N-AgI of poliovirus type 1 by our standard immunization protocol from two different fusions while other investigators failed remains unclear at the moment. A simple explanation for these results might be the criteria applied for the screening and selection procedures of the hybridomas. Another reason could be the fact that the BALB/c mice used for the experiments were derived from different origins.

We thank M. Hilbrig and U. Kuck for excellent technical assistance.

The Heinrich-Pette-Institut is financially supported by the Freie und Hansestadt Hamburg and by the Bundesministerium für Jugend, Familie, Frauen und Gesundheit.

LITERATURE CITED

1. **Blondel, B., O. Akacem, R. Crainic, P. Couillin, and F. Horodniceanu.** 1983. Detection by monoclonal antibodies of an antigenic determinant critical for poliovirus neutralization present

on VP1 and on heat-inactivated virions. Virology **126**:707–710.

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. **Brioen, P., A. A. M. Thomas, and A. Boeye.** 1985. Lack of quantitative correlation between the neutralization of poliovirus and the antibody-mediated pI shift of the virions. J. Gen. Virol. **66**:609–613.
4. **Clark, M. F., and A. N. Adams.** 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. **34**:475–483.
5. **Diamond, D. C., B. A. Jameson, J. Bonin, M. Kohara, S. Abe, H. Itoh, T. Komatsu, M. Arita, S. Kuge, A. Nomoto, A. D. M. E. Osterhaus, R. Crainic, and E. Wimmer.** 1985. Antigenic variation and resistance to neutralization in poliovirus type 1. Science **229**:1090–1093.
6. **Emini, E. A., P. Ostapchuk, and E. Wimmer.** 1983. Bivalent attachment of antibody onto poliovirus leads to conformational alteration and neutralization. J. Virol. **48**:547–550.
7. **Hamann, A., K. J. Wiegers, and R. Drzeniek.** 1977. Isoelectric focusing and 2D-analysis of poliovirus proteins. Virology **78**: 359–362.
8. **Hogle, J. M., M. Chow, and D. J. Filman.** 1985. Three-dimensional structure of poliovirus at 2.9 Å resolution. Science **229**: 1358–1365.
9. **Icenogle, J. P., P. D. Minor, M. Ferguson, and J. M. Hogle.** 1986. Modulation of humoral response to a 12-amino-acid site on the poliovirus virion. J. Virol. **60**:297–301.
10. **Mandel, B.** 1971. Characterization of type 1 poliovirus by electrophoretic analysis. Virology **44**:554–568.
11. **Minor, P. D., D. M. A. Evans, M. Ferguson, G. C. Schild, G. Westrop, and J. W. Almond.** 1985. Principal and subsidiary antigenic sites of VP1 involved in the neutralization of poliovirus type 3. J. Gen. Virol. **65**:1159–1165.
12. **Minor, P. D., M. Ferguson, D. M. A. Evans, J. W. Almond, and J. P. Icenogle.** 1986. Antigenic structure of polioviruses of serotypes 1, 2 and 3. J. Gen. Virol. **67**:1283–1291.
13. **Minor, P. D., G. C. Schild, J. Bootman, D. M. A. Evans, M. Ferguson, P. Reeve, M. Spitz, G. Stanway, A. J. Cann, R. Hauptmann, L. D. Clarke, R. C. Mountford,and J. W. Almond.** 1983. Location and primary structure of a major antigenic site for poliovirus neutralization. Nature (London) **301**:674–679.
14. **Nomoto, A., T. Omata, H. Toyoda, S. Kuge, H. Horie, Y. Kataoka, Y. Genba, Y. Nakano, and N. Imura.** 1982. Complete nucleotide sequence of the attenuated poliovirus Sabin 1 strain genome. Proc. Natl. Acad. Sci. USA **79**:5793–5797.
15. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
16. **Sherry, B., and R. Rueckert.** 1985. Evidence for at least two dominant neutralization antigens on human rhinovirus 14. J. Virol. **53**:137–143.
17. **Uhlig, H., G. Rutter, and R. Dernick.** 1983. Evidence for several unrelated neutralization epitopes of poliovirus type 1, strain Mahoney, provided by neutralization tests and quantitative enzyme-linked immunosorbent assay (ELISA). J. Gen. Virol. **64**:2809–2812.
18. **Van der Werf, S., C. Wychowski, P. Bruneau, B. Blondel, R. Crainic, F. Horodniceanu, and M. Girard.** 1983. Location of a poliovirus type 1 neutralization epitope in viral capsid polypeptide VP1. Proc. Natl. Acad. Sci. USA **80**:5080–5084.
19. **Vrijsen, R., B. Rombaut, and A. Boeye.** 1983. A simple quantitative protein A micro-immunoprecipitation method: assay of antibodies to the N and H antigens of poliovirus. J. Immunol. Methods **59**:217–220.
20. **Wiegers, K. J., and R. Dernick.** 1987. Binding site of neutralizing monoclonal antibodies obtained after in vivo priming with purified VP1 of poliovirus type 1 is located between amino acid residues 93–104. Virology **157**:248–251.
21. **Wiegers, K. J., H. Uhlig, and R. Dernick.** 1986. In vitro stimulation of presensitized mouse spleen cells with poliovirus type 1, Mahoney, and enhancement of poliovirus-specific hybridomas. J. Gen. Virol. **67**:2053–2057.
22. **Wimmer, E., E. A. Emini, and D. C. Diamond.** 1986. Mapping neutralization domains of viruses, p. 159–173. *In* A. L. Notkins and M. B. A. Oldstone (ed.), Concepts in clinical pathogenesis, vol. 2. Springer-Verlag, New York.