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Molecular Basis of Antigenic Structures of Poliovirus: Implications for Their Evolution during Morphogenesis

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Neutralizing monoclonal antibodies against poliovirus type 1 were obtained after conventional immunization or combined in vivo-in vitro immunization. Antibody binding sites were determined by sequence analysis of neutralization-resistant mutants. Site 3 variants had several amino acid substitutions in previously unidentified positions for neutralization resistance. Evidence for a linkage of subsites 3a and 3b is presented. Some site 3b antibodies as defined previously precipitated 14S subunits, although with reduced titers.

The three-dimensional structures of several members of the *Picornaviridae* family, including poliovirus (4, 5), rhinovirus (18), mengovirus (8), and foot-and-mouth disease virus (1), have been resolved at high resolution. Knowledge of the atomic structures of these viruses makes them excellent candidates for the study of the relation between the structure and antigenicity of viral proteins. It also has implications for the understanding of the mechanism of virus neutralization by antibodies and for the development of more efficiently designed new vaccines.

The three major neutralization antigenic sites of poliovirus have been determined by sequencing neutralization-resistant variants selected by a number of monoclonal antibodies (MAbs), predominantly of murine origin. Site 1 was identified by substitutions of amino acid residues 89 to 101, 144, 166, and 253 of VP1 (3, 9–12, 25, 26). Site 2 was identified by substitutions of amino acid residues 164 to 172 and 270 of VP2 and 220 to 226 of VP1 (2, 10, 14, 27). Recently, we and others have presented evidence that the stretch of amino acids 164 to 172 in the E-F loop of VP2 can also function as a linear epitope and can bind neutralizing as well as nonneutralizing MAbs both in the native and in the heat-denatured state (13, 27).

Site 3 was divided into two subsites owing to the lack of cross-reaction of selected variants with a panel of MAbs studied. Site 3a was identified by substitutions of residues 58 to 60, 70, and 73 of VP3 and residues 286 to 290 of VP1 (2, 10, 14). Site 3b was associated with the substitutions of residue 72 of VP2 and residues 76 to 79 of VP3 (2, 10, 14). More recently, we have identified a new site which is recognized by a cross-neutralizing MAb that neutralizes different virus strains of both serotypes 1 and 2. Variants selected with this antibody revealed substitutions in the H-I loops of VP2 and VP3 (21).

Neutralizing MAbs. In Table 1, we have listed seven neutralizing MAbs against site 3 of poliovirus type 1, Mahoney strain, together with their heavy chain isotypes. The evidence for their binding site is given below. In order to avoid any confusion due to the use of different nomenclatures in the literature and due to the results presented here, the designations of antigenic sites in this paper will be restricted to the terms site 1, site 2, and site 3, without considering subsites. One of the seven MAbs is directed against previously defined subsite 3a (see below), whereas the remaining ones are directed against previously defined subsite 3b. Three of them were obtained after combined in vivo-in vitro immunization using infectious virus as antigen.

Selection of neutralization-resistant mutants. A panel of these antibodies was used to select a number of resistant mutants. The mutations conferring neutralization resistance were determined by primer extension sequencing by the dideoxy chain termination method (19). The nucleotide sequence was determined either throughout the polypeptide showing charge alterations in isoelectric focusing, throughout all the regions of each polypeptide known previously to contribute to antigenic sites, or throughout the whole P1 region of the RNA. The nucleotide sequence of the P1 region of the Mahoney strain used in our investigations differed from the published sequence (15) at positions 2257 U → C, 2545 A → G, and 3262 A → U. These three positions, which are silent mutations, also differ from the sequence presented by Kitamura et al. (7). The transition from U → C at position 2133, which is associated with an amino acid change (Phe → Ser) at position 123 of VP3, is also present in the sequence published by Kitamura et al. (7).

The results are summarized in Table 2, in which we have also provided the results obtained by isoelectric focusing for mutant polypeptides. Mutants selected with site 3-specific MAbs had substitutions in VP2 and VP3 which are part of subsites 3a and 3b, respectively. In addition to positions of substitutions already described (2, 10, 14), we found several substitutions which had not been identified before in neutralization release mutants. These include substitutions in VP3, for example at residue 66 of mutant 3, residue 75 of mutants 5, 6, and 19, residue 144 of mutants 13 to 15, and residue 207 of mutants 4 and 7 to 9. Furthermore, we found also a substitution of residue 73 of VP2 (mutants 11 and 12) and of residues 236 and 245 of VP2 (mutants 1 and 21).

Immunological characterization of variants. Representative mutant isolates were tested against all antibodies of this site in a sandwich enzyme-linked immunosorbent assay (ELISA) (20, 25). The results are shown in Fig. 1. Except for MAbs 387 and 581, each antibody revealed its own characteristic binding pattern to site 3 mutants. Pattern 1 is displayed by MAb 50. The binding of this antibody was completely inhibited by the substitution of residue 60 (threonine to alanine) or residue 66 (glutamic acid to aspartic acid) of VP3 or by the substitution of residue 236 (proline to serine) of VP2, which is located in the H strand of this polypeptide. In

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TABLE 1. Isotypes of neutralizing MAbs against poliovirus type 1[a]

MAb code no.	Isotype[b]
50	IgM
387	IgG2a
581	IgG2a
46.5[c]	IgG3
19.2[c]	IgG1
19.9[c]	IgM
21.2	IgG2a

[a] All antibodies were specific for site 3.
[b] Ig, immunoglobulin.
[c] Obtained after combined in vivo-in vitro immunization (24).

addition, the binding of this antibody was partially inhibited by a substitution of residue 75 (lysine to asparagine) of VP3; this inhibition is also demonstrated by an increased neutralization resistance (data not shown). Pattern 2 is displayed by MAbs 387 and 581. Binding of both MAbs was completely inhibited by substitutions of residue 207 (glutamic acid to either aspartic acid or valine) of VP3 and by substitutions of residue 75 (lysine to either asparagine or isoleucine) of VP3. Because the binding patterns of both antibodies were identical in this assay, MAb 387 was omitted in our subsequent studies. Binding of MAbs 19.9 and 46.5 (patterns 3 and 4) was completely inhibited by substitutions of residue 72 (threonine to either lysine or methionine) of VP2 or substitution of residue 73 (lysine to asparagine) and residue 245 (proline to threonine) of VP2. Substitution of residue 245 also inhibited the binding of MAb 7J6 recognizing the intertypic neutralization epitope (data not shown).

Binding of MAb 46.5 was also partially inhibited by substitutions of residue 207 (glutamic acid to aspartic acid) of VP3. Binding of MAb 19.9 was also inhibited by substitution of residue 73 (lysine to arginine) of VP2 and partially inhibited by substitution of residue 75 of VP3 (lysine to asparagine).

Binding of MAb 19.2 (pattern 5) was inhibited by a substitution of residue 144 of VP3 (lysine to either threonine or glutamic acid) but was only partially inhibited by the conservative substitution by arginine in this position. In addition, the binding of this antibody was also inhibited by substitutions of residue 75 (see above) and of residue 73 (serine to cysteine) of VP3; these substitutions also inhibited the binding of MAb 21.2 (pattern 6). Binding of this MAb was also partially inhibited by substitution of residue 72 (threonine to lysine) of VP2.

Development of antigenic sites during morphogenesis. During morphogenesis, poliovirus particles are assembled either directly from pentamers (14S subunits) or through empty capsids or procapsids. For the antigenic characterization of the two different precursors, radioactively labeled virus particles, procapsids, and 14S subunits were isolated from infected cells by sucrose density gradient centrifugation (23). Fractions containing the appropriate particles were pooled and assayed for antibody binding by immunoprecipitation. The results are summarized in Table 3. Obviously, virus particles and procapsids were precipitated by all antibodies listed in the table, with very similar titers. The results demonstrate that all epitopes recognized by our MAbs were also present on procapsids and were equally accessible to the antibody molecules. The binding sites of MAbs 33.5B5 and 35.2B6, which were previously identified as 3b, were found to be very sensitive to denaturation of procapsids (17).

TABLE 2. Nucleotide and amino acid changes of neutralization-resistant mutants of poliovirus type 1

Mutant	Neutralizing MAb	Nucleotide[a]		Amino acid[b]		Charge shift in IEF[c]
		Position	Change	Position	Change	
1[d]	50	1655	C → U	2236	Pro → Ser	—
2	50	1943	A → G	3060	Thr → Ala	—
3	50	1963	A → C	3066	Glu → Asp	—
4	387	2386	G → U	3207	Glu → Asp	—
5	581	1990	A → C	3075	Lys → Asn	VP3 −1
6	581	1989	A → U	3075	Lys → Ile	VP3 −1
7	581	2386	G → C	3207	Glu → Asp	—
8	581	2386	G → U	3207	Glu → Asp	ND[e]
9	581	2385	A → U	3207	Glu → Val	VP3 +1
10	19.9	1164	C → A	2072	Thr → Lys	VP2 +1
11	19.9	1167	A → G	2073	Lys → Arg	—
12	19.9	1168	A → U	2073	Lys → Asn	VP2 −1
13	19.2	2196	A → G	3144	Lys → Arg	—
14	19.2	2196	A → C	3144	Lys → Thr	VP3 −1
15	19.2	2195	A → G	3144	Lys → Glu	VP3 −2
16	19.2	1982	A → U	3073	Ser → Cys	—
17	19.2	1983	G → A	3073	Ser → Asn	ND
18	21.2	1982	A → U	3073	Ser → Cys	ND
19	21.2	1989	A → U	3075	Lys → Ile	VP3 −1
20	46.5	1164	C → U	2072	Thr → Met	—
21	46.5	1682	C → A	2245	Pro → Thr	—

[a] The nucleotide number refers to the position within the poliovirus genome, with the 5′ terminus being nucleotide 1. Change is from wild type to mutant.
[b] The N-terminal amino acids of VP1, VP2, and VP3 are 1001, 2001, and 3001, respectively.
[c] IEF (isoelectric focusing) was done as described previously (27). The charge shifts observed for the viral polypeptides (VP1, VP2, and VP3) are indicated as unit charge alterations towards lower (−) or higher (+) pI values. The absence of any charge alteration (compared with that of the parent virus) is indicated by a dash.
[d] Poliovirus type 1, Mahoney strain, was the parent virus except for mutant 21, which was obtained from the Sabin strain of poliovirus type 1. Antigenic variants were selected according to standard procedures and as described previously (27).
[e] ND, not determined.

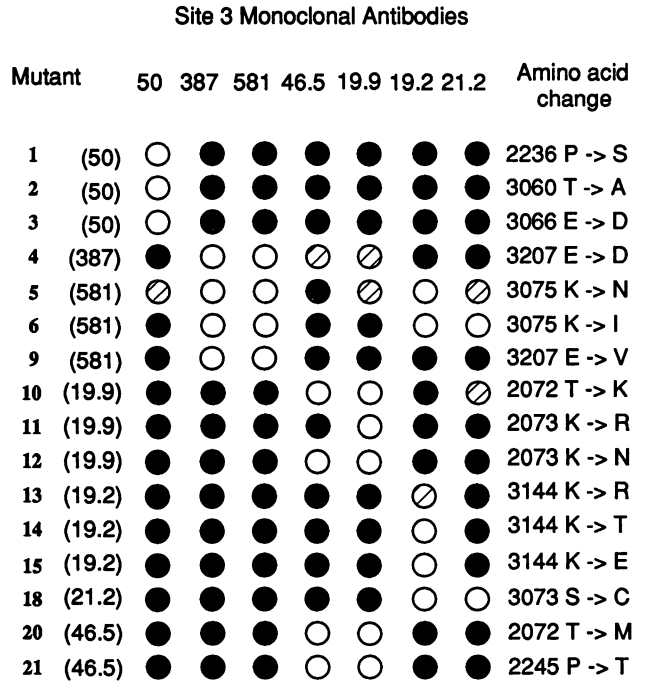

FIG. 1. Antibody binding patterns of resistant mutants of poliovirus type 1. Neutralization-resistant mutants selected with MAbs were assayed in a sandwich ELISA with 10-fold dilutions of hybridoma supernatant (20, 25). Mutants are numbered as in Table 2. MAbs used for the selection are given in parentheses. Symbols: ●, absorbance values >60% of maximum values; ⊘, absorbance values between 20 and 60% of maximum values; ○, absorbance values <20% of maximum values. Included are the amino acid substitutions (single-letter code). The N-terminal amino acids of VP1, VP2, and VP3 are 1001, 2001, and 3001, respectively.

Thus, our results indicated that our procapsid preparation contained predominantly native procapsids.

In contrast to the results just described, 14S subunits were precipitated only by site 1 and site 2 antibodies with titers comparable to those of virus particles or procapsids (data not shown). MAb 50 directed against the previously defined subsite 3a (see above) also precipitated 14S subunits, although with a 100-fold lower titer. Furthermore, in contrast to previous observations which showed that site 3b is created after the assembly of 14S particles (16), MAbs 581 and 21.2, which are directed against this site (see above), precipitated 14S subunits, although with reduced titers. No precipitation of 14S subunits occurred with MAbs 19.2, 19.9, 33.5B5, and 35.2B6.

Discussion. Using our site 3 MAbs, we found in several cases amino acid substitutions in previously unidentified positions responsible for neutralization resistance. Especially, the substitutions of residues 75 and 207 of VP3 were crucial for all of our site 3 antibodies. Both residues are exposed on the surface and presumably are located within the Fab footprints of all our site 3 antibodies, which in addition select for their own characteristic escape mutations. It has been proposed previously that sites 3a and 3b might be linked by appropriate antibodies (14). On the basis of our results, we propose that antigenic site 3 be regarded as a single site. In addition, it has been proposed that site 3b spans the boundary of adjacent (threefold-related) pentamers (14). Evidence for this hypothesis was obtained by analysis of isolated 14S subunits with a panel of site 3b-specific antibodies (16). Here we have demonstrated that 14S subunits were precipitated by some site 3b-specific MAbs. The question of whether the reduction in binding titers is due to the fact that some binding sites are indeed present on 14S subunits, although in a different conformation than is the case with complete capsids, or whether the observed binding is merely due to binding to half sites has yet to be elucidated.

TABLE 3. Immunoprecipitation titers of site 3 MAbs with virus, procapsids, and 14S subunits

MAb	Immunoprecipitation titer[a] ($-\log_{10}$)		
	Virus[b]	Procapsid	14S subunit
50	2.6	2.8	0.6
581	4.5	4.5	1.8
46.5	2.1	1.7	<0
21.2	3.4	3.5	1.0
19.2	3.4	3.2	<0
19.9	2.7	2.3	<0
33.5B5[c]	3.1	2.8	<0
35.2B6[c]	ND[d]	2.8	<0

[a] Immunoprecipitation was on microplates (22, 27). Serial dilutions of ascitic fluids were tested. The dilution precipitating 50% of the antigen (1,000 to 2,000 cpm) was calculated.

[b] Preparations of ^{35}S-methionine-labeled virus, procapsids, and 14S subunits were prepared from infected monolayer cultures of HeLa Ohio cells (27). For the preparation of virus particles, procapsids, and 14S subunits, the cells were thawed and treated with 0.5% Nonidet P-40–0.5% deoxycholate. Cytoplasmic extracts were prepared by centrifugation for 10 min at 10,000 × *g* in an Eppendorf centrifuge at 4°C. Virus particles, procapsids, and 14S subunits were separated by sucrose density gradient centrifugation as described previously (23).

[c] Antibodies were obtained from B. Rombaut and A. Boeyé, Vrije Universiteit Brussel, Brussels, Belgium.

[d] ND, not determined.

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