



5 bh][Yb]WJ UF]Uh]cb`UbX`F Yg]ghUbW`hc`B Yi hf U]nUh]cb`]b`Dc`]cj]fi g`HmdY`%
 5 i h\cf fjt`8Uj]X`7`"8]Ua`cbXž`6f UXZcfX`5`">Ua`Ygcbž`>i hhU`6cb]bž`A]VW]bcf]`? c\ UF Už`G\]bcVi
 5 VYž`<Y]\ UW\]`=hc\ž`Hcg\]\]_c`? ca`Uhgj`ž`A]bYc`5 f]hUž`G\ i gi`_Y`? i [Yž`5_]c`B ca`chcž`5`8`"A`"9`"
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Antigenic Variation and Resistance to Neutralization in Poliovirus Type 1

Abstract. Mutations have been identified in variants of poliovirus, type 1 (Mahoney) on the basis of their resistance to neutralization by individual monoclonal antibodies. The phenotypes of these variants were defined in terms of antibody binding; the pattern of epitopes expressed or able to be exploited for neutralization were complex. Single amino acid changes can have distant (in terms of linear sequence) and generalized effects on the antigenic structure of poliovirus and similarly constituted virions.

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Poliovirus exists in three stable, antigenically distinct serotypes, prototype genomes of which have been sequenced (1-5). The single-stranded, genomic RNA, is enclosed in a naked, tightly packed icosahedral capsid consisting of 60 copies each of four proteins derived by cleavage from a precursor polypeptide (1). Poliovirus is a potent antigen in eliciting a neutralizing response. Neutralizing antigenicity is largely lost when the virion structure is perturbed or completely disrupted (6). However, each of the three large capsid proteins exposed on the surface (VP1, VP2, and VP3) can elicit a weak neutralizing response (7, 8).

The precise definition of the nature and location of those surface features involved in eliciting a neutralizing antibody response to poliovirus type 1 (po-

liovirus 1) (Mahoney) was attempted with neutralizing monoclonal antibodies (N-mcAb's), viral variants resistant to neutralization by individual N-mcAb's, and synthetic peptides derived from the primary sequence of the three major capsid proteins (8-12). Several neutralization antigenic sites (N-Ag's) were identified in poliovirus 1 on all of the major capsid proteins (Fig. 1) by comparison with corresponding synthetic peptides that elicit or prime a neutralizing response in rabbits (11-17). In contrast, there are only two sites in poliovirus 1 against which N-mcAb's have been obtained so far (12, 16, 17).

We have previously characterized a panel of N-mcAb's to poliovirus 1 by their ability to bind, in an enzyme-linked immunosorbent assay (ELISA), to synthetic peptides incorporating capsid sequences. These N-mcAb's were directed against one or the other of two N-Ag's located at amino acids (AA) 70-80 (N-Ag1) and AA 93-103 (N-Ag2) of capsid protein VP1 (12). These N-mcAb's are directed against functionally distinct epitopes as determined by the characterization of nonneutralizable spontaneous variants (11). The N-mcAb's were considered to be directed against the same functional epitope if the same subset of viral variants was resistant to them (16, 17). We sought to further define the various neutralizing epitopes (N-Ep's) by sequencing these variants, following the common assumption that the single point mutations (9, 10) that gave rise to the resistant phenotype would map to the epitope. Here we show that none of these variants had mutations that map to the N-Ag's identified by ELISA, and their phenotypes displayed an unexpected complexity.

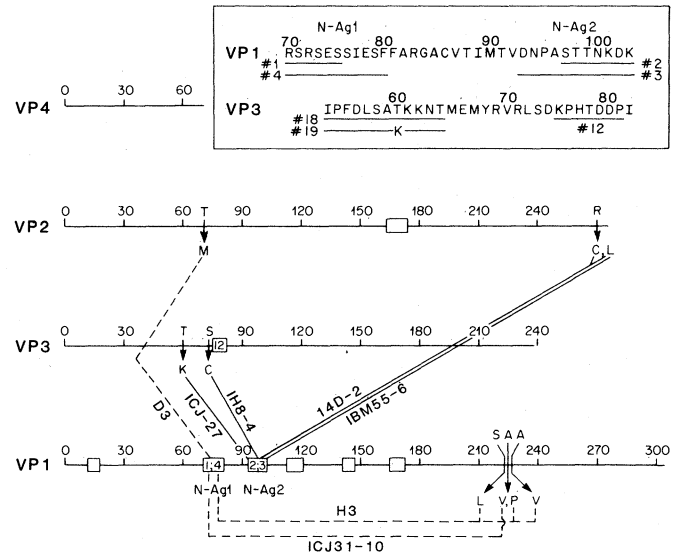
A cluster of mutations at AA 221-223 of VP1 was identified among poliovirus 1 variants resistant to N-mcAb's (H3 and ICJ31-10) directed against N-Ag1 (Table 1 and Fig. 1). This region is one of transition from hydrophilic to hydrophobic character (18) that may not be exposed on the surface of the virion and, therefore, may not be accessible to antibodies. Nonetheless, since this region

varies considerably among the three serotypes (1, 4), it still may influence antigenic structure. The specific amino acid substitutions associated with these resistant phenotypes, serine replaced by leucine (Ser → Leu), and Ala → Val or Ala → Pro, have the potential to significantly alter protein structure, especially in context of the tightly packed virion. Secondary structure analysis of this region (19) predicts that Ala → Pro disrupts the wild-type α helix with a β turn. Although both Ala → Val substitutions increase the probability of a β sheet forming in this region, the α helix is not disrupted and remains in the predicted conformation. More complex interactions between adjoining domains may therefore govern epitope structure. The Ser → Leu substitution, found in conjunction with one of the Ala → Val substitutions (Table 1), seems to be a compensatory mutation in that it is predicted to stabilize the α helix. This suggests that the variant resistant to ICJ31-10 (Table 1) may be the least changed.

The two variants selected on the basis of their resistance to H3 were also resistant to ICJ31-10 while the converse was not the case, a result consonant with the broader binding specificity of H3 (11). Furthermore, immunoprecipitation of [³⁵S]methionine-labeled virions with *Staphylococcus* protein A showed that these variants do not express the epitopes to which the N-mcAb's originally bound (Tables 2 and 3). The three-dimensional structure of VP1 may be modulated by variation in the region AA 221-223 of VP1 such that specific epitopes at AA 70-80 are disrupted. The resistant phenotype of these poliovirus variants (which we refer to as "simple") appears to be due to the loss of antibody binding with no other readily discernible phenotypic alterations.

A variant resistant to D3, another N-Ag1-directed N-mcAb (13), presents a similar case. However, the only mutation we were able to identify in the entire capsid region, a Thr → Met substitution, is in the NH₂-terminal third of VP2 (Fig. 1 and Table 1). The region is also one of transitional hydrophobicity and intertypic variation, and the particular amino acid substitution is predicted to allow the extension of a β sheet. Further evidence that the region surrounding AA 72 in VP2 is not part of the D3 epitope is that the antigen-binding (Fab) fragments of D3 can be specifically cross-linked to VP1 but not to VP2 (10). That amino acid substitutions at sites outside the epitope can alter antibody binding has been demonstrated in tobacco mosaic virus (TMV) (20). Since the TMV capsid consists of a

Fig. 1. The position within the four capsid proteins of neutralization antigenic sites (N-Ag's) and amino acid substitutions found in the variants. Mutations were determined by primer extension and di-deoxy RNA sequencing (34, 35). The N-Ag's are represented as boxes; the numbers within them refer to the synthetic peptides used to define them. Lines, labeled with N-mcAb names, connect the mutations selected by the N-mcAb's with their specific N-Ag's; dashed lines for N-Ag1, solid lines for N-Ag2. (Inset) Segments of VP1 and VP3 amino acid sequence in single letter code (36). The sequences incorporated into the synthetic peptides are underlined. Peptides 1, 2, 3, 4, and 12 are as described (12, 22). Peptides 18 and 19 each contain a tyrosine and glycine residue NH₂-terminal to the indicated sequences that were derived from Mahoney and Sabin strains, respectively, of poliovirus type 1. The peptides were conjugated to bovine serum albumin (BSA) and each injected in complete Freund's adjuvant into four rabbits. No marked neutralizing response was observed to either peptide alone or on subsequent injection of a subimmunogenic dose of either virus (priming). Merrifield synthesis of the peptides, conjugation to BSA, and the schedule of injections were essentially as described (12).



single protein, these effects may be due to interactions within or between individual protein molecules. Expression of the D3 epitope in poliovirus 1 must depend on the interactions between two different capsid proteins.

There are also variants having a simple phenotype among those resistant to N-mcAb's directed against N-Ag2. One variant, resistant to N-mcAb IBM55-6, is similar to the D3-resistant variant in that it has no amino acid substitution in VP1. Instead, it has an Arg → Leu substitution near the COOH-terminus of VP2. An antibody with similar reactivity, 14D2, also selects for variants with amino acid substitutions at this same position; that is, Arg → Cys or Arg → Leu (Table 1 and Fig. 1). This is the only N-mcAb that selected for variants of more than one genotype within the same strain; otherwise these N-Ag2 variants were quite similar to the N-Ag1 variants. The amino acid changes were nonconservative and similarly perturbed the predicted secondary structure. They were located in a region which is neither particularly hydrophilic nor hydrophobic, varied in sequence among the serotypes, and abolished antibody binding.

The N-mcAb's discussed so far neutralize both the Mahoney strain of poliovirus 1 and its derivative, the attenuated type 1 Sabin vaccine strain (21). The remaining N-Ag2 variants are resistant to a family of N-mcAb's directed against determinants specific to the Mahoney strain (functional epitope D, Table 1). Resistance was conferred by either of two mutations, each selected by a particular N-mcAb (Table 1). These amino acid substitutions are in VP3 in a pair of hydrophilic sequences. This suggests that these hydrophilic regions of VP3 may be adjacent to AA 93-103 of VP1 on

the surface of the virion and that the N-Ep's of N-Ag2 include amino acids from some combination of these three regions in a set of discontinuous N-Ep's. A N-Ag is located in the latter of the two sequences, since a synthetic peptide incorporating AA 75-81 of VP3 (Fig. 1) primes for a neutralizing response (22) and a neutralizing antiserum to VP3 (8) binds a longer version of this peptide (23). The other sequence appears not to function as a N-Ag, as synthetic peptides

incorporating AA 53-64 of VP3 (Fig. 1) of either Mahoney or Sabin strain poliovirus 1 do not prime for a neutralizing response in rabbits. We doubt that N-Ag2 contains discontinuous N-Ep's since antibodies elicited by intact virions in mice, rabbits, and humans bind peptides corresponding to the N-Ag2 in VP1 (AA 93-103) but not to peptides representing the N-Ag in VP3 (22, 23).

These variants express a "complex" phenotype. As in the others, these amino

Table 1. Selection and properties of neutralization-resistant variants of poliovirus.

Selecting N-mcAb*	Synthetic peptide bound by the N-mcAb†	Variants			Number of isolates‡
		Mutated protein	Nucleotide change‡	Amino acid change	
D3 (A)	1 (70-75)	VP2	C → U	Thr(72) → Met	2
H3 (B)	1 (70-75) 4 (70-80)	VP1	C → U	Ser(221) → Leu	} 3¶
			C → U	Ala(223) → Val	
			G → C	Ala(222) → Pro	
ICJ31-10 (E)	1 (70-75)	VP1	C → U	Ala(222) → Val	2
1BM55-6 (F)	2 (97-103) 3 (93-103)	VP2	U → C	Silent (in VP1)	} 1
			G → U	Arg(270) → Leu	
14D2 (F)		VP2	C → U G → U	Arg(270) → Cys Arg(270) → Leu	4†† 1††
ICJ27‡‡ (D)	3 (93-103)	VP3	C → A	Thr(60) → Lys	2
ICJ12-9‡‡ (D)	3 (93-103)	VP3	C → A	Thr(60) → Lys	1
IH8-4‡‡ (D)	3 (93-103)	VP3	A → U	Ser(73) → Cys	1
IH8-25‡‡ (D)	3 (93-103)	VP3	A → U	Ser(73) → Cys	1

*The letter in parentheses indicates the functional epitope which the N-mcAb is directed against (9). Antibody preparation was as described (10, 11) or, for 14D2, was done by A.D.M.E.O. †The peptide number is as described (12); numbers in parentheses indicate the amino acids in capsid protein VP1 contained in the peptide (12). ‡U is inserted for 8 of the 11 distinct mutations, four times disrupting the rare dinucleotide CG. §The indicated number of independently derived and plaque-purified isolates (10) were sequenced. The RNA was purified and sequenced directly by primer extension (34). ¶Neutralized both the Mahoney and Sabin strains. †Despite plaque purification all three of these isolates appear to be mixtures of either two single mutant genomes or of a double mutant and wild-type. **This variant was derived from the Sabin strain (34); all others were derived from the Mahoney strain. ††These isolates were not independently derived. ‡‡Neutralized only the Mahoney strain. The brackets indicate double mutants.

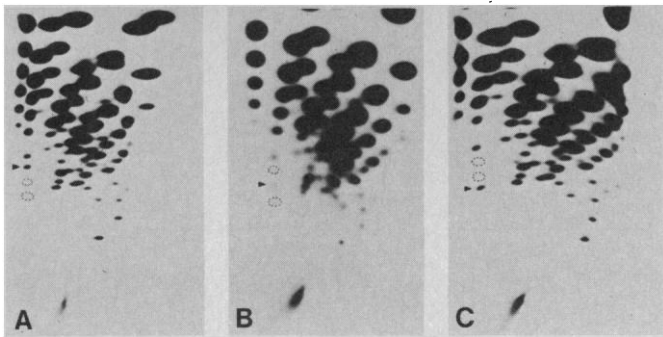


Fig. 2. Fingerprints of genomic RNA's of strains of poliovirus type 1 (25). Mahoney (A); Variant (Thr → Lys) (B); Sabin (C). Oligonucleotide 15' (arrowhead) in fingerprint (A) was derived from the following underlined sequence: CCACAAAAAAAAGAAC. The mutations give rise to ribonuclease-resistant oligonucleotides as follows: CCAAAAAAAAAGAAC Variant; CAAAAAAAAAGAAC Sabin. The positions of the oligonucleotides for the other strains are indicated by dotted circles. The variant (Thr → Lys) fingerprint was similar to that observed with the Mahoney strain at all other positions.

Table 2. Immunoprecipitation (IP) and neutralization (N) of antigenic variants of N-Ag1 (AA 70-80 of VP1). Variants were selected with the N-mcAb's indicated in Table 1.

Antibody	Mahoney		Ala→Val VP1 (222)		Ala→Pro VP1 (222)		Ser→Leu VP1 (221) Ala→Val VP1 (223)		Thr→Met Vp2 (72)	
	IP	N	IP	N	IP	N	IP	N	IP	N
H3	+	+	+	+	-	-	-	-		+
ICJ31-10	+	+	-	-	-	-	-	-		+
D3	+	+	+	+	+	+	+	+	-	-
A13*	+	+	+	+	+	+	+	+		+

*A strain-common N-mcAb directed against another functionally distinct epitope of N-Ag1 (9).

Table 3. Immunoprecipitation (IP) and neutralization (N) of antigenic variants of N-Ag2 (AA 93-103 of VP1). Variants were selected with the N-mcAb's indicated in Table 1.

Antibody	Mahoney		Sabin I		Thr → Lys VP3 (60)		Ser → Cys VP3 (73)		Arg → Leu VP2(270)*	
	IP	N	IP	N	IP	N	IP	N	IP	N
ICJ27	+	+	-	-	-	-	+	-	+	+
ICJ12	+	+	+	-	+	-	+	-	+	+
IH8-4	+	+	-	-	-	-	+	-	+	+
IH8-25	+	+	-	-	-	-	+	-	+	+
1BM55	+	+	+	+	+	+	+	+	-	-
14D2	+	+		+					-	-
1BL25	+	-	+	+	+	+	(+)	-		-
1BA12	+	-	+	+	+	+	+	-	+	-
3D ₈ †	+	-	+	+	+	+	+	-		-
4E ₆ †	-	-	+	+	+	+	-	-		-
1D ₇ †	-	-	+	+	+	+	-	-		-
1D ₄ †	-	-	+	+	+	+	-	-		-

*The Arg → Cys variant had a neutralization pattern similar to that of the Arg → Leu variant and was also not immunoprecipitated by the selecting N-mcAb. †Antibodies prepared by A.D.M.E.O.

Table 4. Neurovirulence testing of the Thr → Lys variant. For in vitro markers (rct, d, and plaque size), days represent days after infection. For neurovirulence markers (lesion score, spread value, and paralysis), cynomolgus monkeys were injected with 10⁷ TCID₅₀ of virus. Observations were made 17 days after infection or at the peak of symptom appearance (25). For lesion score and spread value, units are based on an arbitrary scoring system as described (25).

Virus	rct*	d†	Plaque-size (mm) at 36°C on day				Lesion score‡	Spread value§	Paral- ysis
			3	4	5	7			
Mahoney¶	0.70	0.28	6-8	10-12	17	25			
Mahoney**	0.95	0.79	4-11	6-11	15-20	25	2.19	38.0	3/4
Thr → Lys	0.79	0.48	6-9	10-13	17-18	25	2.09	36.5	4/4
Sabin 1¶	>7.24	4.42	1	3-5	5-8	15			
Sabin 1††	>6.59	4.58	1	1-5	3-8	10-15	0.07	2.0	0/4

*The ratio of virus yield at 36° to 40°C, measured on day 7. †The ratio of virus yield at 0.225 percent to 0.03 percent sodium bicarbonate, measured on day 4. ‡A measure of the severity of the lesions in the central nervous system. §A measure of the spread of infection from the cerebrum through the central nervous system. ||Number of paralyzed monkeys over the total number tested. ¶A reference strain. **Our laboratory strain from which the variant was derived. ††Virus recovered from an infectious complementary clone of the Sabin 1 strain of poliovirus 1 (25).

acid substitutions cause changes in the Chou-Fasman profile. The Thr → Lys change causes an α helix to invade a β turn and the Ser → Cys replacement causes a β sheet to extend into a region of β turns. However, unlike the variants already discussed, resistance to neutralization did not correlate with abolition of antibody binding (Table 4). All four of the selecting N-mcAb's (ICJ27, ICJ12-9, IH8-4, and IH8-25; Table 1) could bind the Ser → Cys variant and one, ICJ12, still bound the Thr → Lys variant. In these cases, however, marked neutralization did not accompany binding.

A Sabin 1-specific N-mcAb will bind, but not neutralize, virus of the Mahoney strain (11, 24). Neutralization can be achieved, however, if the infectious complex of N-mcAb(Sabin) plus virion-(Mahoney) is treated with goat antiserum to mouse immunoglobulin G (IgG) (24). This antibody appears to cross-link the N-mcAb at the surface of the virion, thereby mediating a characteristic shift in the isoelectric point of the complex and neutralization (24). The N-mcAb ICJ12 and the Thr → Lys variant to which it still binds, exhibit this mode of neutralization. That is, the infectious N-mcAb(ICJ12)/ variant(Thr → Lys) complex is neutralized by goat antibodies to mouse IgG. Conversely, the Ser → Cys variant is resistant to this "antiantibody"-mediated neutralization with either the Mahoney- or Sabin-specific N-mcAb's that bind to it.

The variant with the Thr → Lys substitution at AA 60 of VP3 is of further interest as this change is also one of the 21 amino acid replacements found in the attenuated Sabin 1 strain (3). This Thr → Lys variant is susceptible to neutralization by all of the Sabin-specific N-mcAb's in our panel, some of which do not even bind Mahoney strain virions (Tables 2 and 3). The expression of Mahoney- and Sabin-specific N-Ep's are mutually exclusive (11). Thus the Thr → Lys exchange functions like a switch determining which of these two sets of epitopes, Mahoney- or Sabin-

specific, will be expressed. In contrast, the Ser → Cys substitution prevents the neutralization normally accomplished by the binding of these antibodies without changing the repertoire of epitopes expressed. Perhaps a different set of antibodies, indicative of a "new" strain, could bring about neutralization. These data (Tables 2 and 3) indicate that the four N-mcAb's directed against functional epitope D (Table 1) are at least three similar, but different, species.

In light of the limited number of amino acid differences between the attenuated and neurovirulent strains and the ostensibly complete change in characteristic antigenicity exhibited by the Thr → Lys variant it was of interest to see if this variant displayed any degree of attenuation. Both on the basis of *in vitro* markers (rct, d, and plaque size) and direct testing in monkeys (lesion score, spread value, and paralysis) (25) this variant is fully neurovirulent (Table 4). Poliovirus type 1 (Sabin)-specific antigenicity and attenuation are unlinked phenotypes. A serological study of mutants of Sabin 1 virus capable of growth at supraoptimal temperatures has led to the same conclusion (26).

The single nucleotide replacement of cytosine by adenine (C → A) in the ICJ27 variant is one of 55 nucleotide changes observed in the Sabin strain (3). From the known nucleotide sequences of the Mahoney and Sabin RNA's and the variant described here, we predicted and found (Fig. 2) that two-dimensional electrophorograms of oligonucleotides that are resistant to ribonuclease A should display characteristic shifts of a single spot (27).

We have shown that the point mutations conferring resistance to neutralization by N-mcAb's need not be located at the epitope to which the N-mcAb's bind. This runs counter to the common expectation that such single amino acid changes generally cause only minor local distortions of protein structure. Whereas this view has recently been validated for the hemagglutinin of influenza virus, an enveloped virus (28), it has not been demonstrated for the more tightly constrained protein capsids of the nonenveloped viruses, of which poliovirus is, perhaps, an extreme case. Although none of the mutations we found were located at the antibody binding site, this was found to be the case with variants resistant to N-mcAb C3, an antibody that recognizes a denaturation-resistant epitope (29-31).

The prevalence with which mutations conferring antibody resistance occur outside the N-Ag may complicate the

mapping of N-Ep's by nucleotide sequence analysis of variants. For example, the location of the N-Ep's composing an immunodominant N-Ag in poliovirus 3 has been assigned solely on the basis of a clustering of mutations in AA 93-100 of VP1 (32). The N-mcAb's used in that study were similar to ours in that they did not precipitate VP1, and thus it would not be surprising if resistance to them could also be conferred by mutation outside the N-Ag. Perhaps some of the poliovirus 3 N-mcAb's actually bind to epitopes located in a N-Ag other than the one at AA 93-100. We have observed that the immunodominant N-Ag's in poliovirus 1 are rich in β turns while the region containing AA 93 to 100 of poliovirus 3 is largely α helix. Whether the nature of the N-mcAb's [for poliovirus 1, IgG (9); for poliovirus 3, predominantly IgM (32)] or the stability of the variants are major determinants in the selection of neutralization-resistant variants remains unknown. If AA 93-100 in VP1 of poliovirus 3 is the only major N-Ag, the discrepancy in the results for the two serotypes cannot at present be explained.

The existence of epitopes which cannot be exploited for neutralization is not new. However, that a single point mutation can accomplish the interconversion of epitopes through the entire range of neutralization abilities is a novel observation. In addition, these changes do not necessarily alter the primary sequence or local conformation of the individual epitopes. In light of the recent discoveries on the importance of protein backbone flexibility in antigenicity (33) the changes in predicted secondary structure effected by the observed mutations may serve to modulate the stability (flexibility) of the N-Ag's.

That the characteristic antigenicity of the type 1 Sabin strain is not at all associated with its attenuated phenotype is not unexpected. It is alarming, however, that an apparently complete repertoire of Sabin-specific epitopes can be established by a single amino acid change. This restricts the usefulness of these N-mcAb's in measuring the relationship of field isolates to standard strains.

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36. The following abbreviations were used for amino acids: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine.
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