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Three-Dimensional Structure of Poliovirus Serotype 1 Neutralizing Determinants

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Antigenic mutants of poliovirus (Sabin strain, serotype 1) were isolated by the resistance of the virus to anti-Sabin neutralizing monoclonal antibodies. The amino acid replacements within the capsid protein sequence causing the altered antigenicity were identified for each of 63 isolates. The mutations cluster into distinct nonoverlapping peptide segments that group into three general immunological phenotypes on the basis of cross-neutralization analyses with 15 neutralizing anti-Sabin monoclonal antibodies. Location of the mutated amino acid residues within the three-dimensional structure of the virion indicates that the majority of these amino acid residues are highly exposed and located within prominent structural features of the viral surface. Those mutated amino acid residues that are less accessible to antibody interaction are often involved in hydrogen bonds or salt bridges that would stabilize the local tertiary structure of the antigenic site. The interactions of the peptide segments that form these neutralizing sites suggest specific models for the generation of neutralization-resistant variants and for the interaction between the viral surface and antibody.

A virus presents a complex array of antigenic determinants to the immune system of the infected host. Definition of this antigenic structure, with particular emphasis on the neutralizing determinants, is essential for understanding the molecular details of viral pathogenesis and epidemiology and for designing effective therapies and vaccines. Additionally, a thorough description of viral antigenic structure is valuable for the study of viral morphogenesis and virus-host cell interactions.

For poliovirus, the neutralization antigens of the serotype 3 strains have been the most completely analyzed (17, 20). These studies indicate that the neutralizing response in mice to type 3 is dominated by the production of antibodies to a VP1 peptide region that includes amino acid residues 1091 through 1102. (By convention, amino acid residues 1 through 302 from VP1 are designated 1001 through 1302, residues 1 through 272 from VP2 are designated 2001 through 2272, and residues 1 through 237 from VP3 are designated 3001 through 3237.) The dominance of this site has been shown to be due in part to the virus strain used and to the species and strain of the immunized animal (13). In contrast, although a neutralizing monoclonal antibody (MAb) (the C-3 antibody) is known to recognize the corresponding VP1 region in the serotype 1 strains (28, 30), studies with a limited number of serotype 1 neutralizing MAbs and with antisera raised from synthetic peptides have always indicated a more complex antigenic structure (1, 2, 6, 7, 9, 18). Because of this complexity, a complete description of the neutralizing determinants demands the use of a broad panel of neutralizing MAbs and antigenically altered virus variants. We describe here a panel of 15 neutralizing MAbs that were isolated against poliovirus serotype 1 (Sabin strain). These antibodies were used to select and characterize a set of 63 neutralization-resistant variants of the parental virus. Sequence analysis and immunologic characterization of these antigenic isolates identify three neutralization sites in serotype 1 poliovirus. Finally, the sequence analysis of these antigenic variants in the context of the known three-dimensional structure of poliovirus serotype 1 (Mahoney strain) produces a three-dimensional structural characterization for each neutralizing antigenic site. These characterizations demonstrate that two of the three neutralizing sites are formed by peptide segments which are noncontiguous but which are nonetheless located close together on the surface of the virus. In several cases, the structural characterization suggests novel mechanisms for the insensitivity of mutant strains to neutralization by MAbs.

MATERIALS AND METHODS

Cells and media. H1 HeLa cells, SP2/0 myeloma cells, and human fibroblast cells were grown as previously described (25). Medium P5, used for plaque assay, consists of medium A (16) supplemented with 0.0075% DEAE-dextran–0.1% bovine serum albumin–40 mM $MgCl_2$. Unless stated, all dilutions of virus stocks and ascites fluids were made in phosphate-buffered saline containing 0.1% bovine serum albumin (PBSA).

Production of hybridomas. Purified Sabin type 1 poliovirus was used as antigen. Hybridoma 1 was previously described as F7.12 (11). Hybridoma 2 was prepared in a similar manner by J. Icenogle and S.-W. Hong. Hybridomas 3 through 11 were selected as previously described (25), except that the immunization protocol varied. Hybridomas 12 to 15 were from orally immunized mice by a modification of a technique previously described (3) and were kindly provided by Howard Taylor. The antibodies produced by these hybridomas were isotyped by using a peroxidase-based enzyme-linked immunosorbent assay with isotype-specific goat anti-mouse immunoglobulins from Southern Biotechnology Associates, Inc. A total of eight fusions generated the 15 hybridomas used in this study.

Parental virus wild-type strain. The Sabin strain of poliovirus type 1 (LSc-2ab), from E. Seligmann, Jr., Bureau of Biologics, U.S. Food and Drug Administration, was plaque

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purified three times and passaged five times in HeLa cells before preparation of the parental stock used in this study. The parental stock was grown in HeLa cells infected at a multiplicity of infection of 0.6 PFU per cell and harvested after 6.5 h.

Selection of neutralization-resistant variants and preparation of virus stocks. Variants were selected from the wild-type stock by incubating at room temperature for 1 h the parental virus stock (10^4 to 10^6 PFU/ml) with an equal volume of the ascites fluid (1:100 dilution) containing the selecting MAb. Antibody-virus mixtures (200 μl) were inoculated onto HeLa monolayers in 60-mm-diameter tissue culture dishes and allowed to attach for 30 min at room temperature. Monolayers were covered with 2.5 ml of medium P5 containing 0.8% noble agar and then with 2.5 ml of medium P5 containing a 10^{-3} dilution of ascites fluid. After approximately 30 h of incubation at 37°C in 5% CO_2, plaques were visualized with 0.01% neutral red, and agar plugs over well-isolated plaques were transferred to individual tubes containing 1 ml of PBSA. A single plaque was isolated per plate. Virus was released by three cycles of freezing and thawing. For some of the antibodies, two rounds of plaque selection were necessary because of the presence of many tiny wild-type plaques on HeLa plates in the first round.

Isolates 12.1 through 12.4 were selected from individual wild-type plaques. Each plaque (containing about 10^5 PFUs) was mixed with 1/10 volume of a 10^{-1} dilution of ascites fluid containing antibody 12, and the isolates were selected as described above.

Virus stocks were grown on HeLa monolayers in the presence of selecting antibody. Plates were infected with 0.2 ml of the plaque suspension and were incubated under liquid overlay until most of the cells showed evidence of cytopathologic changes. Virus was released from cells by three cycles of freezing and thawing.

Cross-neutralization assay. The titers of antibody-containing ascites fluids were initially determined to ascertain the maximum dilution that protected 5×10^4 HeLa cells from infection by Sabin type 1 poliovirus (multiplicity of infection = 1), and a 10-fold excess of antibody was used in this assay. Ascites fluid diluted in PBSA (50 μl per well) was placed in the wells of a flat-bottomed 96-well tissue culture dish. Mutant virus in medium A (5×10^4 PFU per well, 50 μl) was added to each well, and the dish was incubated for 1 h at 37°C in 5% CO_2. Then 5×10^4 HeLa cells in 50 μl of RPMI 1640 medium were added to each well and incubated at 37°C for 30 h. Wells were stained with crystal violet to demonstrate cell survival. The resistance of each isolate to neutralization was assayed against each of the 15 antibodies.

Preparation of viral RNA. Each virus strain was partially purified from infected cell lysates by high-speed centrifugation and suspended in STE (0.1 M NaCl, 0.01 M Tris hydrochloride [pH 7.9], 0.1 mM EDTA)–0.5% sodium dodecyl sulfate. The RNA was isolated by phenol extraction and ethanol precipitation. Viral RNA isolated in this manner was heavily contaminated with rRNA. This, however, affected neither the clarity nor the specificity of the sequence reactions. RNA from approximately 2×10^8 PFU was used for each sequencing reaction.

RNA sequence reactions. The capsid region was sequenced directly from the RNA genome by a modification of the dideoxy method, using reverse transcriptase. The 14 sequencing primers, spaced at approximately 200 nucleotide intervals throughout the poliovirus capsid sequence, were made by using an Applied Biosystems DNA synthesizer. Sequence reactions using the viral RNA were carried out essentially as those using a DNA template, with empirically determined adjustments of nucleotide concentrations (27). The reactions were performed in batch, using conical-bottomed 96-well microdilution dishes. After the addition of excess deoxynucleotides to chase the reactions, the products were concentrated by evaporation overnight at room temperature. Samples were suspended in 2 μl of formamide-dye mix (80% recrystalized formamide, 0.1% bromophenol blue, 0.1% xylene cyanol FF), heated to 95°C for 3 min, and immediately loaded onto a 5% polyacrylamide sequencing gel.

A few of the sequences obtained by the dideoxynucleotide method were not sufficiently unambiguous for a determination of the amino acid replacement. These were identified by direct sequence analysis of primer-extension products. Primers selected from among the 14 used for the initial sequence analysis were labeled to high specific activity with T4 polynucleotide kinase. The labeled primers were annealed to RNA templates and extended with reverse transcriptase. The products were purified by phenol extraction and ethanol precipitation and used as substrates in chemical degradation sequencing reactions (15).

Polyacrylamide gel electrophoresis. The gel configuration displayed a highly resolved sequence in the range of 50 to 250 bases from the primer with a single loading of each sample and without acid-fixing or drying the gel. The gel composition was 5% acrylamide–0.25% bisacrylamide–7 M urea–1× TBE (0.09 M Tris base, 0.09 M boric acid, 0.002 M EDTA). Gel dimensions were 100 by 33 cm, the upper 50 cm was prepared with a spacer of 0.2-mm thickness, and the lower 50 cm was prepared with a wedge-shaped spacer increasing in thickness from 0.2 to 0.6 mm. Sequence reactions from 10 variants and a parental wild-type control were run together. To emphasize single-base differences among the variants and parental sequences, the sequence reactions were grouped and loaded by nucleotide, i.e., all the G reactions were loaded next to one another, all the A reactions were loaded next to one another, etc. The gels were electrophoresed for 500 W · h, covered with Saran Wrap, and autoradiographed under R-5 X-ray film (35.56 by 91.44 cm; Kodak). Generally the autoradiograms provided a sequence between 30 and 300 bases from the primer. In some cases more than 400 bases of readable sequence was obtained. In all cases the sequences obtained were extensive enough to overlap from one primer to the next, and in most cases they were enough to reconfirm mutant identifications by sequence from two different primers.

Calculations of surface accessibility. Surface accessibility calculations for the poliovirus structure were kindly provided by J. Tainer and E. Getzoff, Research Institute of Scripps Clinic. In preliminary calculations the atomic coordinates for the Mahoney strain of type 1 poliovirus were used to construct the surface which is accessible to a 0.14-nm-radius probe sphere by using the program MS (5). This surface was expressed as a densely sampled set of points. For each point, the location, surface normal, and identity of the generating atoms were determined and tabulated. The surface accessibility for each point on the surface was then evaluated by using a program developed by J. Tainer and E. Getzoff (manuscript in preparation). Briefly, this program uses an efficient algorithm for the analytical determination at each surface point of the largest probe sphere that can be placed tangentially to the surface (i.e., centered along the surface normal) which does not intersect the surface at any other point. The surface accessibility of each amino acid residue was taken to be the largest such

TABLE 1. Origin and isotype of hybridomas

MAb[a]	Immunization protocol	Fusion no.	Antibody isotype[b]
1	3 ip[c] injections	1	IgG2a
2	3 ip injections	2	IgG2a
3	4 ip injections	6	IgG2a
4	4 ip injections	7	IgG2b
5	4 ip injections	6	IgG2a
6	2 ip injections	8	IgG2a
7	2 ip injections	8	IgG2a
8	2 ip injections	9	IgG2a
9	2 ip injections	9	IgG3
10	2 ip injections	9	IgG2a
11	2 ip injections	9	IgG2a
12	Oral	10	IgG2a
13	Oral	11	IgG2a
14	Oral	11	IgA
15	Oral	10	IgG2a

[a] All MAbs used SP2/0 cells as the myeloma fusion partner, with the exception of antibodies 1 and 2, which used P3-NS-1/1Ag4-1. Cells for all fusions were from spleens, with the exception of antibodies 13 and 14 which were isolated from fusions with Peyer's patch and mesenteric lymph node cells.

[b] IgG, Immunoglobulin G.

[c] ip, Intraperitoneal (5 μg per injection).

sphere which could be associated with any of the surface points generated by any atom in the residue.

RESULTS

Isolation of neutralizing MAbs and of neutralization-resistant virus mutants. (i) Selection and isotype of MAbs. Fifteen mouse hybridomas secreting neutralizing MAbs were selected after fusion of myeloma cells with either immune mouse spleen or Peyer's patch and mesenteric lymph node cells (Table 1). These antibodies, derived from a total of eight fusions, were isolated from seven different animals; fusions 10 and 11 used different tissues from the same mouse. This procedure minimized the probability that any of the hybridomas would be siblings.

(ii) Selection of neutralization-resistant viruses. Isolates capable of multiplication in the presence of a single neutralizing MAb were selected from a stock of poliovirus which had been prepared at low multiplicity. These spontaneously arising variants were isolated with frequencies ranging from 10^{-3} to 10^{-6} mutants per PFU. These frequencies are similar to those reported previously for poliovirus (1, 8, 18) and for rhinovirus 14 (25).

Sequence and antigenic characterization of isolates. All the resistant virus strains could be organized into three identical antigenic groups on the basis of two independent lines of evidence: (i) the sequence analysis and (ii) the immunological behavior of each mutant in cross-neutralization assays.

(i) Sequence identification of neutralization-resistant variants. The complete nucleotide sequence for the capsid proteins of each strain was determined, thus identifying all altered amino acids and silent (third-position) base changes in the four capsid proteins (Table 2). Identical amino acid changes were often observed in isolates selected by different MAbs. Different amino acid substitutions were also observed in isolates selected by the same MAb. Twelve of the isolates contained silent third-base changes, and several of the isolates were identified with multiple amino acid substitutions. The majority of the variants showed only single-amino-acid differences from the parental strain, so that the neutralization-resistance phenotype was clearly determined by that specific amino acid substitution. The mutations clustered in discrete regions of the capsid protein sequence: in VP1, residues 1221 to 1226; in VP2, residues 2072, 2164 to 2170, and 2270; and in VP3, residues 3058 to 3060 and 3076.

(ii) Immunological characterization of variants. The resistance of each viral strain to neutralization by each MAb was assessed qualitatively in cross-neutralization assays (Fig. 1). The strains and MAbs were organized into three major groups: group A, variants selected by MAbs 1, 9, 11, and 12; group B, variants selected by MAbs 10, 13, and 15; and group C, variants selected by MAbs 2 through 8. With the exception of isolate 7.3, the variants within each group were defined antigenically by their neutralization resistance to other MAbs within the group and by neutralization sensitivity to MAbs outside the group. Within each resistance group, subpatterns were observed. Because mutations identified in different subpatterns sometimes varied only by the amino acid substituted and not in the position of the mutation (see below), the presence of these subpatterns probably reflects differences in the interactions of specific antibodies with the mutant antigen.

Description of neutralizing antigens. (i) Mutant group A: variants selected by MAbs 1, 9, 11, and 12. The identified mutations cluster at amino acid residues 3058 through 3060. The wild-type Mahoney strain (serotype 1) is also resistant to these MAbs, reflecting the presence at 3060 of a Thr (Mahoney) rather than Lys residue (Sabin) (6). Although single-amino-acid changes are often observed for each neutralization-resistance pattern, the same antibody-resistance pattern can also be observed as the result of two interacting mutations, as seen in isolate 12.1. A change at 3060 from a Lys to a Glu residue is observed in isolates 1.6, 1.7, 11.5, and 12.1. Consequently, virus isolates 1.6, 1.7, and 11.5 are resistant to all four MAbs within this group (subpattern A-1). However, because of the additional change at 3059 from Ala to Gly, isolate 12.1 now is neutralized by MAb 1. This pattern of neutralization (sensitivity to MAb 1 and resistance to MAbs 9, 11, and 12; subpattern A-2) is also observed with isolates 9.1 to 9.5, 11.1 to 11.4, and 12.2 to 12.4; these isolates characteristically show a single-amino-acid substitution of the Lys-3060 residue to an uncharged, polar amino acid (Thr, Gln, or Asn). Thus, the same immunological phenotype can be observed with a single mutation at 3060 (Lys to polar amino acid) or a double mutation at 3059 (Ala to Gly) and at 3060 (Lys to Glu).

(ii) Mutant group B: variants selected by MAbs 10, 13, and 15. This group of variants displays a single neutralization phenotype, resistance to all MAbs in this group (MAbs 13 to 15 and 10). Mutations were identified at one of two locations, Thr-3076 or Pro-2072. Two of the three antibodies (MAbs 10 and 13) select for substitutions at either 2072 or 3076, suggesting that these amino acids from different capsid proteins contribute to the same antigenic site. Thus, this antigenic site is probably formed only within the three-dimensional structure of the virus and satisfies the classical immunological definition of a conformational determinant.

(iii) Mutant group C: variants selected by MAbs 2 through 8. The heterogeneity of neutralization patterns within this group suggests that several peptide segments form a complex site and that a variety of amino acid substitutions are possible. Amino acid substitutions are identified in two capsid proteins at 1221 through 1226, 2164 through 2170, and 2270. Several of the group C isolates contain multiple-amino-acid substitutions (3.1, 8.2, 7.4, 5.4, 2.1, 6.3, and 7.3). However the amino acid substitution responsible for the antibody-resistant phenotype can be identified for the major-

TABLE 2. Nucleotide and amino acid substitutions in poliovirus type 1 (Sabin) neutralization-resistant isolates

Selecting MAb	Isolate no.[a]	Nucleotide change			Amino acid residue[b]	Amino acid substitution		
		Position[c]	WT[d]	Mutant		WT	Mutant	Silent
1	1.1	1957	A	C	3058	Ser	Arg	
	1.3	1959	U	A	3058	Ser	Arg	
	1.4	1957	A	C	3058	Ser	Arg	
	1.6	1963	A	G	3060	Lys	Glu	
	1.7	1963	A	G	3060	Lys	Glu	
9	9.1	1963	A	C	3060	Lys	Gln	
	9.2	1963	A	C	3060	Lys	Gln	
	9.3	1963	A	C	3060	Lys	Gln	
	9.4	1963	A	C	3060	Lys	Gln	
	9.5	1965	A	C	3060	Lys	Asn	
11	11.1	1963	A	C	3060	Lys	Gln	
	11.2	1963	A	C	3060	Lys	Gln	
	11.3	1963	A	C	3060	Lys	Gln	
	11.4	1963	A	C	3060	Lys	Gln	
	11.5	1963	A	G	3060	Lys	Glu	
12	12.1	1963	A	G	3060	Lys	Glu	
		1961	C	G	3059	Ala	Gly	
	12.2	1965	A	C	3060	Lys	Asn	
	12.3	1965	A	C	3060	Lys	Asn	
	12.4	1965	A	C	3060	Lys	Asn	
10	10.1	1184	C	U	2072	Thr	Met	
	10.2	2011	C	U	3076	Pro	Ser	
	10.3	2011	C	U	3076	Pro	Ser	
	10.4	2011	C	U	3076	Pro	Ser	
	10.5	2011	C	U	3076	Pro	Ser	
13	13.1	2011	C	U	3076	Pro	Ser	
	13.2	2011	C	U	3076	Pro	Ser	
	13.3	2011	C	U	3076	Pro	Ser	
	13.4	1184	C	A	2072	Thr	Lys	
	13.5	2011	C	U	3076	Pro	Ser	
15	15.1	2011	C	U	3076	Pro	Ser	
	15.2	2011	C	U	3076	Pro	Ser	
	15.3	2011	C	U	3076	Pro	Ser	
	15.4	2011	C	U	3076	Pro	Ser	
	15.5	2011	C	U	3076	Pro	Ser	
2	2.1	1777	C	A	2270	Arg	Ser	
		2332	A	G	3183	Ser	Gly	
		3114	U	C	1205			Tyr
	2.3	3170	C	U	1223	Ala	Val	
	2.5	3172	C	A	1224	Leu	Ile	
4	4.2	1778	G	A	2270	Arg	His	
	4.3	3170	C	U	1223	Ala	Val	
	4.4	3179	A	U	1226	Asp	Val	
	4.5	3179	A	U	1226	Asp	Val	
5	5.1	1475	C	U	2169	Ser	Leu	
		2879	A	U	1127	Tyr	Phe	
		2817	A	U	1105			Thr
	5.2	1471	A	G	2168	Thr	Ala	
	5.3	1472	C	U	2168	Thr	Ile	
		3216	U	C	1237			Gly
	5.4	1472	C	U	2168	Thr	Ile	
		3164	C	U	1221	Ser	Leu	

Continued on following page

TABLE 2—*Continued*

Selecting MAb	Isolate no.[a]	Nucleotide change			Amino acid residue[b]	Amino acid substitution		
		Position[c]	WT[d]	Mutant		WT	Mutant	Silent
3	3.1	3179	A	G	1226	Asp	Gly	
		2503	U	A	1002	Leu	Ile	
		1548	C	U	2193			Phe
	3.2	1472	C	U	2168	Thr	Ile	
		3354	G	A	1284			Val
	3.3	3179	A	G	1226	Asp	Gly	
		1140	C	U	2057			Asp
		1896	C	U	3037			Pro
	3.4	1471	A	G	2168	Thr	Ala	
		3165	G	A	1221			Ser
	3.5	3179	A	G	1226	Asp	Gly	
8	8.2	1465	A	G	2166	Asn	Asp	
		1016	U	C	2016	Leu	Ser	
	8.3	1478	C	A	2170	Pro	His	
	8.4	1459	G	A	2164	Asp	Asn	
	8.5	1459	G	C	2164	Asp	His	
7	7.1	1471	A	G	2168	Thr	Ala	
	7.3	1964	A	C	3060	Lys	Thr	
		1460	A	C	2164	Asp	Ala	
		2865	U	C	1122			Leu
	7.4	1465	A	G	2166	Asn	Asp	
		1464	C	A	2165	Asp	Glu	
		1554	C	U	2195			His
	7.5	1460	A	U	2164	Asp	Val	
6	6.1	1459	G	A	2164	Asp	Asn	
	6.2	1465	A	G	2166	Asn	Asp	
	6.3	1472	C	U	2168	Thr	Ile	
		1961	C	A	3059	Ala	Glu	
		3036	A	G	1179			Ser
	6.4	1471	A	C	2168	Thr	Pro	
		2943	G	U	1148			Ser
	6.5	1465	A	G	2166	Asn	Asp	

[a] Isolates are named with a code of the form *N.x*, where *N* identifies the number of the selecting antibody and *x* identifies the plaque number.
[b] Each amino acid residue within the capsid protein sequence is identified by a single four-digit number. The first digit refers to the capsid protein in which the residue is found, and the last three digits refer to the location of the amino acid residue within that protein.
[c] The nucleotide number refers to the position of the mutation within the poliovirus RNA genome, with the 5′ terminus being nucleotide 1.
[d] WT, Wild type.

ity of these multiply substituted isolates because other isolates with the same pattern of antibody resistances possess only one of the amino acid substitutions. For example, amino acid substitutions at 1002 and 1226 were identified for variant 3.1 (antigenic subpattern C-4). However, only the Gly substitution at Asp-1226 is observed for variants 3.3 and 3.5 (also antigenic subpattern C-4). Thus the single Asp to Gly substitution at 1226 can produce the observed immunological phenotype and the mutation at 1002 (which is located on the inside of the virus capsid [10]) is probably antigenically silent. Similarly, the Asn to Asp change at 2166 is identified as the resistance-generating mutation for isolates 8.2 and 7.4.

Variant 2.1 (subpattern C-1) is a double mutant with substitutions of Ser at Arg-2270 and of Gly at Ser-3183. Because neither of these specific amino acid substitutions was found as a singly substituted isolate, it is not possible to identify definitively the relevant amino acid. However, a substitution at Arg-2270 is observed in 4.2, indicating that substitutions at this position can lead to a resistant phenotype. In addition, it is probable from the location of residues 3183 (which is at the base of the depression surrounding the protusion at the fivefold axis and is inaccessible to antibodies) and the position and interactions of 2270 within the three-dimensional structure of the virus (see the Discussion) that it is the substitution of Ser at 2270 which is responsible for the resistant phenotype.

Isolate 5.4 contains substitutions in both the VP2 (Thr-2168) and the VP1 (Ser-1221) components of this antigenic site. The single substitution of Ile at Thr-2168, as observed in isolates 3.2 and 5.3, confers resistance to neutralization by MAbs 5 through 8 (subpattern C-8). Thus, the Leu substitution at Ser-1221 confers a partial resistance of isolate 5.4 to neutralization by MAbs 2 and 4. Because isolates containing mutations in 1223 to 1226 can be resistant to neutralization by MAbs 3 and 5 (subpatterns C-1, C-3, and C-4), a singly substituted Leu at Ser-1221 is also likely to be resistant to MAbs 3 and 5. Thus, the presence of two mutations within one antigenic site again (like isolate 12.1 in group A) contributes to the observed resistance pattern such that strain 5.4 is to some extent now resistant to all the group C MAbs. This underscores the importance of determining the entire capsid sequence to identify all altered amino acid residues that could potentially contribute to the observed antigenic phenotype.

Variant 6.3 possesses a phenotype identical to that of variants 3.2 and 5.3 (subpattern C-8). Consistent with the observed sequence changes of strains 3.2 and 5.3, 6.3 also

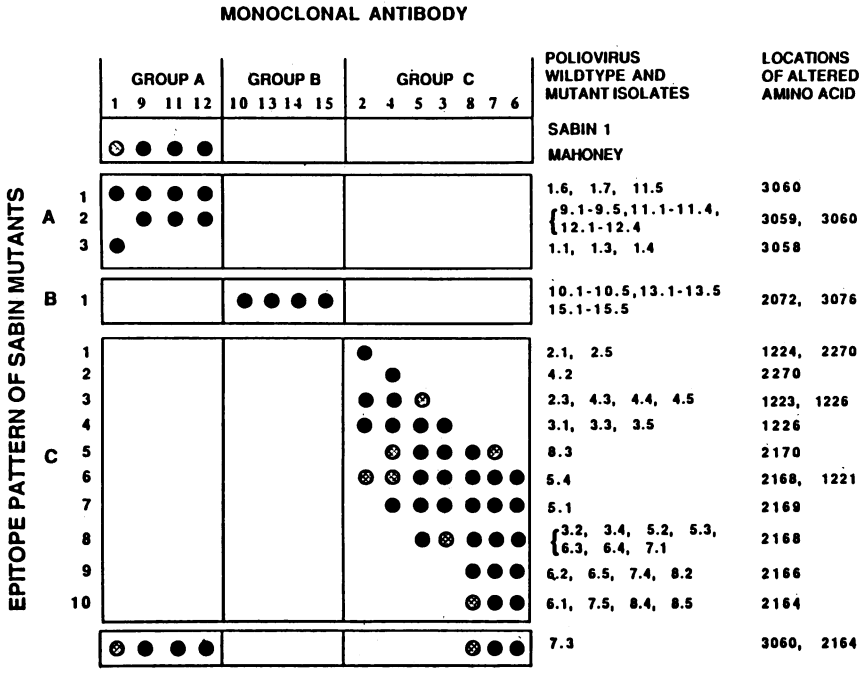


FIG. 1. Antibody-resistance patterns of poliovirus serotype 1 isolates. Antigenic variants were tested for the ability to kill HeLa cell monolayers in the presence of each of the 15 MAbs described in Table 1. Individual isolates showing the same antibody-resistance patterns were grouped into classes. Neutralization is indicated by the absence of a circle, resistance by ●, and partial resistance by ⊛

shows a Thr to Ile change at 2168. Thus, the antigenic profile of 6.3 can be completely defined by this single-amino-acid change at 2168. Surprisingly, 6.3 also shows an Ala to Glu change at 3059. Mutations in 3058 through 3060 have been associated with group A isolates and are resistant to MAbs 1, 9, 11, and 12. Yet strain 6.3 is neutralized by MAbs 1, 9, 11, and 12. Thus, the Ala to Glu change at 3059 is not detected by these group A MAbs and is apparently antigenically silent.

Isolate 7.3 is also a double mutant, showing substitutions of an Ala at Asp-2164 and a Thr at Lys-3060. The behavior of 7.3 to neutralization by MAbs 1, 9, 11, and 12 is identical to that observed with Mahoney which also has a Thr residue at 3060. In addition, the neutralization pattern of isolate 7.3 by MAbs 2 through 8 is identical to that of isolates 6.1, 7.5, 8.4, and 8.5, all of which possess an amino acid substitution at 2164. Thus, mutations in 3058 to 3060 and in 2164 to 2170 appear to behave as independent antigens, and strain 7.3 has a phenotype that is a composite of two antigenic groups.

The variants selected by and those resistant to MAbs 2 and 4 show mutations in VP1 and at 2270. Those virus strains resistant to MAbs 8, 6, and 7 show substitutions in 2164 to 2170. MAbs 3 and 5 select for and recognize mutations in both the VP1 and VP2 regions. The apparent interactions of MAbs 3 and 5 with both the VP1 and VP2 regions indicate that amino acids 1221 to 1235, 2164 to 2170, and 2270 form a single antigenic site.

DISCUSSION

A panel of 15 neutralizing MAbs against the Sabin strain of serotype 1 poliovirus was used to select and characterize a total of 63 antigenic variants. Altered amino acids within the capsid proteins were identified by determining the sequence of the capsid protein region from the mutated RNA genome. Mutations mapped into distinct oligopeptide regions: 1221 to 1226, 2072, 2164 to 2170, 2270, 3058 to 3060, and 3076. Cross-neutralization and sequence analyses define three antigenic domains that correspond to three major neutralizing antigenic determinants for serotype 1 poliovirus. In addition, two of the three mutant groups could be further divided into subgroups on the basis of the pattern of resistances observed in the cross-neutralization assays, thus associating particular amino acid substitutions with specific immunological phenotypes.

By using MAbs made against several different type 1 strains, similar studies had identified substitutions at amino acid residues 1221 to 1223, 2169 to 2170, 2270, 3058 to 3060, 3071, and 3073 (1, 6, 18). However, the limited availability of cross-neutralization data and the incompleteness of sequence characterizations made it difficult to determine which amino acid residues operationally formed segments of the same neutralizing determinant. The mutations described here indicate that certain of the antigenic segments from VP1 and VP2 (1221 to 1223 and 2169 to 2170) are more extensive than previously described. The extensive sequence and immunological analyses described here combined with the aggregate observations from the other studies more clearly define the amino acid segments that form the parts of three neutralizing sites. These sites are distinct from the immunodominant site previously described for serotype 3 (residues 1091 to 1102). To develop a consistent naming of sites, the immunodominant site of type 3 will be defined as site 1. The site defined by the mutations in amino acid residues 1221 to 1226, 2164 to 2170, and 2270 will be defined as neutralizing site 2; the site defined by mutations in amino acid residues at 3058 to 3060, 3071, and 3073 will be named site 3A; and the new site defined by mutations in amino acid residues at 3076 and 2072 will be named site 3B.

Sites 2, 3A, and 3B have been repeatedly observed with several panels of MAbs that were generated from fusions

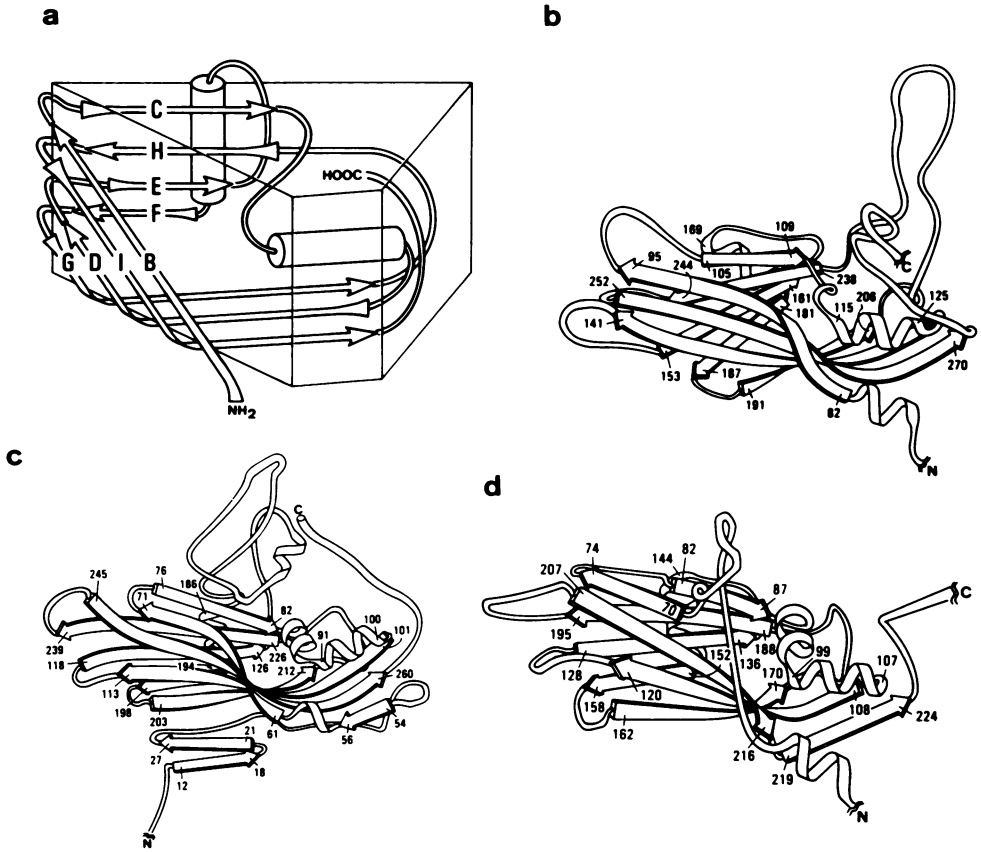


FIG. 2. Schematic representation of poliovirus capsid proteins. (a) Simplified diagram showing the folding pattern of the structurally conserved core. Beta strands are represented as arrows, and the flanking helices are represented as cylinders. Ribbon diagrams show VP1 (b), VP2 (c), and VP3 (d). In general, portions of the proteins shown at the top of each diagram are located at the outer surface of the virus. The amino- and carboxyl-terminus extensions of VP1 and the amino-terminal extension of VP3 have been truncated for clarity (adapted from reference 10; ribbon diagrams drawn by E. Getzoff, Research Institute of Scripps Clinic).

using different tissues, several different immunization protocols, and two different serotype 1 poliovirus strains (Mahoney and Sabin). The reproducible identification of amino acid residues contributing to sites 2, 3A, or 3B in each of the poliovirus serotype 1 studies suggests that these three sites represent the dominant neutralizing sites for the virus in the mouse.

Several sites observed in other studies were not seen here. For serotype 3 poliovirus strains, mutations at 1286 to 1290 have been isolated by using Sabin type 3 MAbs. Cross-neutralization studies have shown that these mutations are linked to site 3A (3058 to 3060) (17, 18). Mutations in the 1286 to 1290 region have not been observed in any of the serotype 1 studies. This is consistent with the suggestion that this component of site 3A is Sabin type 3 specific (18). Additionally, no serotype 1 neutralization-resistant variants were isolated in region 1091 to 1102 (site 1), which is the immunodominant site observed for type 3 strains in mouse sera and which is recognized by the serotype 1 MAb C-3 (28, 30). Consistent with the sequence analyses, the C-3 MAb neutralized all variants described here (unpublished observations). Recently, other MAbs have been isolated that also recognize site 1 (19). However, each of the MAbs that recognized site 1 was isolated from mice immunized with an unusual protocol (e.g., direct intrasplenic injection of the native virus) or with altered viral antigens (e.g., the C-3 antibody was raised against heat-denatured virus). Thus, MAbs recognizing site 1 in serotype 1 poliovirus have been obtained only under unusual circumstances. This is consistent with the observation that mouse sera exhibit only low levels of antibodies that bind to the 1091 to 1102 sequence after intraperitoneal immunization with native serotype 1 virus (13).

Structural descriptions of neutralizing sites. The poliovirus capsid proteins are derived from a large polyprotein by posttranslational proteolytic processing. The capsid precursor P1 (23) or the set of capsid proteins VP4-VP2-VP3-VP1 derived from P1 by proteolytic cleavages, constitute a structural unit called the protomer. During assembly, five protomers associate and rearrange to form a pentameric complex which is apparently an obligatory intermediate in viral assembly (22). Subsequently, 12 pentamers, thus 60 identical copies of the protomer, assemble to enclose the viral RNA in a roughly spherical, icosahedrally symmetric protein shell.

The molecular structure of poliovirus type 1 Mahoney strain has been determined at 0.29 nm resolution (10). The extensive interactions observed among particular capsid proteins have permitted an unambiguous identification of the subunits which constitute the protomer and pentamer substructures. The three major capsid proteins (VP1, VP2, and VP3) share a common structural motif, a wedge-shaped, eight-stranded, antiparallel beta barrel (Fig. 2). These core motifs pack together, with the narrow ends of the wedge pointing toward the particle fivefold and threefold axes,

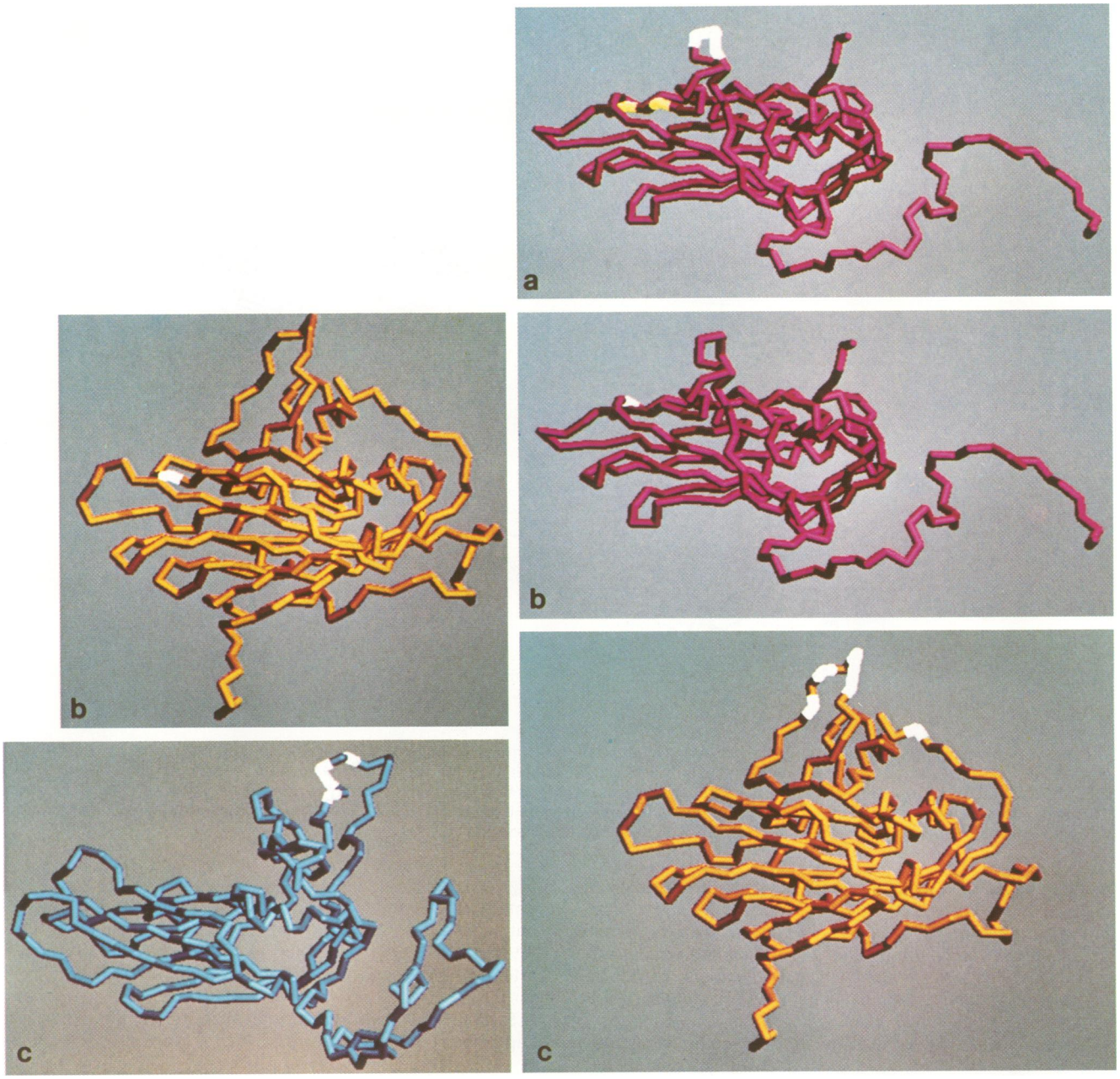


FIG. 3. Alpha carbon models of the major capsid proteins in type 1 poliovirus, showing the location of mutations that confer resistance to MAbs. VP1, VP2, and VP3 are shown in blue, yellow, and red, respectively, in orientations similar to those in Fig. 2. Mutation sites are highlighted in white. (a) Site 3A (yellow highlights indicate residues 3071 and 3073, which were not seen in this study but which have been linked to site 3A in previous studies [see text]). (b) Site 3B. (c) Site 2.

FIG. 4. Location of the immunodominant neutralizing antigenic sites of type 1 poliovirus. (a) A simple geometric figure showing the symmetry and approximate shape of the poliovirus particle. The positions of VP1, VP2, and VP3 that form a single protomer are indicated in relation to the twofold, threefold, and fivefold axes of symmetry of the particle. Vertices of the geometric figure correspond to the prominent protusions which occur at the fivefold and threefold axes of the virion. (b) Space-filling representation of the outer surface of the poliovirion. VP1 is blue, VP2 is yellow, and VP3 is red. Antigenic sites are highlighted in white. (c) Alpha carbon models of four protomers superimposed on the geometric figure. VP1, VP2, and VP3 are blue, yellow, and red, respectively. Each colored circle marks the position of a monoclonal release mutation. Amino acid residues from sites 2, 3A, and 3B are indicated by white, yellow, and cyan (turquoise) circles, respectively. Residue 3071 and 3073, which were not seen in this study but which have been shown in previous studies to be linked to site 3A, are indicated in magenta. The alpha carbon model of an Fab (26) is shown in white to indicate its relative size. The antigen-binding site of the Fab is at the end of the roughly cylindrical Fab which is shown in contact with the schematic representation of the particle. (d) Stereo pictures showing the same four protomers on an expanded scale, to detail the locations of the antigenic sites.

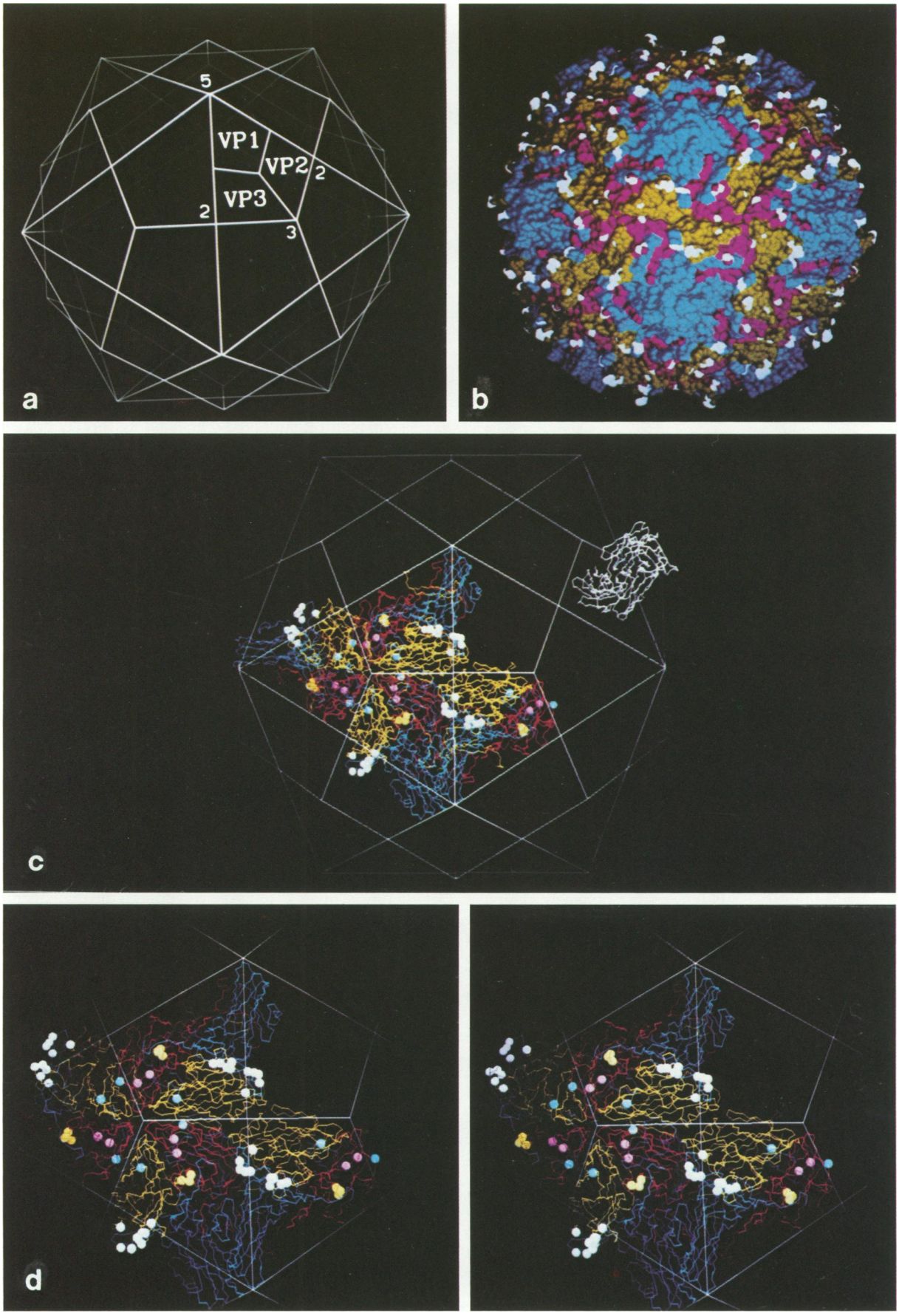

5
VP1
VP2
2
VP3
2
3
a
b
c
d

TABLE 3. Surface accessibility of altered residues in poliovirus I neutralization-resistant variants

Protein	Position	Amino acid	Exposure[a]
VP1	1221	Ser	3.00
	1222	Ala	3.00
	1223	Ala	3.00
	1224	Leu	3.00
	1226	Asp	1.05
VP2	2072	Thr	2.75
	2164	Asp	0.91
	2166	Asn	3.00
	2168	Thr	3.00
	2169	Ser	3.00
	2170	Pro	1.50
	2270	Arg	3.00
VP3	3058	Ser	1.01
	3059	Ala	3.00
	3060	Lys	3.00
	3071	Arg	1.45
	3073	Ser	1.14
	3076	Pro	3.00

[a] The diameter (in nanometers) of the largest hard spherical probe that can be centered on the amino acid residue before touching any of the neighboring residues within the three-dimensional structure of the Mahoney strain of poliovirus serotype 1. The dimensions of an Fab footprint are approximately 3 × 2 nm. Calculations using probes larger than 3 nm diameter were not determined (see Materials and Methods).

forming the continuous protein shell of the virion. The outer surface of the particle is decorated by solvent-exposed loops that connect the polypeptide segments of the conserved core motifs. The mutations that confer resistance to neutralizing MAbs are located in these loops (Fig. 3 and 4).

Three of the largest features on the surface of the virus are (i) a major surface protrusion at each fivefold axis in which the loops connecting the top three pairs of strands at the narrow end of the beta barrel of VP1 are exposed. (ii) A large protrusion near the particle twofold axes formed by the loop connecting the E and F strands (as defined in Fig. 2a) of the beta barrel of VP2 and the other loop connecting the G and H strands of the beta barrel of VP1. (iii) A smaller protrusion near the particle threefold axes in which the loops connecting the top two strands at the narrow end of the beta barrels of VP2 and VP3 and a small loop (residues 3050 and 3070) which precedes the B strand of VP3 are exposed. The most highly solvent-exposed portion of each of these surface features is about the same size as the antigen-binding site of the Fab portion of an antibody molecule (Fig. 4c). These features are separated from one another by deep depressions in the particle surface that are inaccessible to antibody-sized probes.

All of the mutations in serotype 1 poliovirus identified in this study are contained within surface features 2 and 3. (Surface feature 1 includes the major antigenic site of serotype 3 poliovirus.) These mutation sites are described in detail from a structural perspective (see below). Relevant to this description are the location and interactions among amino acid residues from the same antigenic group and the exposure of these residues to solvent or to antibody-sized probes (Table 3). These structural observations are consistent with the information derived from cross-neutralization and sequence analyses, provide insight into the interaction between the virus with neutralizing antibodies, and suggest mechanisms by which some of the variants might escape neutralization.

(i) Site 3A. Residues 3058 to 3060 are located at the top of the small loop (3050 to 3070) which is part of the major surface feature near the threefold axes (Fig. 3a and 4). These three residues are part of a class I beta turn which is stabilized by a hydrogen bond between the carbonyl oxygen of 3058 and the peptide nitrogen of 3061. The side chains of 3058 and 3060 extend out from one face of the loop, whereas the side chain of Ala-3059 extends out from the top of the loop (Fig. 5a). This difference in orientation may be relevant to the observation that mutations in residues 3058 and 3060 play a direct role in escape from neutralization, whereas mutations at 3059 (which have only been seen in multiply substituted variants) modulate the effects of other mutations in the cross-neutralization studies. Residues 3071 and 3073, which are also part of site 3A, are positioned on the same side of the 3050 to 3070 loop as the side chains of 3058 and 3060 (Fig. 5a).

The side chains of 3059 and 3060 are highly exposed and are thus readily accessible to an antibody molecule (Table 3). The side chain of serine 3058 is somewhat less exposed to spherical probes. However, the small size of this fingerlike surface feature might be expected to produce a greater exposure of 3058 (and of nearby residues such as 3071 and 3073) to an irregular probe such as the antigen-binding site of an antibody. In addition, all of the mutations at 3058 result in the substitution of a large side chain (Arg) for Ser at this position, suggesting that the side chain would be more exposed in the variant strains.

The positive charge of the Lys at 3060 may play an important role in the recognition of the Sabin strain by neutralizing antibodies. A substitution of Glu for Lys at this position (isolates 1.6, 1.7, and 11.5) results in escape from neutralization by all four antibodies in group A (MAbs 1, 9, 11, and 12), whereas substitutions of the polar uncharged amino acids Gln (11.1 to 11.4), Asn (12.3 and 12.4), or Thr (Mahoney and 7.3) result in escape from three of the four antibodies in this group (MAbs 9, 11, and 12). As noted above, the role played by residue 3059 in this site is particularly interesting. The rather drastic substitution of a Glu for an Ala (in the multiply substituted isolate 6.3) is antigenically silent for all site 3A specific antibodies, whereas the nominally more conservative substitution of Gly for Ala (in the double mutant 12.1) enables MAb 1 to neutralize isolate 12.1 despite the additional substitution of Glu for Lys at 3060. A glycine at the second position of the beta turn provides increased freedom to the mainchain of the turn. The substitution of a Gly at 3059 might allow a conformational adjustment that eliminates the unfavorable effect of a Glu at 3060 on the interaction of strain 12.1 with MAb 1.

The kinetics and stoichiometry of neutralization by MAb 1 have been extensively studied (12). These investigators showed that approximately four antibody molecules are required to neutralize each virus and that the antibody neutralizes the monomeric fraction of virus under conditions wherein both antigen-binding sites of an antibody molecule are attached to a single virion. In addition, they suggested that the antibody binds to a pair of sites related by a particle twofold axis and proposed that this antibody neutralizes virus by cross-linking pentamers. In the virus, the twofold related copies of site 3A are located approximately 11 nm apart, which is within the allowed range of separation of the two antigen-binding sites in an antibody molecule. Modeling studies indicate that there are no obstacles to bivalent attachment at this site (A. J. Olson and J. M. Hogle, unpublished observations).

(ii) Site 3B. The mutations which occur in site 3B are the least diverse group in this study. MAbs 10, 13, and 15 are sensitive to mutations at either 2072 or 3076. Residue 2072 is located in the loop that connects the outermost pair of strands (strands B and C) of the beta barrel of VP2, and residue 3076 occurs in the corresponding loop in the beta barrel of VP3 (Fig. 3b). Thus, these residues make up part of the prominent surface feature near the threefold axis (Fig. 4). Both side chains are sufficiently exposed to be completely accessible to an antibody-sized probe (Table 3). It is interesting that in the intact virus the distance between 2072 and 3076 from the same protomer (approximately 3.0 nm) is large with respect to the size of an antigen-binding site of an Fab. In contrast the distance between 2072 and 3076 from adjacent (threefold-related) pentamers is much shorter (approximately 1.2 nm) (Fig. 4d). This suggests the likelihood that site 3B may actually span the boundary between pentamers in the virus.

As defined by the mutations within this study, the sites recognized by groups 3A and 3B antibodies are separated by 2.5 to 3.0 nm in the virion. This distance is about the same as the diameter of the antigen-binding site of an Fab and is thus at the upper limit allowed for subcomponents of a single antigenic site. Residues 3071 and 3073, which have been shown to be part of site 3A by cross-neutralization studies (6, 18), are located midway between sites 3A and 3B in the structure (Fig. 4d), indicating that these two sites may eventually be linked by the appropriate antibody. However, despite the number of mutations (18 group 3A mutants and 15 group 3B mutants) and of MAbs (four group 3A and four group 3B) used in the study reported here, we have yet to identify a virus variant which is capable of simultaneously conferring resistance to both a group 3A and a group 3B antibody. Thus, at least for the present, sites 3A and 3B are operationally defined as distinct antigenic sites.

(iii) Site 2. The site recognized by group 2 antibodies is complex. It is formed by three distinct polypeptide segments, namely residues 1221 to 1226, residues 2164 to 2170, and residue 2270 (Fig. 3c). Residues 1221 to 1226 occur in the large loop that connects the G and H strands of VP1. Residues 2164 to 2170 are situated at the top of the large double loop (residues 2127 to 2185) that connects the E and F strands of the beta barrel of VP2. Residue 2270 is located near the exposed carboxyl terminus of VP2. In the native virus these peptide segments lie close together and form much of the highly exposed surface of the large feature near the particle twofold axis (Fig. 4).

Most of the residues in this site (1221, 1223, 1224, 2166, 2168, 2169, and 2270) that have been identified as mutations are highly exposed and thus are likely to participate directly in antibody binding (Table 3, Fig. 5b). Residues 2166 to 2169 form a beta turn that is closed by a hydrogen bond between the carboxyl oxygen of 2166 and the peptide nitrogen of 2169 (Fig. 5c). It may be significant that the side chains of Asn-2166, Thr-2168, and Ser-2169 (which all have been observed as mutation sites) are all located on the same face of the beta turn, whereas the highly exposed side chain of Gln-2167 (which has not yet been identified as an escape mutation) extends out from the other face of this turn (Fig. 5b and c). The side chains of 2166, 2168, and 2169 may define the face of this loop that is preferred for interactions with antibodies. It is also noteworthy that several of the escape mutations (specifically the change of Thr-2168 to Ile in variants 3.2, 5.3, 5.4, and 6.3 and the change of Ser-2169 to Leu in variant 5.1) would place large hydrophobic side chains in positions that are highly exposed to solvent in the native structure. Although these changes may affect antibody binding directly, it is also possible that they cause substantial local conformational changes within the loop which allow the hydrophobic side chains to be buried, thus disrupting the structure of the antigenic site.

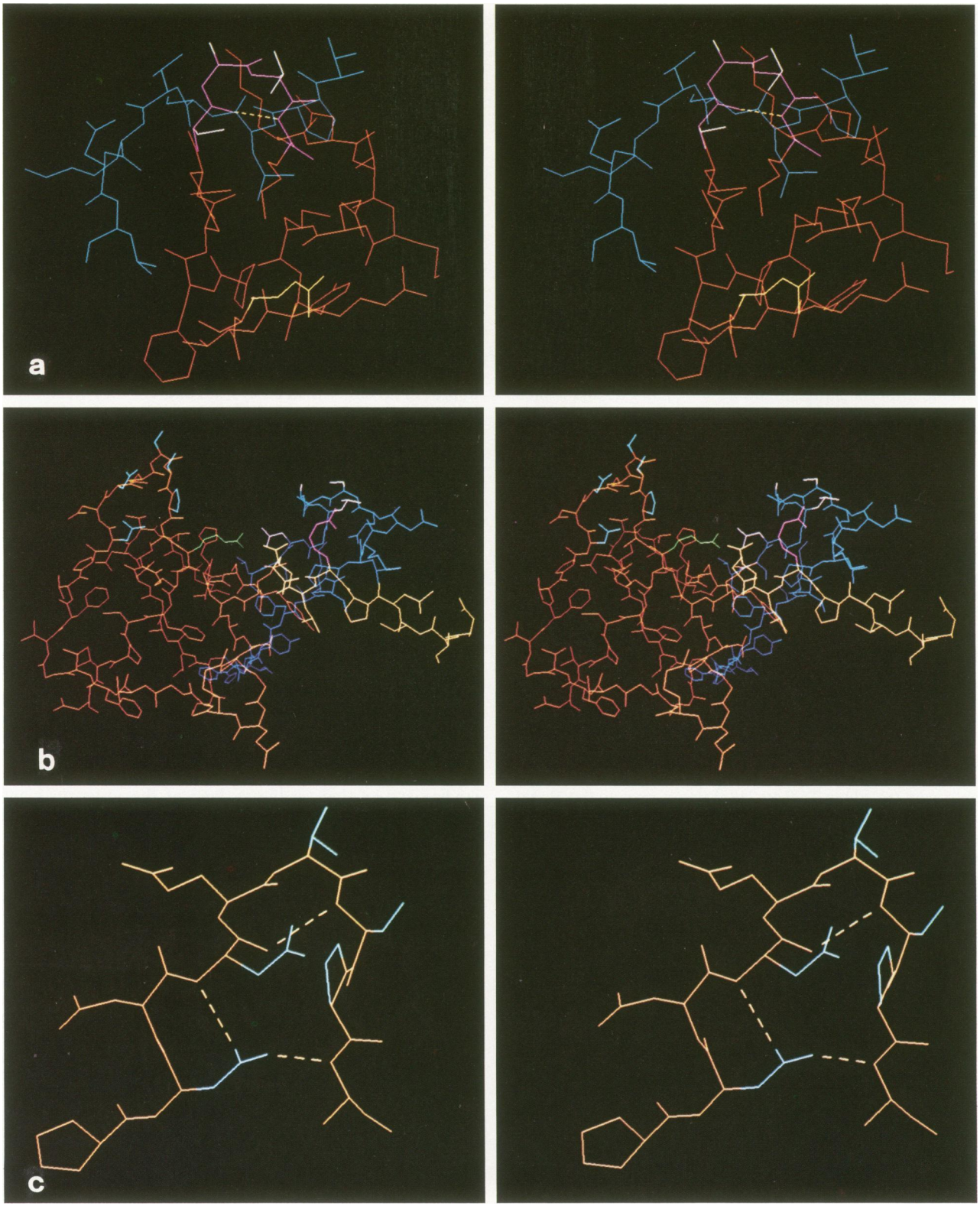
Residues 1221 to 1226 form a single turn of irregular helix (Fig. 5d). This helix is immediately preceded by an unusual beta turn between 1217 and 1220. The unusual geometry of this turn results in a close approach of the carbonyl oxygens of residues 1217, 1218, and 1220 on one face of the turn. The side chain of Arg-2270 approaches this face of the turn, and the guanidinium nitrogens participate in a network of hydrogen bonds with the carbonyl oxygens (Fig. 5d). Arg-2270 thus appears to play a critical role in the stabilization of the local conformation of VP1 in this region. Mutations in 2270 may result in escape from neutralization either by altering the interactions of 2270 with antibodies or by destabilizing the structure of other residues in this site.

Several of the variants in site 2 have mutations in residues with only limited exposure to antibodies. These include Asp-2164, Pro-2170, and Asp-1226 which would be exposed to probes no larger than 0.90, 1.50, and 1.04 nm in diameter, respectively (Table 3). While we cannot rule out the possibility that these residues would be accessible to an irregular probe such as the antigen-binding site of an Fab, each of these residues participates in interactions that might provide alternative explanations for their role in escape mutations. Specifically, the carboxylate group of Asp-2164 accepts hydrogen bonds from the peptide nitrogens of 2166 and 2171 (Fig. 5c). These hydrogen bonds link the two sides of the large loop of VP2 and may be essential for maintaining the conformation of this loop and, hence, for the correct presentation of residues 2166, 2168, and 2169. Similarly, the proline at position 2170 may place restrictions on the mainchain conformation that are important for the structure of this loop. Finally, Asp-1226 participates in a salt bridge with Arg-2172 that links two separate peptide segments of site 2 (Fig. 5b) and might also be necessary for maintaining the structure of this complex conformational determinant.

Neutralization resistance and antigenic variation. All of the poliovirus mutations identified in this and other MAb release studies are located on the surface of the virus, in highly exposed loops that are readily accessible to antibody binding. Similarly, these exposed loops are foci for differences in the length and sequence seen for different serotypes of poliovirus as well as for the largest structural differences among various picornaviruses (10, 14, 21). Indeed, related proteins generally exhibit considerably greater variability in their solvent-exposed loops than they do in their more internal core regions.

The localization of antigenic sites in exposed loops has previously been observed in the hemagglutinin and neuraminidase proteins of influenza virus (4, 29), and a distribution of sites similar to those of poliovirus has also been observed in rhinovirus 14 (21, 24). The decoration of a virus surface with loops that can accommodate mutation thus appears to be a common mechanism by which animal viruses escape immune surveillance without disrupting portions of the structure that are necessary for the integrity of the virus.

In this context, it is worth noting that the characterization of neutralization-resistant mutants is not necessarily capable of identifying every amino acid in the antibody-binding site. In particular, any mutation in the antibody-binding site which would interfere with capsid stability, virus assembly, receptor recognition, or any other requirement of the virus life cycle would not be viable and thus not detected. The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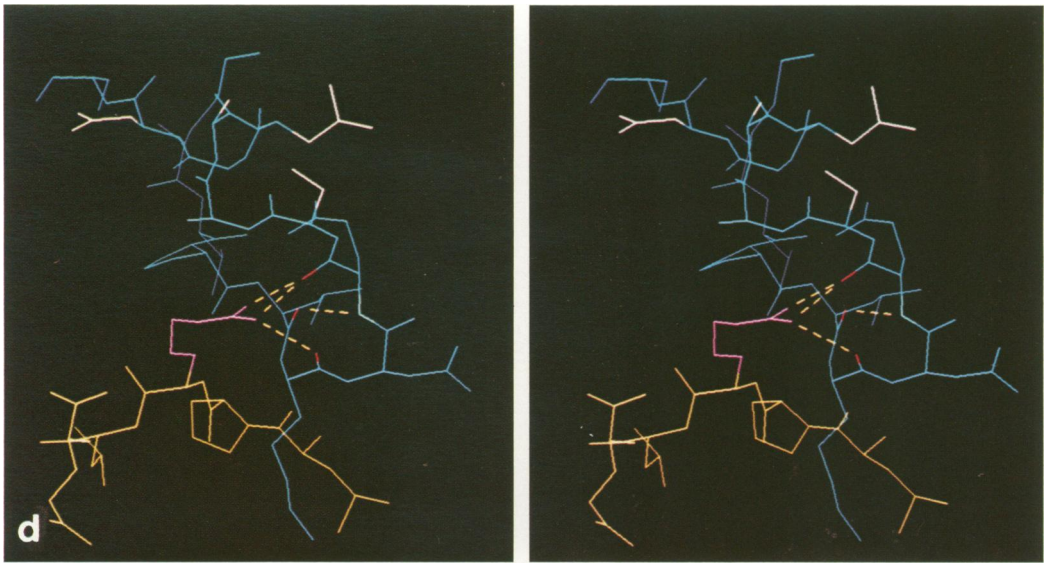

FIG. 5. Stereo pictures showing details of antigenic sites 3A and 2 in the atomic model of type 1 poliovirus. In panels a, b, and c, the uppermost residues in the figure are the outermost in the virus. (a) Site 3A. Residues 3054 to 3071 of VP3 are shown in orange, except as noted. The beta turn from 3058 to 3061 is magenta. Proceeding clockwise across the top of the beta turn, the side chains of Ser-3058, Ala-3059, and Thr-3060 are shown in white. The side chain of Arg-3071 is yellow. Residues 1292 to 1299 of VP1 are shown in blue. Although residue 3060 is shown as a Thr (because the coordinates from which the figure is derived are from the Mahoney strain of type 1), model building studies indicate that a Lys (in the Sabin strain) could readily be accommodated in this position. Note that the side chain of Arg-3071 (which has been linked to site 3A in previous studies) is located on the same side of the 3050 to 3070 loop as the side chains of 3058 and 3060. In panels b, c, and d, antigenic site 2 is shown in various degrees of detail. In general, residues 2130 to 2178 of VP2 are shown in orange, residues 2265 to 2272 of VP2 are shown in yellow, and residues 1206 to 1228 of VP1 are shown in blue. Side chains of particular interest are indicated in other colors: Asp-2164, Asn-2166, Thr-2168, Ser-2169, and Pro-2170 are cyan; Arg-2172 is green; Arg-2270 is magenta; Ser-1221, Ala-1223, Leu-1224, and Asp-1226 are white. Panel b shows all of site 2. Note the salt bridge between the side chains of Arg-2172 and Asp-1226 which links the VP2 and VP1 components of site 2 together. (c) An expanded view of residues 1263 to 2171. Residue numbers increase clockwise around the loop from the lower left to the lower right. Note that the side chains of 2166, 2168, and 2169 all lie on the same side of this large loop from VP2. Dotted lines indicate hydrogen bonds of particular interest. The hydrogen bond between the carbonyl oxygen of 2166 and the peptide nitrogen of 2169 defines the beta turn at the top of the loop. Hydrogen bonds between the carboxylate group of Asp-2164 and the peptide nitrogens of 2166 and 2171 apparently help to stabilize the loop structure. Any mutation that substituted another amino acid for Asp at 2164 (including the mutation to Asn in strain 6.1) would be unable to make this stabilizing interaction and would result in an altered conformation. (d) Stabilizing interactions made by the side chain of Arg-2270. (The orientation of the figure has been changed from that shown in panel b for clarity.) The positively charged guanidinium group of Arg-2270 makes hydrogen bonds with the carbonyl oxygens of 1217, 1218, and 1220 (shown in red), thus stabilizing the unusually close approach of the three oxygens to one another. The hydrogen bond between the carbonyl oxygen of 1217 and the peptide nitrogen of 1220 that defines the beta turn at the end of this loop from VP1 is also shown. Proceeding clockwise across the top of the figure, the side chains of Asp-1226 (upper left), Ala-1223, Leu-1224, and Ser-1221 are shown in white.

small number of mutations observed in site 3B is consistent with the idea that the potential for viable mutations in this region is highly limited.

One of the significant results of this study is the striking correlation between the structural definition of antigenic sites and the operational definition provided by the cross-neutralization studies. Mutations that are linked by cross-neutralization studies lie close together on the surface of the virus in discrete well-separated areas that are each no larger than the footprint of the antigen-binding site of an Fab. This correlation increases the confidence in the reliability of using cross-neutralization and sequencing studies to define antigenic sites in viruses whose three-dimensional structures have not yet been determined.

In addition, each mutation has provided a possible model for escape from neutralization. Most of the neutralization escape mutations are in prominently exposed positions in which they can interact directly with antibody. However, a significant number of mutations are not so prominently exposed. Interestingly, these less-exposed residues are observed to participate in interactions that contribute to the stability of the conformation of the loops. Sequence changes in these residues provide a particularly clear indication that mutations may also result in escape from neutralization by causing local conformational adjustments in the loops that form the antigenic sites. There is, however, no evidence that mutations distant from an antigenic site can confer neutralization resistance and thus no evidence for long-range propagation of conformational changes to distant parts of the structure.

This study has provided a detailed characterization of the neutralizing antigenic sites in serotype 1 poliovirus in the context of its known three-dimensional structure. The definition of these sites will be valuable for characterization of the antibody-antigen interaction and for studies on the mechanisms of antibody-mediated neutralization. The structural characterization of the antigenic sites together with the general correlation between the surface exposure of a residue and its inclusion in an antigenic site predict additional residues which may form part of these or other new sites. Although these residues have not been observed as MAb-selected mutations, it may be possible to introduce mutations in these residues by site-specific methods. The existing panel of MAbs will be useful for characterizing these constructs. Finally, the results of this study together with the recent determination of the structure for the Sabin strain of

type 3 poliovirus (D. J. Filman et al., manuscript in preparation) and with the previous extensive sequencing and cross-neutralization studies on type 3 (17, 18, 20) will eventually provide a structural basis for understanding the observed serotypic differences between types 1 and 3 poliovirus.

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