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Mutations Conferring Resistance to Neutralization with Monoclonal Antibodies in Type 1 Poliovirus Can Be Located Outside or Inside the Antibody-Binding Site

BRUNO BLONDEL,[1] RADU CRAINIC,[1]* ODILE FICHOT,[2] GABRIELLE DUFRAISSE,[1] ADINA CANDREA,[1] DAVID DIAMOND,[3] MARC GIRARD,[2] AND FLORIAN HORAUD[1]†

Unité de Virologie Médicale[1] and Unité de Virologie Moléculaire,[2] Institut Pasteur, 75724 Paris Cedex 15, France, and Department of Microbiology, School of Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794[3]

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Antigenic variants resistant to eight neutralizing monoclonal antibodies were selected from wild (Mahoney) and attenuated (Sabin) type 1 infectious poliovirions. Cross-immunoprecipitation revealed interrelationships between epitopes which were not detected by cross-neutralization. Operational analysis of antigenic variants showed that seven of eight neutralization epitopes studied were interrelated. Only one neutralization epitope, named Kc, varied independently from all the others. This latter, recognized by C3 neutralizing monoclonal antibody, was present not only on infectious virions but also on heat-denatured (C-antigenic) particles and on isolated capsid protein VP1. Loss of the neutralization function of an epitope did not necessary result from the loss of its antibody-binding capacity. Such potential, but not functional, neutralization epitopes exist naturally on Mahoney and Sabin 1 viruses. Their antibody-binding property could be disrupted by isolating antigenic variants in the presence of the nonneutralizing monoclonal antibody and anti-mouse immunoglobulin antibodies. Single-point mutations responsible for the acquisition of resistance to neutralization in the antigenic variants were located by sequence analyses of their genomes. Mutants selected in the presence of C3 neutralizing monoclonal antibody always had the mutation located inside the antibody-binding site (residues 93 through 103 of VP1) at the amino acid position 100 of VP1. On the contrary, antigenic variants selected in the presence of neutralizing monoclonal antibodies reacting only with D-antigenic particles had mutations situated in VP3, outside the antibody-binding site (residues 93 through 103 of VP1). The complete conversion of the Mahoney to the Sabin 1 epitope map resulted from a threonine-to-lysine substitution at position 60 of VP3.

Several reasons justify the renewal of interest in the study of poliovirus antigens. Poliomyelitis still remains an important public health problem in some areas of the world (1), despite efficient and intensive use of inactivated and live-attenuated vaccines in industrialized countries (35). Through application of our present knowledge on the molecular biology and replication of poliovirus, the safety and efficacy of these vaccines can be improved or replaced by new, synthetic, vaccines. Due to its simplicity, poliovirus is a valuable model for studying the interaction of antibodies and antigenic determinants expressed at the surface of the virion and its precursors. Such investigations have a twofold purpose: (i) elucidation of the still-obscure mechanisms of virus neutralization by antibodies and (ii) identification, in molecular terms, and location of viral capsid structures which induce neutralizing antibodies.

The relationship between distinct neutralization epitopes (N-Eps) can be examined by operational analysis of viral mutants resistant to neutralization by neutralizing monoclonal antibodies (N-mcAbs). Two epitopes are considered as belonging to the same operationally defined group if the mutation affecting the reactivity of one epitope affects the reactivity of the second (48). With this approach, only a single operationally defined group of N-Eps was discerned in type 3 poliovirus, despite the use of many N-mcAbs of varied origins. All mutations were found to cluster in amino acid positions 93 to 100 on structural polypeptide VP1 (24, 37). A different situation was encountered in type 1 poliovirus. Several neutralization antigenic sites (N-Ags) (46) were described on capsid polypeptides VP1, VP2, and VP3 based on the capacity of synthetic peptides to induce or to prime for neutralizing antibodies (7, 8, 18–20). Two of them were located on capsid protein VP1, at amino acid positions 70 through 80 (N-Ag1) and 93 through 103 (N-Ag2), by a binding assay of N-mcAbs with virus-specific synthetic peptides (18). All type 1 N-mcAbs so far tested are directed against N-Eps which cluster in one or the other of these two N-Ags. At this time it is not known whether the two VP1 amino acid sequences identified as N-Ags represented all, or only part, of a more complex N-Ag with the eventual participation of other sequence(s) of VP1 or of other capsid polypeptides. By analyzing mutants resistant to neutralization by N-mcAbs, several operationally defined groups of N-Eps have been defined (4, 17, 21). Intriguingly, so far genome sequencing of such antigenic variants revealed that the mutations were always located outside the mcAb-binding site (16).

The complexity of N-Ags in type 1 poliovirus requires further investigation to clarify how their conformation is influenced by other, nearby or distant, polypeptide sequences on the viral capsid. Taking advantage of the N-Ep map which we established by using eight type- and strain-specific N-mcAbs (13), we studied the interdependence of these epitopes by analyzing neutralization-resistant mutants derived from the Mahoney virus and from its attenuated derivative, Sabin 1 strain. The results of the operational analysis were substantiated by sequencing mutant viral

* Corresponding author.
† Formerly Horodniceanu.

genomes. It will be shown that mutations affecting N-Eps of the N-Ag2 of VP1 can indeed be located outside, as well as inside, the antibody-binding site. Relevant information on the molecular basis of mutations affecting Mahoney and Sabin 1 virus antigenic specificity is also given. The data presented in this study contribute to a better understanding of the structure and antigenic modulation of the N-Ag2 in type 1 poliovirus.

MATERIALS AND METHODS

Cells and media. HEp-2 cells were grown in Eagle basal medium with 5% aseptic calf serum (Medical and Veterinary Supplies, Slough, United Kingdom). Vero cells were cultured in Dulbecco modified Eagle minimum essential medium (MEM) supplemented with 5% aseptic calf serum.

Virus. Type 1 poliovirus was grown in HEp-2 cell monolayers in Eagle basal medium containing 2% fetal bovine serum, at 37°C for the wild reference type 1 Mahoney strain and at 34°C for the LSc2ab Sabin type 1 attenuated vaccine strain.

Titration of the virus. The infectivity of virus suspensions was titered either by the endpoint micromethod (36) on HEp-2 cells or by plaque assay on Vero cells. For plaque assay, confluent monolayers of Vero cells in 24-well (2-cm^2) Costar plates were inoculated, in duplicate, with 0.2 ml of fourfold serial dilutions of virus suspension. After adsorption, the inoculum was discarded; cells were washed twice and overlaid with Dulbecco modified Eagle MEM containing 2% fetal calf serum and 0.8% carboxymethyl cellulose. Plaques were counted after 3 days of incubation at 37°C (Mahoney virus) or 34°C (Sabin 1 virus) in a humid 5% CO_2 atmosphere, followed by fixation and staining with 0.05% methylene blue.

Hybridomas. The hybridoma cell lines were obtained from mice immunized with either Mahoney or Sabin 1 purified, infectious virus and selected for poliovirus neutralizing activity against the immunizing strain. Their preparation and growth have been described elsewhere (9, 14). An unusual N-mcAb, C3, was obtained from mice immunized with C-antigenic, heat-denatured Mahoney virions (2). The C3 antibody neutralizes homologous (Mahoney) and heterologous (Sabin) type 1 poliovirus and reacts in immunoprecipitation with C-antigenic particles and with isolated VP1 of the homologous virus. Hybridoma culture medium or mouse ascites fluid (34) was used as the mcAb source, as specified below.

Preparation of radiolabeled virions. Confluent monolayers of HEp-2 cells (about 2.5×10^7 cells per 75 cm^2) were washed and inoculated with virus at a multiplicity of infection of 50 in 2 ml of Eagle basal medium without calf serum. After 30 min of incubation at 37°C for virus adsorption, 13 ml of Eagle basal medium supplemented with 2% calf serum was added without discarding the inoculum, and incubation was continued at 37°C for Mahoney virus or Mahoney-derived strains and at 34°C for Sabin 1 virus or Sabin 1-derived mutants. At 2 h postinfection medium was replaced with methionine-free Eagle MEM. At 2.5 h postinfection, [^{35}S]methionine (150 μCi) was added, and the medium was adjusted to 2% fetal calf serum. After 18 h of incubation (complete cytopathic effect), the culture was frozen and thawed three times, cell debris was removed by low-speed centrifugation (4,500 × *g* for 30 min), and virions were purified by isopycnic CsCl gradient centrifugation as previously described (2).

NI assay. To determine the presence of an N-Ep on a viral strain, we use the neutralization index (NI) assay as previously described (13). The virus titer (VT) on Vero cells was determined in parallel in the absence (VTo) and in the presence (VTa) of a given dilution of the hybridoma supernatant containing the N-mcAb. The NI was calculated according to the formula NI = log VTo − log VTa.

Reactions were considered positive (N-Ep present) for an NI value of ≥2.00 and negative (N-Ep absent) for an NI of <2.00 (in most cases no detectable neutralization occurred). The working dilution of an mcAb was chosen from prior NI assays as the highest dilution of hybridoma supernatant giving at least 3 NI units (NI of >3.00) with the reference poliovirus strain, i.e., the strain which served for the selection of the respective N-mcAb. The reference virus strains were included in each test as a control.

Immunoprecipitation. Immunoprecipitation was carried out as described by Kessler (28) with some modifications (3). Briefly, 25 μl of ascites fluid containing mcAb was added to the suspension of purified [^{35}S]methionine-labeled poliovirions (20,000 cpm). Immune complexes were precipitated with *Staphylococcus aureus* (Cowan 1) and assayed for radioactivity. An epitope was considered present (positive reaction) when the immunoprecipitated fraction was >20% of the input radioactivity (usually >60%). When an epitope was absent, 0 to 2% of the radioactivity was precipitated. No value between 2 and 20% was encountered.

Viral mutants resistant to neutralization by mcAb. Tenfold serial dilutions of virus were incubated at 37°C with hybridoma supernatant containing the N-mcAb at a concentration at least 100 times greater than its endpoint titer in neutralization tests. Confluent monolayers of Vero cells (10-cm^2 wells) were seeded with virus-antibody mixtures. After 1 h of incubation at 37°C, the inoculum was discarded, and the unadsorbed virus was washed off. Cells were then overlaid with 3 ml of 0.9% agar in phenol red-free MEM containing 2% fetal bovine serum and 10% selecting hybridoma supernatant and incubated at 37°C for Mahoney virus and at 34°C for Sabin 1 virus. After 3 days, cultures were stained with 0.02% neutral red in 1 ml of agar overlay. Plaques were picked up and suspended in 1 ml of serum-free MEM. After three cycles of freezing and thawing, each viral clone was passaged once in the presence of the selective mcAb. A 0.5-ml sample of virus was preincubated for 1 h at 37°C with the same volume of the corresponding mcAb and then seeded onto a 25-cm^2 sheet of HEp-2 cells. After 1 h of incubation at 37°C, 7 ml of MEM containing 2% fetal calf serum and 10% hybridoma supernatant was added. The virus was harvested after complete cytopathic effect, 24 or 36 h after incubation at the optimum growth temperature for each virus.

The frequency of mutation toward resistance to neutralization with an N-mcAb was calculated as the log virus titer (PFU) difference in the absence and in the presence of the respective mcAb as determined by plaque assay.

Nucleotide sequence determination. Viral RNA was phenol extracted and ethanol precipitated from concentrated, CsCl gradient-purified virus. Oligonucleotide VP1 primer d(CCTCCGTAACTGGAC) was prepared by J. Igolen (Pasteur Institute, Paris), and the oligonucleotide VP3 primer d(TATGCTTGGAGAAAT) was kindly supplied by E. Wimmer. Nucleotide sequences were obtained by the dideoxy chain termination method (41). DNA primer (20 ng) was mixed with viral RNA (1 μg) in 4 μl of 200 mM Tris buffer (pH 3) containing 280 mM KCl and 20 mM $MgCl_2$. For each reaction 1 μl of the latter solution was combined with 1 μl of a mixture containing 800 μM each dGTP, dCTP, and

dTTP and 160 μM dATP, 3 μCi of [alpha-^{32}P]dATP (about 3,000 Ci/mmol; Amersham Corp.), and 7 U of reverse transcriptase (Stehelin, Switzerland) in distilled water. The concentration of dideoxynucleoside triphosphate was 50 μM ddGTP, ddCTP, and ddTTP and 5 μM ddATP. Reactions were carried out for 15 min at 37°C under 4 μl of paraffin and then chased with 1 μl of 1.5 mM dATP in distilled water. Incubation was continued for 15 min and then stopped with 1 μl of 60 mM EDTA. Paraffin was eliminated with ether, and 3 μl of sample buffer (41) was added. After heating for 2 min at 95°C, 3-μl samples were analyzed by electrophoresis on 6 to 8% acrylamide–7 M urea gels (35 by 23 cm by 0.04 cm) in 75 mM Tris-boric acid buffer (pH 8.3) containing 3 mM EDTA (33). Before casting the gel, the unnotched plate was treated with gamma-methacryloxypropyl-trimethoxy silane (a gift from Wacker Chemie, Munich, Federal Republic of Germany) to bind the gel to the plate. The notched plate was coated with trimethylchlorosilane to repel the gel. After electrophoresis, the gel adhering to the treated plate was fixed, and urea was removed by soaking in 10% acetic acid for 15 min. After washing with water, the gel was dried at 80°C and autoradiographed on X-ray film (Fuji).

RESULTS

Nomenclature and binding sites of N-mcAbs. We have previously shown that each N-mcAb used in this study recognizes a distinct N-Ep, by testing them against a large panel of type 1 poliovirus field isolates (13).

In the present paper, we adopted the following nomenclature, in correspondence (Table 1) with that of previous publications in which the same N-mcAbs were used (2, 4, 9, 10, 12, 13, 16, 18, 22, 23, 45, 47).

N-Eps were designated according to the neutralization specificity group to which they belonged: (i) common for both Mahoney and Sabin 1 strains (K N-Ep), (ii) Sabin-specific (S N-Ep), and (iii) Mahoney-specific (M N-Ep). Three N-Eps were found in the K group (Ka, Kb, and Kc), one was found in the S group (S), and four were found in the M group (Ma, Mb, Mc, and Md). Most of the N-Eps analyzed in this study (Ka, Kc, Ma, Mb, Mc, and Md) were shown by N-mcAb binding to synthetic peptides to cluster in N-Ag2 (residues 93 through 103 of VP1) (18). The Kb N-Ep was shown to be part of N-Ag1 (residues 70 through 80 of VP1) (18). The localization of the S epitope is not yet known.

TABLE 1. Nomenclature of mcAbs and corresponding epitopes of type 1 poliovirus

Neutralizing mcAb		Binding site (N-Ag)[b]	Neutralization epitopes	
Original name[a]	Present name		Mahoney strain	Sabin 1 strain
1BM55 (1c)	αKa	N-Ag2	Ka	Ka
ICJ31 (Ib)	αKb	N-Ag1	Kb	Kb
C3	αKc	N-Ag2[c]	Kc	Kc
1BA12 (Io)	αS	ND[d]		S
ICJ27 (Io)	αMa	N-Ag2	Ma	
IH8-4 (Ic)	αMb	N-Ag2	Mb	
IH8-25 (Id)	αMc	N-Ag2	Mc	
ICJ12 (Ia)	αMd	N-Ag2	Md	

[a] Names from references 2, 4, 9, 10, 12, 13, 16, 18, 23, 45, and 47.

[b] The antibody-binding sites were determined by reaction with synthetic peptides mimicking amino acid sequences of Mahoney virus capsid polypeptide VP1 in enzyme-linked immunosorbent assay. N-Ag1 is located at amino acid residues 70 through 80; N-Ag2 is located at amino acid residues 93 through 103 (18).

[c] The antibody-binding site of αKc (C3) was determined by immunoprecipitation of an increasingly truncated VP1 beta-lactamase fusion protein expressed in *Escherichia coli* (45, 47).

[d] ND, Not determined.

N-mcAbs corresponding to each of the N-Eps were designated by the same names used for the N-Ep preceded by the symbol α (αKa, αKb, αMa, etc.).

Neutralization-resistant mutants were designated by the name of the target N-Ep of selection preceded by the letter of the parental virus (M for Mahoney and S for Sabin 1 virus) and followed by the superscript r (resistant) (e.g., M-Ka^r is a Mahoney-derived variant resistant to neutralization with αKa N-mcAb).

Operational mapping of N-Eps. N-Eps were classified into operationally defined groups by isolating antigenic variants from either Mahoney or Sabin 1 virus. This was done by selecting mutants that were resistant to neutralization by each N-mcAb by direct cloning of virus under an agar overlay containing N-mcAb, as described in Materials and Methods. The frequency of antigenic variants in Mahoney and in Sabin 1 virus populations ranged between $10^{-2.9}$ and $10^{-5.2}$, which indicates that a single-point mutation in each case accounts for acquisition of the resistance to neutralization.

Four viral clones selected against each N-mcAb were examined by neutralization and antibody binding (immunoprecipitation) with each of the eight mcAbs of the panel (Table 2). One representative variant of the four clones examined is shown for each class of mutants, except when two different phenotypes were found in the same class. Modification of K epitopes never affected the reactivity of virus with the αS or αM mcAbs. Within the group of K N-Eps, Kc was independent; Ka and Kb were independent from each other on Mahoney virus, whereas on Sabin 1 strain resistance to neutralization by αKa (S-Ka^r mutant) was accompanied by resistance to Kb antibody.

When we selected from Mahoney virus mutants resistant to Mahoney-specific αMa, αMb, αMc, and αMd N-mcAbs, two phenotypes were obtained. The first phenotype, represented by mutants M-Ma^{r}1, M-Mb^{r}1, M-Mc^{r}1, and M-Md^{r}1, was most frequently encountered (14 of 16 clones examined). All of these mutants were resistant to neutralization not only by the N-mcAb against which they were selected, but also by the other three αM (Mahoney-specific) mcAbs. The Ma, Mb, and Mc epitopes were lost in terms of neutralization as well as in terms of antibody binding. The Md epitope always retained its capacity to bind homologous antibody in spite of the loss of its function in virus neutralization. An interesting observation was that these variants acquired the property of being neutralized by the Sabin-specific (αS) mcAb while losing their sensitivity to neutralization by all Mahoney-specific antibodies. Moreover, they failed to react with αKb antibody in immunoprecipitation, exactly as did Sabin 1 strain. Through a single point mutation, a complete conversion of the N-Ep map occurred from the Mahoney pattern to that of Sabin 1. This confirmed our preliminary results (4) and recent observations of Diamond et al. (16).

The second phenotype (mutants M-Mc^{r}2 and M-Md^{r}2 in Table 2) differed from the first one in the following ways: (i) loss of the neutralization function of the Ma, Mb, and Mc epitopes was not accompanied by loss of their antibody-binding capacity, and (ii) the mutants conserved the S and Kb epitope patterns of the parental virus. Antigenically similar variants were independently obtained by Diamond et al. (16) with the αMb and the αMc N-mcAbs.

TABLE 2. Epitope pattern of neutralization-resistant mutants derived from Mahoney and from Sabin type 1 poliovirus strains

Virus[a]	mcAbs[b]															
	αKa		αKb		αKc		αS		αMa		αMb		αMc		αMd	
	NI	Ip	NI	Ip	NI	Ip	NI	Ip	NI	Ip	NI	Ip	NI	Ip	NI	Ip
Mahoney	+	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+
M-Ka^r	0	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+
M-Kb^r	+	+	0	+	+	+	0	+	+	+	+	+	+	+	+	+
M-Kc^r	+	+	+	+	0	0	0	+	+	+	+	+	+	+	+	+
M-Ma^{r}1	+	+	+	0	+	+	+	+	0	0	0	0	0	0	0	+
M-Mb^{r}1	+	+	+	0	+	+	+	+	0	0	0	0	0	0	0	+
M-Mc^{r}1	+	+	+	0	+	+	+	+	0	0	0	0	0	0	0	+
M-Md^{r}1	+	+	+	0	+	+	+	+	0	0	0	0	0	0	0	+
M-Mc^{r}2	+	+	+	+	+	+	0	+	0	+	0	+	0	+	0	+
M-Md^{r}2	+	+	+	+	+	+	0	+	0	+	0	+	0	+	0	+
Sabin 1	+	+	+	0	+	+	+	+	0	0	0	0	0	0	0	+
S-Ka^r	0	+	0	0	+	+	+	+	0	0	0	0	0	0	0	+
S-Kb^r	+	+	0	0	+	+	+	+	0	0	0	0	0	0	0	+
S-Kc^r	+	+	+	0	0	+	+	+	0	0	0	0	0	0	0	+
S-S^r	+	+	+	0	+	+	0	+	0	0	0	0	0	0	0	+

[a] One representative variant is shown among the four viral clones isolated for each mcAb, except for M-Mc^r and M-Md^r mutants, where two different phenotypes were encountered and the number of the clone examined is mentioned.

[b] Viral clones were tested against the whole panel of mcAbs used in this study by neutralization (NI) and immunoprecipitation (Ip) as described in Materials and Methods: +, positive rection; 0, negative reaction, according to quantitative criteria presented in Materials and Methods. Results obtained with the mcAb used for the selection of the respective mutant are boxed with full lines. Dotted lines box indicate modifications of the epitope(s) other than the target N-Ep in selection.

Antigenic variants obtained from Sabin virus resistant to neutralization by S (S-S^r in Table 2) showed no modification of K or M epitopes in any of the clones tested, as might have been expected from Mahoney-to-Sabin antigenic conversion in Mahoney mutants resistant to αM mcAbs.

Antigenic variation of neutralization-resistant mutants was never followed by modification of the migration pattern of viral capsid polypeptides in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (results not shown). This was even true for variants in which a complete Mahoney-to-Sabin antigenic conversion occurred. In addition, such conversion was not accompanied by the loss of neurovirulence in the M-Mb^{r}1 variant (G. C. Schild [National Institute for Biological Standards and Control, London], results not shown).

Although αKb mcAb neutralized both the original Sabin virus and Mahoney-derived mutants having Sabin 1 antigenic phenotype, it did not immunoprecipitate these strains (Table 2). On the contrary, αKb mcAb reacted fully in immunoprecipitation with Mahoney virus. This particular mechanism of neutralization cannot be explained based on our present knowledge, since neutralization is generally considered to result from a stable association of antibody with the virus, leading to a shift in the surface charge of the virion (27, 32). We are currently running experiments to elucidate the particular mechanism by which the αKb mcAb is able to neutralize without binding in immunoprecipitation.

Another phenomenon which drew our attention in a previous study (13) was that, in some instances, mcAbs neutralizing for one strain bound to, but did not neutralize, another strain. This was the case for Sabin-specific (αS) N-mcAb and Mahoney virus and for Mahoney-specific (αMd) N-mcAb and Sabin strain (Table 2). The latter phenomenon was also observed with some in vitro-derived variants, like the Ka epitope of M-Ka^r or S-Ka^r mutants, the Kb epitope of M-Kb^r mutants, all M and S epitopes of M-Mc^{r}2 and M-Md^{r}2 clones, and the Kc epitope in S-Kc^r variants (Table 2). Interestingly, the Kc epitope, although it functioned as a neutralization epitope on both Mahoney and Sabin 1 strains, behaved in a different manner when mutants resistant to αKc N-mcAb were selected from one or from the other strain.

Operational analyses carried out by neutralization assay showed that the eight N-Eps examined fell into apparently three operationally defined groups: the Ka-Kb group, the M-S group, and the Kc epitope. However, antibody-binding tests revealed a clear relationship between the M-S group and the Kb epitopes on Mahoney virus that was not apparent in terms of neutralization.

Mutants resistant to αIg-mediated neutralization. We focused further our attention on αS and αMd mcAbs which bound to Mahoney and Sabin 1 virus, respectively, without affecting viral infectivity. The fact that binding and neutralization of S and Md epitopes are discrete functions on the virion was demonstrated by the conservation of the binding properties of these epitopes in all mutants resistant to αS or αM antibodies. We therefore asked whether the region(s) of capsid proteins controlling the binding capacity of such epitopes was the same as that controlling their function in neutralization. To answer this question, we tried to obtain a new class of viral mutants incapable of binding αS and αMd mcAbs. It is self-evident that an antibody capable of binding without affecting viral infectivity cannot in itself be used as a selective agent. We therefore used neutralization of infectious virus-antibody complexes by antiglobulin (αIg) antibodies (32) to select viral mutants resistant to αIg-mediated neutralization and consequently which would no longer bind to nonneutralizing mcAbs. αIg-mediated neutralization has already been demonstrated for type 1 poliovirus (16, 23, 31).

Anti-mouse immunoglobulin antibodies could reduce infectivity of Sabin 1 virus previously sensitized with nonneutralizing αMd mcAb (Fig. 1A). Such αIg-mediated neutralization also occurred with the Mahoney virus-αS mcAb combination (Fig. 1B).

We were thus able to obtain antigenic variants which no longer bound αS or αMd mcAb (Table 3). The M-S(αIg)^r mutant was derived from Mahoney virus by using the Sabin-specific (αS) mcAb and αIg antibodies as selective

pressure. The S-Md(αIg)^r variant was obtained from the Sabin 1 virus in the presence of Mahoney-specific αMd mcAb and αIg serum. In contrast to their respective parents, both mutant viruses were unreactive in immunoprecipitation with the corresponding nonneutralizing mcAb. This loss of antibody-binding was accompanied by the loss of virus neutralizability in the McAb-αIg system. The frequency of mutation was $10^{-2.9}$ for M-S(αIg)^r and $10^{-3.6}$ for S-Md(αIg)^r, indicative of single-point mutations in both cases. The epitope map of mutant resistant to αIg-mediated neutralization is given in Table 4. The target epitopes were not the only

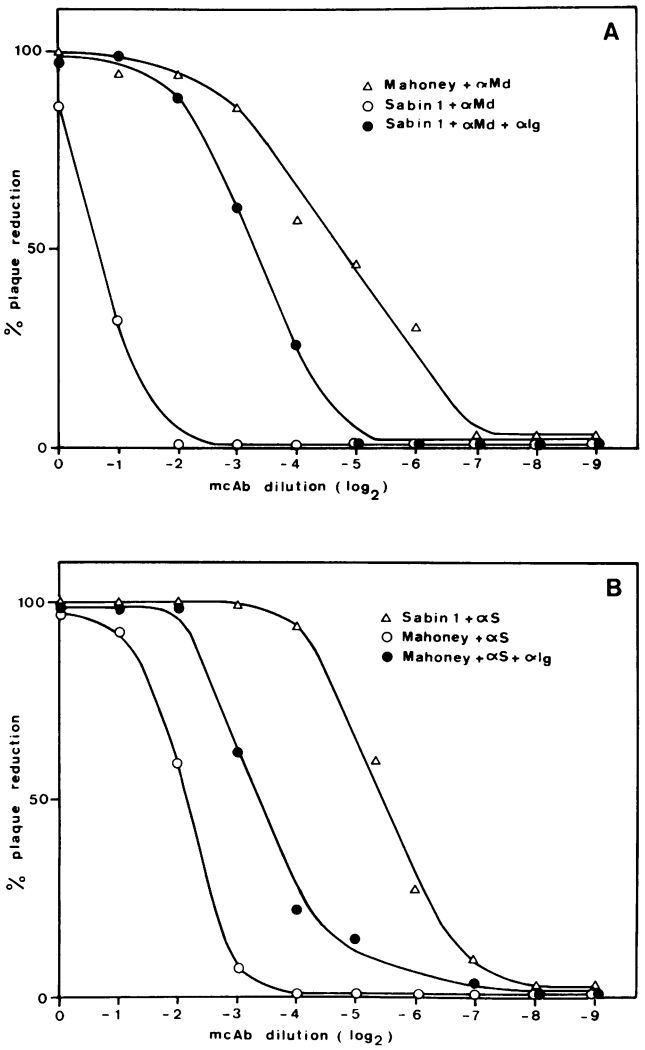

FIG. 1. Antiglobulin-mediated neutralization of poliovirus sensitized by nonneutralizing mcAb. Equal volumes (0.2 ml) of virus suspension (Mahoney or Sabin 1 strain) containing about 200 PFU and serially diluted hybridoma supernatant fluid (αS or αMd) were kept in contact for 2 h at 37°C. A sample (0.4 ml) of rabbit anti-mouse Ig (αIg) serum (Cappel Laboratories) diluted 1/200 or 0.4 ml of diluent was added to each virus-mcAb mixture and further incubated for 1 h at 37°C. Portions (0.2 ml) of each mixture were plaque assayed for residual virus infectivity as described in Materials and Methods, using four wells per mixture. Four control wells received 0.2 ml of virus suspension similarly incubated but in the absence of any antibody. The percent plaque reduction was plotted against the dilution of hybridoma supernatant fluid for each specified virus-mcAb combination.

TABLE 3. Loss of antibody-binding capacity by viral mutants resistant to αIg-mediated neutralization

Virus	NI[a] with:				Immunoprecipitation[b] with:	
	αS	αS + αIg	αMd	αMd + αIg	αS	αMd
Mahoney	0.50	2.75			+	
M-S(αIg)^r	0.25	0.25			−	
Sabin 1			0.75	2.50		+
S-Md(αIg)^r			0.75	0		−

[a] The NI was calculated as described in Materials and Methods at a dilution of 1/16 of the mcAb containing hybridoma supernatant fluid and of 1/400 of the rabbit anti-mouse serum in the reaction mixture.

[b] Mouse ascites fluid was used as source of mcAb in the immunoprecipitation reaction carried out as described in Materials and Methods.

ones affected by the mutation. In the M-S(αIg)^r variant, loss of the antibody-binding capacity of S epitope was accompanied by a conversion of Mahoney-specific (M) group of epitopes from the Mahoney- to the Sabin-like pattern. Moreover, the αKa antibody, although it still neutralized the mutant virus, no longer reacted with virions in immunoprecipitation. On the other hand, mutation rendering the Sabin-derived S-Md(αIg)^r variant unable to bind αMd antibody also entailed the loss of virus susceptibility to neutralization with Sabin-specific αS N-mcAb.

αIg-mediated neutralization is not an universal property of epitopes which retain their capacity to bind antibody without functioning in neutralization. This was demonstrated in previous studies by Diamond et al. (16) as well as in the S-Kc^r–αKc antibody system. The S-Kc^r strain is a Sabin-derived mutant resistant to neutralization with αKc mcAb (Table 2) which still reacts in immunoprecipitation with αKc antibody. For the given concentrations of αKc mcAb, the addition of αIg antibodies had no effect on the S-Kc^r–αKc virus-antibody complex, whereas it increased the neutralization of the Sabin virus–αKc complex (Fig. 2).

Amino acid substitutions accounting for the acquisition of N-Ep modifications. We sequenced two of the antigenic variants derived from Mahoney virus which demonstrated a complete conversion of their antigenic phenotypes toward that of Sabin 1 strain, namely, M-Md^{r}1 and M-Mb^{r}1. In both cases, the point mutation was located at amino acid position 60 of VP3, where Lys was substituted for Thr (Fig. 3), thus reproducing the Sabin-specific sequence in this region of the capsid polypeptide. Similar results were recently obtained by Diamond et al. (16) with our αMa and αMd N-mcAbs. That the Lys at position 60 of VP3 is critical for expression of the Sabin-specific (S) N-Ep was also confirmed by the replacement of Lys by Glu in the Sabin-derived S-S^r variant. These results provide molecular support for the phenomenon of mutual exclusiveness of S N-Ep and M group of N-Eps found here and in our earlier studies of natural antigenic variants of poliovirus type 1 (12, 13).

Since the loss of neutralization function of S and Md epitopes was not accompanied by the loss of their antibody-binding capacity, it was interesting to find out which region of capsid proteins controls their three-dimensional conformation. For this purpose, we sequenced the M-S(αIg)^r and the S-Md(αIg)^r mutants selected by αIg-mediated neutralization, which did not bind the αS and the αMd mcAbs, respectively (Table 3). Sequencing results (Fig. 3) showed that the mutation was located in the same VP3 region as that conferring resistance to neutralization with αS and αMd N-mcAbs, namely, at position 71 (Arg to Gln) for

TABLE 4. Epitope pattern of αIg-mediated neutralization-resistant mutants derived from Mahoney and Sabin type 1 poliovirus strains

Virus[a]	mcAbs[b]															
	αKa		αKb		αKc		αS		αMa		αMb		αMc		αMd	
	NI	Ip	NI	Ip	NI	Ip	NI	Ip	NI	Ip	NI	Ip	NI	Ip	NI	Ip
Mahoney	+	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+
M-S(αIg)^r	+	0	+	+	+	+	0	0	0	0	0	0	0	0	0	+
Sabin 1	+	+	+	0	+	+	+	+	0	0	0	0	0	0	0	+
S-Md(αIg)^r	+	+	+	0	+	+	0	+	0	0	0	0	0	0	0	0

[a] See footnotes *a* of Table 2.
[b] See footnote *b* of Table 2.

M-S(αIg)^r mutants and position 58 (Ser to Arg) for S-Md(αIg)^r mutants.

Type 1 poliovirus antigenic variants examined so far here and by others (16) showed mutations situated outside of the antibody-binding site. They were all selected by using N-mcAbs raised with infectious virus and recognizing operationally interdependent N-Eps. It was thus of interest to sequence mutants resistant to αKc (C3) N-mcAb, because the Kc epitope is functionally independent and also the only one conserved on heat-denatured virions and on isolated VP1 (2).

We sequenced six Kc^r variants: three derived from Mahoney virus (M-Kc^{r}1, M-Kc^{r}2, and M-Kc^{r}3) and three derived from Sabin 1 strain (S-Kc^{r}1, S-Kc^{r}2, and S-Kc^{r}3). In all Kc^r variants, irrespective of the parental virus strain

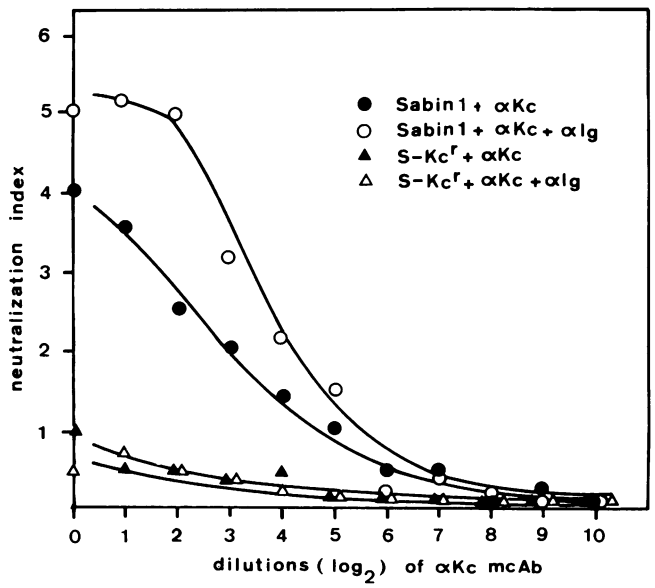

FIG. 2. Testing of αIg-mediated neutralization of the S-Kc^r mutant. Serial 10-fold dilutions of virus were mixed in equal volumes (50 μl) with 2-fold serial dilutions of αKc mcAb containing hybridoma supernatant fluid. The virus-antibody mixture was incubated in duplicate in 96-well Falcon plates for 2 h at 37°C before adding, where specified, rabbit anti-mouse Ig(αIg) serum at a final dilution of 1/400. Vero cells were added only after a further incubation of 1 h at 37°C. The neutralization index was calculated as described in Materials and Methods. The neutralization of the S-Kc^r virus by αKc mcAb was tested in the absence and in the presence of αIg antibodies and was compared with the neutralization of the parental Sabin 1 virus by the same αKc mcAb in the absence or in the presence of αIg serum.

(Mahoney or Sabin 1), the mutation responsible for resistance to neutralization by αKc antibody involved amino acid 100 of VP1 (Fig. 3). This is the first time that a mutation in type 1 poliovirus has been found to map within the mcAb binding site (N-Ag2 for αKc antibody).

All three Mahoney-derived M-Kc^r clones which we sequenced showed the same guanine-for-adenine substitution at base position 2777 of the poliovirus type 1 genome (base numbering according to Nomoto et al. [38]) imposing an Asn-to-Asp substitution at amino acid residue 100 of VP1. In contrast, in all three Sabin-derived S-Kc^r clones the uracil base 2779 was substituted with either adenine (S-Kc^{r}1 in Fig. 3) or guanine (S-Kc^{r}2 in Fig. 3; S-Kc^{r}3, not shown). Both nucleotide changes resulted in the same Asn-to-Lys missense mutation at residue 100 of VP1.

Although all Kc^r mutants resisted neutralization by αKc antibody, only S-Kc^r clones, derived from Sabin 1 virus, still bound the selecting mcAb in immunoprecipitation. Mahoney and Sabin 1 parental viruses both reacted with αKc antibody in neutralization as well as in immunoprecipitation, despite amino acid differences existing between these two viruses inside the antibody-binding site at positions 95 and 99 of VP1 (38) (Fig. 3). It was thus rather interesting to find that a mutation within the amino acid sequence determining the Kc N-Ep might elicit a modification such that the epitope still retained its ability to bind the selecting antibody while no longer functional in neutralization.

A double mutant (M-Mb^{r}Kc^r in Fig. 3) was derived from Mahoney virus by selecting first against the αMb N-mcAb (M-Mb^r clone) and then against αKc N-mcAb. Despite the Sabin-like antigenic phenotype, elicited by the Thr-to-Lys substitution at the position 60 of VP3, this double mutant showed exactly the same Asn-to-Asp amino acid substitution at the position 100 of VP1 as αKc resistant mutants selected from the original Mahoney virus.

DISCUSSION

Mutations affecting N-Ep and their reaction with mcAbs. It could have been thought that the acquisition of resistance to neutralization results only from the loss of antibody-binding capacity by an N-Ep. However, as shown here and elsewhere (13, 16, 21, 23), viral mutants escaping neutralization by an mcAb can have two different phenotypes. In one, the correct conformation of the epitope is lost and the mcAb no longer binds to the virion, whereas in the other, antibody-binding capacity is conserved. The presence of non-neutralization epitopes on the surface of poliovirus has already been demonstrated on type 1 poliovirus (7, 13, 23, 43) as well as on type 3 poliovirus (5, 26). It has become clear that such nonneutralization epitopes, or at least some of them, can actually function as neutralization epitopes in certain antigenic variants.

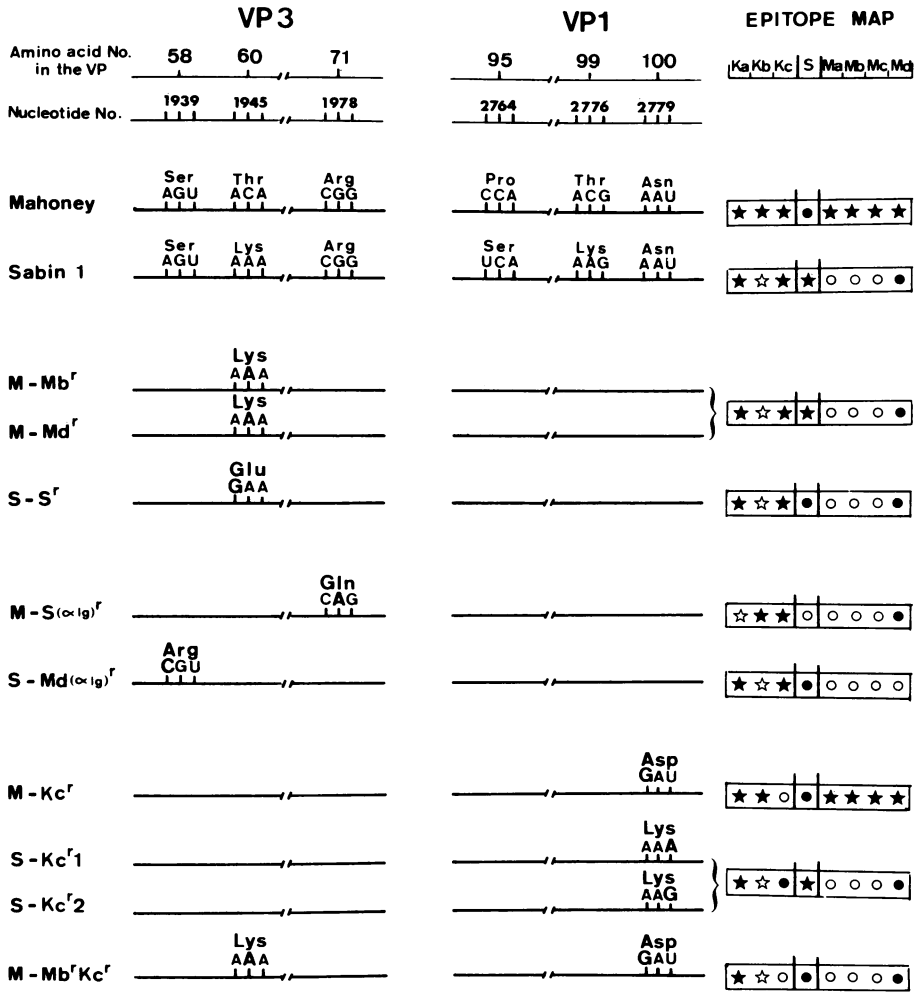

FIG. 3. Amino acid substitutions in capsid polypeptides of type 1 poliovirus antigenic variants. Sequencing of virus genomes in the specified coding regions was carried out as described in Materials and Methods. The base numbering system was that of Nomoto et al. (38) for type 1 poliovirus genome. The number of amino acids in the respective capsid protein and the number of the last nucleotide in the corresponding codon are specified. Substituting nucleotides and amino acids in mutated genomes are printed in boldface type. The epitope map was constructed according to combined results obtained by neutralization (NI) and immunoprecipitation (Ip). Each virus was tested with all mcAbs: ○, NI^-, Ip^-; ●, NI^-, Ip^+; ★, NI^+, Ip^+; ☆, NI^+ Ip^-.

Loss of the neutralization function by an epitope which conserves its antibody-binding capacity is puzzling since the conformational integrity of the antigenic site is apparently unaltered. So far the mechanism for such a phenomenon remains obscure. Since this type of mutation has not yet been reported for poliovirus type 2 or 3, one wonders whether it is a property peculiar to type 1 poliovirus or to all poliovirus serotypes.

Epitope interdependency. The eight N-Eps that we have described for type 1 poliovirus were previously classified according to their strain specificity into Mahoney specific (M), Sabin 1 specific (S), and common (K) for both strains. The operational analysis of antigenic variants by cross-virus–N-mcAb reactions showed interrelationships among N-Eps belonging to different groups of strain specificities. Cross-immunoprecipitation brought about some information concerning epitope relationship that was not revealed by cross-neutralization. It thus seems evident that both of these tests should be used to obtain a more complete picture of epitope interdependency.

A peculiar phenomenon was revealed when the results of operational analyses of the eight distinct epitopes were compared with those of antibody binding of the respective mcAbs. Epitopes mapping within the same neutralization antigenic site of VP1 were not always functionally interrelated. This was the case of the Kc epitope which, despite its belonging to the N-Ag2, was functionally independent of all of the other N-Eps belonging to the same N-Ag. On the other hand, epitopes belonging to two different N-Ags were found to be functionally interdependent. Indeed, the Kb epitope of N-Ag1 was interrelated in functional analysis with epitopes belonging to N-Ag2 (Ka and the group of M epitopes). Moreover, priming animals with synthetic peptides mimicking N-Ag1 gives rise, after boosting with viruses, to neutralizing antibodies cross-reacting with N-Ag2 and vice versa (D. Diamond, unpublished). This suggests that N-Ag1 and N-Ag2 are either contiguous on the virion surface or are both under the influence of another, distinct region of the virus capsid.

Mahoney versus Sabin antigenic phenotype. In a previous study, we observed a reversion of the epitope map of Sabin virus toward that of its parental Mahoney strain upon

multiplication in the human gut (13). We have also observed the reciprocal transition of the Mahoney epitope map toward that of the Sabin virus when mutants resistant to Mahoney-specific N-mcAbs were isolated from Mahoney virus (4). Using αMa and αMd N-mcAbs to derive neutralization resistant mutants, Diamond et al. (16) also recently reported transition from Mahoney to Sabin antigenicity. Here, a total of six Mahoney virus epitopes showed altered behavior such that their reaction with the corresponding mcAb became similar to that of the original Sabin virus. The prominent finding was that a single-point mutation was responsible for the new phenotype. Results of sequencing (16) (Fig. 3) showed that the unique replacement of a Thr residue (Mahoney) by a Lys residue (Sabin) at position 60 of VP3 was responsible for this pleiotropic phenotypic effect. That the Lys at position 60 of VP3 is the critical amino acid conferring Sabin specific antigenicity was also confirmed here by sequencing a Sabin virus resistant to neutralization with its specific N-mcAb αS (S-S^r mutant in Fig. 3). The Lys residue was replaced in this case by a Glu residue and not by the Mahoney Thr residue, which might explain why the Sabin-derived S-S^r mutants did not revert to a full Mahoney phenotype. This assumption was reinforced by demonstrating that an Lys-to-Thr substitution in Sabin-derived mutants gives rise to a Mahoney-like antigenic phenotype (D. Diamond, unpublished data). It thus seems probable that the Thr-to-Lys substitution at position 60 of VP3 in the original Sabin 1 strain was selected during in vivo passages of Mahoney virus performed by Li and Schaeffer on monkey skin (30). It is reasonable to presume that neutralizing antibodies developed by inoculated monkeys were the selective factor for the appearance of the Sabin 1 antigenic phenotype in the nonneurotropic variant used by A. B. Sabin to develop the type 1 oral poliovirus vaccine (40). Since the Mahoney-to-Sabin antigenic conversion in the M-Mb^{r}1 variant was not accompanied by attenuation of neurovirulence in monkeys, it is clear that the absence of the Mahoney-specific group of N-Eps does not correlate with attenuation of virulence, confirming our recent results (11) and those of Diamond et al. (16). Similarly, conservation of the Sabin epitope pattern in neurovirulent mutants selected from Sabin 1 virus at high temperature (11) shows that the presence of Sabin-specific N-Ep is not involved in attenuation. These conclusions should be taken into consideration in interpreting the results of studies of the natural antigenic variation of Sabin virus excreted by vaccinees.

Of the phenotypes found among natural antigenic variants of type 1 poliovirus (13), a particular one was encountered in which all Mahoney- and Sabin-specific epitopes were present in terms of antibody-binding but not in terms of neutralization. The same phenotype was obtained as an alternative in the selection of Mahoney mutants resistant to Mahoney-specific N-mcAbs (16) (M-Md^{r}2 and M-Mc^{r}2 in Table 2). The corresponding mutation was localized to position 73 of VP3 (16). It therefore seems evident that it is the amino acid position affected in VP3 rather than the target Mahoney epitope used in selection, which is essential for generating one or the other mutant antigenic phenotype.

The strain-specific Md and S epitopes, which function as neutralization epitopes on Mahoney and Sabin virus, respectively, were antibody-binding but not neutralization epitopes on the reciprocal strain. They were never lost in antibody-binding terms in any of the neutralization-resistant mutants selected with mcAbs alone. αIg-mediated neutralization allowed us to select viral mutants in which the spatial conformation of such nonneutralization epitopes was disturbed so as to render them nonreactive with the corresponding antibody. We were thus able to show that mutations located outside of the capsid region controlling the conformation of these nonfunctional N-Eps (positions 58 and 71) were located in the same region of VP3 (Fig. 3) which controls their neutralization function (positions 60 and 73), outside of the antibody-binding site. Amino acid substitutions on VP3 at positions 58 (Sabin) and 71 (Mahoney) had a similar, but not identical, pleiotropic effect on the strain-specific antigenic phenotypes as the Thr (Mahoney) or Lys (Sabin) substitution at position 60 of VP3 (Fig. 3). It could thus be concluded that the four amino acid positions on VP3, namely, 58, 60, 71 (Fig. 3), and 73 (16), can modulate independently the neutralization function and three-dimensional conformation of strain-specific epitopes situated in the N-Ag2 of VP1.

Epitope Kc. N-mcAbs raised with infectious, D-antigenic type 1 poliovirus did not react with heat-denaturated C-antigenic particles or with isolated structural polypeptides. All neutralization-resistant mutants selected against these N-mcAbs and which were analyzed here or elsewhere (16) had amino acid substitutions outside of the antibody-binding sites. αKc (C3) antibody, raised with C particles, had the unique trait of recognizing a configuration, located in N-Ag2 on infectious D virions, which was conserved on heat- or detergent-denatured virions. It may thus be assumed that the conformation of the Kc N-Ep is not influenced by neighboring epitopes mapping at the same antigenic site. This assumption was confirmed by the operational mapping of N-Eps, which showed that the Kc epitope was not influenced by mutations affecting epitopes recognized by mcAbs raised with infectious virions. Moreover, the neutralization-resistant mutants selected against αKc N-mcAb had mutations at residue position 100 of VP1, within the antibody-binding site (N-Ag2).

The difference in the amino acid substitutions at position 100 of VP1 between Mahoney (Asn to Asp) and Sabin (Asn to Lys) virus in Kc^r mutants had a decisive influence on the capacity of αKc antibodies to bind and immunoprecipitate the neutralization-resistant virions. Whereas Mahoney-derived Kc^r variants gave a negative binding test, the Sabin-derived Kc^r mutants were still able to bind αKc mcAbs. It might be that the substitution of the Asn residue at position 100 of VP1 with different amino acids in Mahoney- and Sabin-derived variants is imposed by amino acid sequence differences existing between these two viruses at positions 95 and 99 in N-Ag2 (Fig. 3). Some of our results plead in favor of the latter assumption. When αKc neutralization-resistant mutants were isolated from Sabin virus, two different mutant codons were obtained, both of which, however, coded for Lys at position 100 of VP1. This supports the idea that only mutants which have Lys at position 100 of VP1 survived selection, other mutants either having lethal mutations or being still susceptible to neutralization with αKc antibody. The results obtained by sequence analysis of the doubly neutralization-resistant mutant M-Mb^{r}Kc^r also support this explanation. The amino acid change consequent to the second Kc selection was an Asp to Asn substitution at position 100 of VP1, exactly the same as that encountered in Kc^r mutants derived directly from Mahoney virus.

Modulation of N-Ag2 (amino acids 93 through 103) of type 1 poliovirus. The neutralization function and three-dimensional conformation of N-Ag2 thus appear to be modulated by several distinct regions of the viral capsid. Different amino acids, situated either outside (residue 270 on VP2 and residues 58, 60, 71, and 73 on VP3) (16) (Fig. 3) or inside the

antibody-binding site (residue 100 of VP1) (Fig. 3), are key positions in determining the conformation of N-Ag2. The definition of N-Ag2 as an antibody-binding site was shown in enzyme-linked immunosorbent assays by N-mcAb binding of synthetic peptides carrying VP1 sequences (18). However, it is still not definitely proven that regions of capsid proteins other than residues 93 through 103 of VP1 do not effectively participate in the construction of N-Ag 2. High-resolution X-ray crystallography will elucidate the precise topology of neutralization immunogenic sites on poliovirus.

The complexity of N-Ag2 may explain why, so far, synthetic peptides reproducing amino acid sequences of type 1 poliovirus were not capable of efficiently eliciting neutralizing antibodies (7, 18). These peptides were poor immunogens, as are isolated structural polypeptides themselves (5, 6, 15, 44). Recently, however, it was reported that a synthetic peptide consisting of the amino acid sequence 89 through 104 of type 3 poliovirus VP1 (25) induced a significant level of neutralizing antibodies in rabbits. It may be that the corresponding N-Ag present on residues 93 through 103 of type 3 poliovirus has a less complex structure than that of type 1 poliovirus. It has been shown that mutations critical for conformation in this type 3 capsid region may be localized to eight amino acid positions, all situated inside of the antibody-binding site. It is intriguing to see that two viruses, probably of common ancestry, possessing the same general physical and biological properties and many sequence homologies throughout their genome (29, 38, 39, 42), carry such structurally and functionally different immunodominant sites in the same region of VP1.

The way in which N-Ag2 is functionally and structurally modulated seems to be much more complex in type 1 poliovirus than in type 3 poliovirus. This complexity benefits the capacity of the virus to survive in nature and spread among humans. It should be recalled that the main outbreaks of poliomyelitis have been due to type 1 poliovirus, whereas type 3 poliovirus has only been responsible for a small number of paralytic cases.

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ADDENDUM IN PROOF

After submission of this manuscript, the atomic resolution structure of two picornaviruses, rhinovirus (M. G. Rossamnn, E. Arnold, J. W. Erickson, E. A. Frankenberger, J. P. Griffiths, H. J. Hecht, J. E. Johnson, G. Kamer, M. Luo, A. G. Mosser, R. R. Rueckert, B. Sherry, and G. Vriend, Nature (London) **317**:145–153, 1985) and poliovirus (J. M. Hogle, M. Chow, and D. J. Filman, Science **229**:1358–1365, 1985), was published. The data clearly indicate that residue 270 on VP2 and residues 58, 60, 71, and 73 on VP3 in Mahoney type 1 poliovirus are topographically distant from N-Ag2 (amino acids 93 through 103 of VP1).

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