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Molecular Basis for Linkage of a Continuous and Discontinuous Neutralization Epitope on the Structural Polypeptide VP2 of Poliovirus Type 1

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We obtained neutralizing monoclonal antibodies against a continuous neutralization epitope on VP2 of poliovirus type 1 strain Mahoney by using a combined in vivo-in vitro immunization procedure. The antibody-binding site was mapped to amino acid residues within the peptide segment (residues 164 through 170) of VP2 by competition with synthetic peptide and sequencing of resistant mutants. Cross-neutralization of these mutants with another neutralizing monoclonal antibody revealed a linkage of the continuous epitope and a discontinuous neutralization epitope involving both loops of the double-loop structure of VP2 at the twofold axis on the surface of the virion.

The characterization of antigenic structures of animal viruses at the molecular level has made great progress in the last few years. Especially in the picornavirus group the use of neutralizing monoclonal antibodies (MAbs) and rapid sequencing techniques of RNA genomes of neutralization-resistant mutants have been valuable tools for the mapping of neutralization sites (2, 7, 21–23, 25, 29, 40, 41). These results in context with the known three-dimensional structure of several members of the picornavirus group provided a detailed, although certainly still incomplete, picture of viral surface structures involved in a specific immune response (16, 27). The limitations are probably due to the small number of MAbs and to the screening and selection procedures used. In general, three to four neutralizing antigenic sites have been described, involving residues of all three major polypeptides. The existence of neutralization sites on the three major polypeptides of poliovirus type 1 was first demonstrated by immunization of rabbits with purified polypeptides (6).

The sites recognized by murine MAbs have been mapped to amino acid residues 91 through 101 of VP1 (N-AgI, site 1) and to residues 221 through 226 of VP1 and 164 through 170 and 270 of VP2 (N-AgII, site 2). N-AgIII, site 3, was mapped to amino acids 58 through 60 and residues 70 through 73 and residues 76, 77, and 79 of VP3 and to residues 286 through 290 of VP1, respectively (7, 21–23, 25, 40–42). During these studies, an apparent immunodominance of site 1 over sites 2 and 3 in serotypes 2 and 3 was suggested despite the close structural relationship of poliovirus serotypes. This paradox has been attributed to the restricted immune response of inbred mouse strains and to the subtle structural differences of serotypes 1 and 3 at this site (17). However, using the same mouse strains, we have shown recently that site 1 of poliovirus type 1 is immunogenic by using conventional immunization protocols (33, 40). Moreover, we described the molecular basis for the linkage of a continuous and a discontinuous epitope involving this site of poliovirus type 1 (41).

In the present study, we describe the linkage of a new continuous and discontinuous neutralization epitope formed by residues of VP2 of poliovirus type 1. Both neutralizing MAbs recognizing either the continuous or the discontinuous epitope were obtained after combined in vivo-in vitro immunization (39). Previously, this procedure resulted in the isolation of hybridomas secreting neutralizing MAbs against the continuous epitope within site 1 of poliovirus type 1 (38). Neutralizing MAbs reacting with isolated polypeptides, thus recognizing continuous neutralization epitopes, are very rare among picornaviruses. To our knowledge, the two cases described in the literature include one antibody against an epitope also within site 1 of poliovirus type 1 and another antibody against an epitope between residues 153 and 164 of VP2 of human rhinovirus 2 (1, 31, 35). However, a combination of antibodies directed against continuous and discontinuous epitopes within the same site may become very useful for the detection of structural changes of virus particles.

MATERIALS AND METHODS

Growth of virus and cells. Poliovirus type 1 strain Mahoney was propagated in HeLa S3 cells and purified as described previously (8). The Sabin strain of poliovirus type 1 (Lsc2ab) was grown in suspension cultures of HeLa Ohio cells (Flow Laboratories, Inc., McLean, Va.). Infected cells were harvested after 8 h at 35°C. Cells were lysed by one cycle of freezing and thawing and Dounce homogenization. Cellular debris was removed by low-speed centrifugation. The supernatant was treated with 0.5% sodium deoxycholate and clarified by centrifugation for 10 min at 10,000 × *g*. From this supernatant, which was adjusted to 0.3 M NaCl and treated with 1% sarcozyl, virus was pelleted by high-speed centrifugation and further purified by cesium chloride density gradients. Virus-containing fractions were stored at −20°C. Virus was pelleted from the fractions by high-speed centrifugation for isoelectric focusing (IEF) and preparation of RNA. HeLa Ohio cells were also grown as monolayer cells and used for microneutralization tests and for the selection and propagation of antigenic variants.

Preparation of viral polypeptides for immunization. Pure poliovirus polypeptides were obtained by reversed-phase high-pressure liquid chromatography in 60% formic acid and a gradient of acetonitrile as detailed previously (14). After

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freeze-drying they were dissolved in 8 M urea and 0.1% dithioerythritol.

Preparation of MAbs. For in vivo immunization, mice were primed subcutaneously with 20 μg of polypeptide in complete Freund adjuvant. Four weeks later they were boosted intraperitoneally three times with the same amount of antigen without adjuvant on 3 successive days, and fusion was carried out on day 4. For in vitro stimulation, mice were primed as above. They were boosted once or twice at 4-week intervals also with 20 μg of antigen. At 2 to 6 months after boosting, the animals were sacrificed and the spleens were removed. In vitro cultivation of splenocytes was done in the presence of 5 μg of poliovirus per ml as described previously (39). Fusion of spleen cells after conventional immunization of BALB/c mice or after in vitro stimulation of primed spleen cells was done as described previously (18, 39). Hybridoma supernatants were initially screened in a solid-phase enzyme-linked immunosorbent assay with poliovirus type 1 (Mahoney) per ml as the antigen bound either directly to microdilution plates or by polyclonal rabbit anti-poliovirus immunoglobulins. The antibodies were typed by using isotype-specific anti-mouse immunoglobulins.

Virus neutralization. Virus neutralization was determined in a microneutralization assay (33). Dilutions of ascites fluids (100 μl) were incubated with wild-type or mutant virus (50 PFU in 50 μl) for 2 h at 37°C. Then 100 μl of trypsinized HeLa cells (5×10^5/ml) was added. After 3 days the cells were stained with 0.1% crystal violet–20% ethanol in phosphate-buffered saline. In competition experiments with synthetic peptides, antibody dilutions were preincubated with peptide (100 μg/ml) for 1 h at 37°C before the addition of virus.

Immunoprecipitation. Protein A-aided immunoprecipitation was carried out in microdilution plates (36). Briefly, [^{35}S]methionine-labeled virus (10 μl) was incubated with 100 μl of serial twofold dilutions of ascites fluids and incubated for 1 h at room temperature. Then anti-mouse immunoglobulins (10 μl) were added, and the plates were kept for 1 h at 4°C. Finally, Formalin-fixed cells of the Cowan 1 strain of *Staphylococcus aureus* were added. After 30 min at 4°C, the plates were centrifuged for 15 min at 260 × *g*, and 100 μl of supernatant was removed for determination of radioactivity. In the competition experiments with synthetic peptides, the antibodies were preincubated with peptide (40 μg/ml) for 1 h before the addition of virus.

Selection of mutants. Antigenic variants were selected by standard procedures (2, 29, 30). Briefly, 10-fold dilutions of wild-type virus were incubated with ascites fluid dilutions in MEM-FCSH (minimal essential medium containing 2% fetal calf serum and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.2]) containing an approximately 100-fold excess of antibody above endpoint titers as determined in a microneutralization test. After incubation for 2 h at 37°C, the virus-antibody mixture was plated onto HeLa monolayer cells in six-well plates (Greiner, Nürtingen, Federal Republic of Germany). After 1 h at 37°C, the inoculum was removed and cells were overlaid with 2 ml of 0.9% agar in minimal essential medium containing 5% fetal calf serum and a 10^{-2} or 10^{-3} dilution of the ascites fluid. After 2 days, plaques were stained with Thiazolyl blue (Serva, Heidelberg, Federal Republic of Germany) in 1.8% agar (19). Mutant plaques were picked and transferred into separate tubes containing MEM-FCSH. After freezing and thawing, each viral clone was passaged once in separate wells of a six-well plate in the presence of a 10^{-2} dilution of ascites fluid in minimal essential medium containing 5% fetal calf serum. The virus was harvested after 48 h and used as mutant stock.

Purification of variants. Each virus strain was purified by low-speed and high-speed centrifugation of infected cell lysates. After solubilization of the high-speed pellet (0.1 M NaCl, 10 mM Tris [pH 7.4], 1 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate), the virus was further purified by sucrose density gradient centrifugation. Purified virus was pelleted from pooled fractions by high-speed centrifugation and served as material for IEF and for preparation of viral RNA.

IEF. IEF was done in horizontal slab gels (12). The slab gel was prepared in a 23- by 11.5- by 0.1-cm glass chamber (LKB Instruments, Freiburg, Federal Republic of Germany). One plate was treated with 1% Silane A 174 (3-methacryloxypropyl-trimethoxysilane) (26) in chloroform to assure tight adherence of the gel. A thin Mylar sheet (0.08 cm) bearing slots was included in the chamber assembly. The gel mixture containing 5% acrylamide, 0.13% *N,N'*-methylen-bisacrylamide, 9 M urea, and 2% ampholines (pH 3.5 to 10) (LKB Instruments) and 0.03% ammonium persulfate was poured into the chamber, and the chamber was left overnight at room temperature. After the chamber was disassembled, the plate with the adhering gel was placed onto a cooling block of the horizontal slab gel apparatus with a thermostat (Multiphor II; LKB Instruments) with the temperature set at 10°C. Paper electrode strips containing 0.02 N H_3PO_4 (cathode) and 0.04 N NaOH (anode) were placed on top of the edges of the gel. During pre-electrophoresis (400 V, 30 min), the gel was soaked with 8 M urea, 100 mg of dithiothreitol per ml. Virus samples were dissociated for 1 h at 25°C in 9 M urea and 100 μg of RNase A per ml; dithiothreitol (0.5%) was added after 15 min. After sample application, voltage was increased stepwise at 10-min intervals from 50 to 100 to 200 to 400 V. Then power was set to 25 W and focusing was continued for 3,600 Vh. The gel was fixed with 20% trichloroacetic acid and silver stained (15).

Sequencing of viral RNA. Viral RNA was extracted with phenol-chloroform in the presence of 1% sodium dodecyl sulfate and ethanol precipitated. Wild-type and variant RNA (1 to 2 μg) and the appropriate primer (10 to 20 ng) were annealed, and the nucleotide sequences were determined by the dideoxy-chain termination method (28) with avian myeloblastosis virus reverse transcriptase (Promega Biotec Co., Madison, Wis.). The samples were loaded to a 6% polyacrylamide sequencing gel containing 8 M urea. The RNA of the mutants was sequenced at least throughout the regions of VP2 and VP1 known to contribute to N-AgII or site 2 (25). No further sequencing was done when the amino acid substitutions found were compatible with the results obtained by IEF.

Preparation of peptide antiserum. The peptide from the VP2 region 164 through 170 of poliovirus type 1 (Mahoney), Cys-Asp-Asn-Asn-Gln-Thr-Ser-Pro, was synthesized by Bio-Chrom (Berlin, Federal Republic of Germany). The additional cysteine at the N terminus was added to provide a coupling group for the heterobifunctional maleimidobenzoyl-*N*-hydroxysuccinimidoester (20) (Serva, Heidelberg, Federal Republic of Germany). Bovine serum albumin (BSA) (0.5 μmol) in 1.1 ml of 10 mM sodium phosphate (pH 7.5) was reacted for 30 min at room temperature with maleimidobenzoyl-*N*-hydroxysuccinimidoester (5 μmol) in dimethylformamide. Excess reagent was removed by filtration through a Bio-Gel P6 column (Bio-Rad Laboratories, Richmond, Calif.) in 10 mM sodium acetate (pH 5.5) at 4°C. This

TABLE 1. Binding and neutralization titers of MAbs obtained after in vivo or combined in vivo-in vitro immunization with purified VP2 of poliovirus type 1

MAb		Binding titer[a]	Neutralization titer[a]	Polypeptide specificity[b]	Antibody isotype
No.	Code				
1	2.4[c]	64	<10	VP2	IgG2
2	2.5[c]	16	<10	VP2	IgM
3	2.6[c]	16	<10	VP2	IgM
4	2.27[c]	8	<10	VP2	IgG3
5	10.26[d]	32	<10	VP2	IgM
6	27.77[d]	128	<10	VP2	IgG1
7	27.108[d]	1,280	1,280		IgM
8	27.138[d]	64	<10	VP2	IgM
9	27.165[d]	160	160	VP2	IgM
10	34.3[d,e]	160	40	VP2	IgG1

[a] Reciprocals of ascites fluid dilutions precipitating 50% of input radioactivity or giving 100% protection in a microneutralization test.
[b] Determined by immunoblotting.
[c] Obtained after in vivo immunization.
[d] Obtained after combined in vivo-in vitro immunization.
[e] Neutralization titer after the addition of rabbit anti-mouse immunoglobulins.

resulted in the introduction of four maleimidobenzoyl groups per molecule of BSA as determined with Ellman reagent. For cross-linking of peptide, 0.1 μmol of BSA-maleimidobenzoyl group in 1 ml of 10 mM sodium phosphate (pH 5.5) was reacted with 1 μmol of peptide at room temperature for 3 h.

Rabbits were immunized subcutaneously with 0.5 mg of BSA-peptide complex in complete Freund adjuvant. They were boosted twice at 3-week intervals with the same amount of BSA-peptide complex in incomplete Freund adjuvant. Three weeks after the last boost, the animals were bled and the serum was collected.

RESULTS

Isolation of MAbs. In our in vivo immunization protocol, aimed at the isolation of MAbs against continuous neutralization epitopes, mice were primed and boosted with purified polypeptide. Mice were also primed and boosted with purified polypeptide in our combined in vivo-in vitro immunization system; however, splenocytes were stimulated in vitro with infectious virus as the antigen for 6 days before fusion (39). Screening of hybridomas from both protocols was primarily done by indirect enzyme-linked immunosorbent assay with poliovirus as antigen bound either directly to the solid phase or by rabbit anti-poliovirus immunoglobulins. In neutralization tests carried out at this stage we very rarely found protection of HeLa cells from poliovirus infection by hybridoma supernatants obtained from both immunization protocols. Moreover, we could never establish a stable hybridoma cell line producing neutralizing antibodies from a microculture well showing initial neutralizing activity. Therefore we added an additional screening step. Hybridomas were selected according to their reaction pattern in the enzyme-linked immunosorbent assay and further tested by microimmunoprecipitation with labeled virus as the antigen. Cells from microculture wells precipitating a considerable amount of radioactivity were cloned and expanded and used for production of ascites fluids.

Comparison of MAbs obtained from immunization with VP2 of poliovirus type 1. In Table 1, we have summarized a comparison of virus-binding and neutralization titers of 10 MAbs obtained by conventional in vivo immunization (nos. 1 through 4) or by combined in vivo-in vitro immunization (nos. 5 through 10). Antibodies with titers exceeding 2^{-3} in the microimmunoprecipitation test were included in Table 1. Antibody 5 was derived from a fusion after in vitro stimulation of spleen cells after a single boost in vivo, whereas antibodies 6 through 10 were derived from mice boosted twice in vivo with purified polypeptides. Obviously hybridomas resulting from this protocol yielded antibodies that had higher virus-binding titers and also in some cases higher neutralizing activity. Except for antibody 7, all antibodies reacted with VP2 in an immunoblot (Table 1). These results provide direct evidence for the presence of a continuous epitope on VP2 of poliovirus type 1, which epitope is also expressed on the surface of infectious virus particles. From both immunization procedures antibodies of the immunoglobulin M (IgM) isotype were obtained, suggesting that in both cases they could have originated from primary as well as secondary immune responses. The failure of antibody 7 to react with isolated VP2 indicated recognition of a discontinuous epitope. More evidence for this assumption is given below.

TABLE 2. Inhibition of virus binding and neutralization by MAb with synthetic peptides

MAb		Binding titer[a] after preincubation with peptide:		Neutralization titer[a] after preincubation with peptide:	
No.	Code	A[b]	B[c]	A	B
1	2.4	<2	64	NT	NT
5	10.26	<2	32	NT	NT
6	27.77	<2	128	NT	NT
7	27.108	1,280	1,280	320	640
8	27.138	<2	64	NT	NT
9	27.165	<10	160	10	160
10	34.3[d]	<10	160	<10	20
11	95[e]	320	<10	640	10

[a] Reciprocals of ascites fluid dilutions precipitating 50% of input radioactivity or giving 100% protection in a microneutralization test. Antibodies were preincubated with peptide for 1 h at room temperature or 37°C. NT, Not tested.
[b] Peptide A (VP2 residues 164 through 170): Cys-Asp-Asn-Asn-Gln-Thr-Ser-Pro.
[c] Peptide B (VP1 residues 93 through 104): Cys-Asp-Asn-Pro-Ala-Ser-Thr-Thr-Asn-Lys-Asp-Lys-Leu-Cys. Peptide B was circularized by the two additional cysteines.
[d] Neutralization titers after addition of rabbit anti-mouse immunoglobulins.
[e] MAb against N-AgI.

Inhibition of virus binding and neutralization by MAbs with synthetic peptides. Several of the antibodies listed in Table 1 were tested in competition experiments with a synthetic peptide comprising residues 164 through 170 of VP2 of poliovirus type 1 (peptide A). A similar peptide corresponding to amino acids 167 through 173 of VP2 has been used for the induction of a neutralizing response in rabbits (10). In addition, this segment of VP2 is part of a surface loop that is exposed near the twofold axis on the surface of the virion (16). As a control in our experiments we used a peptide representing amino acid residues 93 through 104 of VP1 (peptide B) and a neutralizing MAb (no. 95) directed against this site (N-AgI) (38). The results of the competition experiments are summarized in Table 2. Virus binding and virus neutralization of all antibodies recognizing isolated VP2 (Table 1) were blocked by preincubation with peptide A. Preincubation with peptide B had no effect; however, it abolished reactivity of antibody 95.

These results demonstrated that the peptide representing

TABLE 3. Cross-neutralization of resistant mutants

Virus or mutant	Selecting MAb	Neutralization titer[a]		
		MAb		Antipeptide antibody[b]
		27.108	27.165	
Virus				
PV1 Mahoney		320	320	128
PV1 Sabin		<10	10	32
Mutants				
1	27.108	<10	<10	16
2	27.108	<10	<10	<4
3	27.108	<10	160	<4
4	27.108	<10	640	<4
5	27.108	<10	80	128
6	27.165	10	<10	<4
7	27.165	10	<10	<4
8	27.165	40	<10	<4

[a] Reciprocals of ascites or serum dilutions giving 100% protection in a microneutralization test.

[b] The peptide (VP2 residues 164 through 170) Cys-Asp-Asn-Asn-Gln-Thr-Ser-Pro was coupled to BSA and used for immunization (for details, see Materials and Methods).

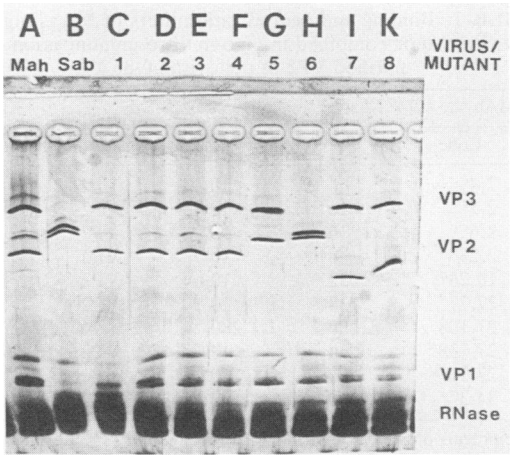

FIG. 1. IEF patterns of poliovirus type 1 strains. Polypeptides of poliovirus type 1 Mahoney and Sabin strains are shown in lanes A and B. Mutant viruses are analyzed in lanes C through K. The numbers refer to their designations used in Tables 3 and 4. Positions of wild-type polypeptides are indicated.

amino acid residues 164 through 170 of VP2 contained a continuous epitope that was recognized by all antibodies reacting with isolated VP2 included in this table. However, virus binding and neutralization of antibody 27.108 were not competed by preincubation with peptide A or B, providing additional evidence for recognition of a discontinuous epitope.

Cross-neutralization of resistant mutants. Eight antibody-resistant variants were selected in the presence of antibodies 27.108 and 27.165, respectively. Titers of all mutants were determined in a cross-neutralization test in comparison to wild-type Mahoney and the Sabin strain of poliovirus type 1 (Table 3). We also included a rabbit antiserum that was raised against peptide A (Table 2) coupled to BSA. Both MAbs and also the polyclonal anti-peptide antibodies exhibited a strong specificity for the Mahoney strain. This specificity is due to an amino acid substitution within this peptide segment of VP2 of the Sabin strain. The mutation of nucleotide 1462 (A → G) in the Sabin strain resulted in a substitution of the asparagine by aspartate in position 165 of VP2 (24). Mutants 1 and 2, which were selected in the presence of antibody 27.108, were also resistant to antibody 27.165, demonstrating a linkage between the discontinuous epitope and the continuous neutralization epitope. This linkage was also found with mutants 6 to 8 selected by antibody 27.165. Mutants 3 and 4, although still sensitive to monoclonal antibody 27.165, were nevertheless resistant to neutralization by polyclonal anti-peptide antibodies. Although mutations have occurred within the peptide segment representing residues 164 through 170 of VP2, obviously they were tolerated by the MAb. In contrast, the phenotype of mutant 5 was most compatible with a mutation outside of this segment of VP2. Determination of the sequence changes conferring the resistant phenotype of the mutants should provide insight into the molecular basis of the linkage of both epitopes.

Sequence determination of antigenic variants. Before our sequencing analysis, the phenotype of all mutants was analyzed by IEF of their structural polypeptides in comparison with wild-type virus (Fig. 1). The pattern of the Mahoney and Sabin strains is shown in lanes A and B of Fig. 1. The differences found in the positions of VP1, VP2, and VP3 of the Sabin strain are due to amino acid substitutions that resulted from the 22 point mutations in the three major capsid polypeptides (24). Mutants 1 through 4 (lanes C through F, respectively) showed no charge alteration of their polypeptides, as compared with wild-type virus. However, mutants 5 through 8 (lanes G through K, respectively) had charge alterations of VP2. Mutant 6 (lane H) also had an altered VP3, which, however, is irrelevant in this context. The mutation in VP2 of mutant 5 and the results of our previous cross-neutralization tests suggested that the discontinuous epitope must be also formed by residues of VP2. Therefore we first sequenced the RNAs of all mutants through the region encoding for VP2 and, if necessary, also through other parts of the capsid region of the genome (Table 4 and Fig. 2).

All amino acid substitutions resulting from point muta-

TABLE 4. Sequence changes of neutralization resistant mutants

Virus or mutant	Selecting MAb	Nucleotide change[a]	Amino acid change[b]
Viruses			
PV1 Mahoney			
PV1 Sabin		1462 A → G	2165 Asn → Asp
Mutants			
1	27.108	1463 A → U	2165 Asn → Ile
2	27.108	1466 A → G	2166 Asn → Ser
3	27.108	1471 A → G	2168 Thr → Ala
4	27.108	1471 A → U	2168 Thr → Ser
5	27.108	1393 C → A	2142 His → Asn
		2991 U → C	1164 Ala silent
6	27.165	1465 A → G	2166 Asn → Asp
		1964 C → A	3060 Thr → Lys
7	27.165	1469 A → G	2167 Gln → Arg
8	27.165	1470 G → C	2167 Gln → His

[a] The nucleotide sequence was determined by the dideoxy-chain termination method with avian myeloblastosis virus reverse transcriptase and synthetic oligonucleotide primers. The nucleotide number refers to the position within the poliovirus genome (32).

[b] The amino acid sequence was deduced from the nucleic acid sequence. The N-terminal amino acids of VP1, VP2, and VP3 are identified by the four-digit numbers 1001, 2001, and 3001, respectively.

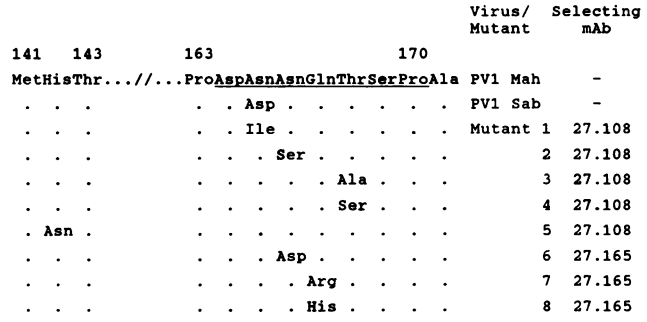

FIG. 2. Amino acid substitutions found in the Sabin strain of poliovirus type 1 and antibody-resistant mutants. Neutralization resistant mutants were selected by neutralizing MAbs 27.108 (mutants 1 through 5) and 27.165 (mutants 6 through 8). The amino acids are numbered from the N terminus of VP2. The sequence of the peptide used in competition experiments and for the preparation of the rabbit hyperimmune serum is underlined.

tions of the mutants were found between amino acid residues 165 through 168 of VP2 except for mutant 5. In this case, a point mutation was detected about 70 nucleotides upstream, resulting in a substitution of an asparagine for a histidine at position 142 of VP2. This substitution of the weakly basic histidine by asparagine is also demonstrated by the appropriate charge shift of VP2 of the mutant (Fig. 1). Sequencing of the RNA encoding for the VP3 region of mutant 6 revealed a substitution of residue 60 of VP3 (Thr → Lys), explaining the charge shift of VP3 of this mutant (Fig. 1). This substitution (Thr → Lys) is also found in VP3 of the Sabin strain of poliovirus type 1 (24).

The substitutions of threonine 168 either by alanine or serine (mutants 3 and 4) were tolerated by antibody 27.165, although both mutants became resistant to anti-peptide antibodies. The question of whether these changes are simply tolerated by the antibody or are outside the combining site is left open at the moment.

DISCUSSION

MAbs. Detailed knowledge of antigenic structures of viral polypeptides, especially of structures involved in the induction of a protective immune response, is of fundamental interest for future vaccine development. Because of its known three-dimensional structure, poliovirus type 1 is one of the best-suited candidates for structural and immunologic analysis among naked animal viruses. For the study of antigenic structures of poliovirus polypeptides contributing to antigenic sites either on the mature virion or only on isolated polypeptides and immature precursor particles, it seemed useful to us to raise a panel of MAbs by using isolated structural polypeptides as immunogens. After immunization with VP1 in our in vivo system we obtained antibodies from 19 hybridomas. These antibodies neither neutralized virus nor bound to virus particles. In contrast, after immunization with VP2, all eight hybridomas selected and propagated secreted antibodies that bound to virus particles but also failed to neutralize virus. In addition, the antibodies against VP1 were directed against different parts of the polypeptide chain determined by their reaction with proteolytic fragments in Western blots, whereas all VP2 antibodies bound to the same fragment after cleavage of VP2 (data not shown). Since in both cases identical screening assays and selection procedures had been applied, we assume that VP2, in contrast to VP1, retained a partial native structure even after separation by reversed-phase high-pressure liquid chromatography (14). An indication for this assumption was provided by the observation that electrophoresis of poliovirus polypeptides in high concentrations of formic acid resulted in an inversion of their mobilities in polyacrylamide gels (13). Obviously in the in vivo situation this residual structural element of VP2 was predominantly recognized by the immune system.

TABLE 5. Immunoprecipitation titers of MAbs with virus or heated virus (1 h, 56°C) as the antigen

MAb	Immunoprecipitation titer[a]		Specificity
	Virus	Heated virus	
27.108	512	<8	N-AgII
27.165	256	8,192	N-AgII
197	2,048	<8	N-AgII
70	1,024	128	N-AgI
95	256	1,024	N-AgI

[a] Reciprocal of antibody dilution precipitating 50% of input radioactivity.

As shown previously, we could increase the number of specific hybridomas 6- to 20-fold by in vitro stimulation of primed spleen cells (39). Using this protocol and a refined screening and selection procedure of specific hybridomas, we succeeded in the detection of neutralizing MAbs against a continuous epitope on VP1 (38). As described in the present study, this procedure resulted also in the detection of neutralizing MAbs against a continuous and a discontinuous epitope on VP2. Apparently in vitro stimulation of primed spleen cells yielded hybridomas that secreted high-affinity antibodies, indicated by increased titers in virus-binding assays and in the appearance of virus neutralization activity. It is likely that during in vitro stimulation of primed spleen cells by virus particles a subset of memory cells recognizing cross-reacting antigens is stimulated, giving rise to these antibody secreting hybridomas. At this point, however, we are quite aware that the number of antibodies is too small to draw definite conclusions regarding the mechanisms underlying our immunization procedures.

Continuous and discontinuous neutralization epitopes. Circumstantial evidence for continuous neutralization epitopes on poliovirus polypeptides was obtained by immunization of animals with isolated polypeptides purified by a variety of different procedures (3, 4, 6, 34, 37). In some cases, synthetic peptides selected on the basis of hydrophilicity profiles of viral polypeptides were also found to induce a low neutralizing response (5, 9–11, 43). Since the determination of the three-dimensional structure, it has become evident that peptides that could induce a neutralizing immune response were parts of loops extending from the conserved β-barrel structure of the individual polypeptides (16).

A discontinuous neutralization epitope involving the peptide segment of residues 164 through 170 of VP2 of poliovirus type 1 has been described previously (22, 25). This site on the virus had been defined as N-AgII or site 2 (22, 25, 42). The epitopes described by Page et al. (25) also involved residues 221 through 226 of VP1 and the arginine 270 of VP2. The discontinuous epitope described here is also linked to residues 164 through 170 of VP2, but in addition it involves residue 142 in the adjacent loop of the double-loop structure near the twofold axis of the virion. To date, with our collection of mutants we have not been able to detect a linkage of the peptide segment to residues 221 through 226 of VP1 or to residue 270 of VP2. All mutants described here

were still sensitive to an antibody (no. 197, Table 5) that also selected for a substitution of residues 270 of VP2 and 223 of VP1 (manuscript in preparation). Mutants selected by this antibody, which was obtained after immunization with infectious virus, were still sensitive to the antibodies described in this paper.

Linkage of the continuous and discontinuous epitopes. In a previous report, we have described a linkage of continuous and discontinuous epitopes involving the loop of residues 96 through 104 (N-AgI) and residues 141 through 152 of VP1 of poliovirus type 1. In analogy to human rhinovirus 14, we have called the discontinuous epitope N-AgIB (41). In VP2 we have now demonstrated a similar situation. In this case both loops are not separated by β-strands but rather are parts of a double loop structure with a short intervening α-helix (16).

At the moment, the discontinuous epitopes both on VP1 and VP2 are characterized by a single mutant, in each case with a single amino acid substitution in the loop adjacent to the continuous epitope. However, both mutations occurred in nonconserved regions between serotypes, indicating a possible role of these sites also in vivo.

It has to be emphasized that the linkage of continuous and discontinuous neutralization epitopes described here and in our previous reports provides the first direct evidence for localization of binding sites of antibodies recognizing discontinuous epitopes (40, 41). In this case, long-range effects of the mutations on the conformation of their binding sites can be ruled out.

Finally, the combination of antibodies recognizing continuous and discontinuous epitopes within the same site provides us with sensitive probes for monitoring structural changes during virus morphogenesis or after virus-cell interactions. A first example for an application is the stability of the epitopes after the well-known conformational change which accompanies the transition of N to H particles (Table 5). Evidently during this transition the discontinuous epitopes involving two adjacent loops are readily destroyed.

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