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Poliovirus Sabin Type 1 Neutralization Epitopes Recognized by Immunoglobulin A Monoclonal Antibodies

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Immunity to poliomyelitis is largely dependent on humoral neutralizing antibodies, both after natural (wild virus or vaccine) infection and after inactivated poliovirus vaccine inoculation. Although the production of local secretory immunoglobulin A (IgA) antibody in the gut mucosa may play a major role in protection, most of information about the antigenic determinants involved in neutralization of polioviruses derives from studies conducted with humoral monoclonal antibodies (MAbs) generated from parenterally immunized mice. To investigate the specificity of the mucosal immune response to the virus, we have produced a library of IgA MAbs directed at Sabin type 1 poliovirus by oral immunization of mice with live virus in combination with cholera toxin. The epitopes recognized by 13 neutralizing MAbs were characterized by generating neutralization-escape virus mutants. Cross-neutralization analysis of viral mutants with MAbs allowed these epitopes to be divided into four groups of reactivity. To determine the epitope specificity of MAbs, virus variants were sequenced and the mutations responsible for resistance to the antibodies were located. Eight neutralizing MAbs were found to be directed at neutralization site N-AgIII in capsid protein VP3; four more MAbs recognized site N-AgII in VP1 or VP2. One IgA MAb selected a virus variant which presented a unique mutation at amino acid 138 in VP2, not previously described. This site appears to be partially related with site N-AgII and is located in a loop region facing the VP2 N-Ag-II loop around residue 164. Only 2 of 13 MAbs proved able to neutralize the wild-type Mahoney strain of poliovirus. The IgA antibodies studied were found to be produced in the dimeric form needed for recognition by the polyimmunoglobulin receptor mediating secretory antibody transport at the mucosal level.

Despite the dramatic decrease of poliomyelitis achieved with the efficient and intensive use of inactivated and live attenuated vaccines in industrialized countries, the disease still remains an important public health problem in some areas of the world (8, 17, 35, 45). Detailed knowledge of antigenic determinants, especially neutralizing determinants of the viruses, is essential for understanding the mechanisms of antibody-mediated virus neutralization and protection from disease.

In both natural poliovirus infection and oral immunization, the virus replicates in oropharyngeal and intestinal mucosa, eliciting both a humoral antibody response and a strong local immunity which is correlated with the production and intraluminal release of secretory immunoglobulin A (IgA) antibody (15, 33, 40). This appears to be consistently conferred by the oral poliovirus vaccine, although the parenterally administered inactivated vaccine has been occasionally reported to also elicit mucosal poliovirus-specific immunoglobulin secretion (5).

The mucosal tissues underlying the gut epithelium are heavily populated with cells of the immune system (21). To elicit a mucosal immune response, antigens are transported across the epithelium by M cells and are subsequently processed and presented to effector B lymphocytes, mostly in the Peyer's patches. Stimulated B cells leave the gut-associated lymphoid tissues and migrate to distant mucosal or glandular sites, where they differentiate and produce polymeric IgA. Secretory IgA is a polymeric immunoglobulin which is actively transported through the mucosal epithelia by a mechanism, mediated by the polymeric immunoglobulin receptor (pIgR), which is not available to monomeric immunoglobulins like IgG (42).

Poliovirus is a picornavirus of the *Enterovirus* genus. The virion consists of a single strand of messenger-sense RNA enclosed in an icosahedral capsid composed of 60 copies of proteins VP1, VP2, VP3, and VP4. The precise structure of the capsid proteins for infectious virus of the serotype 1 wild-type Mahoney has been established by X-ray crystallography (16).

The antigenic structure of poliovirus has been extensively studied by characterization of neutralizing IgG class monoclonal antibodies (MAbs) elicited in mice after parenteral immunization. By nucleic acid sequencing of neutralization-resistant variants selected by murine MAbs, three major neutralization antigenic sites have been mapped to the three major polypeptides, VP1, VP2, and VP3, composing the virion surface. These results were confirmed by the construction of chimeric viruses and analysis by X-ray crystallography (2, 9, 29–31, 34). Site 1 (N-AgI) consists of amino acid residues 91 to 101 and 144 of VP1 (hereafter designated 1091 to 1101 and 1144); site 2 (N-AgII) consists of residues 221 to 226 of VP1 (1221 to 1226) and 164 to 170 and 270 of VP2 (2164 to 2170 and 2270); and site 3 (N-Ag III) consists of residues 58 to 60, 70 to 73, 76, 77, and 79 of VP3 (3058 to 3060, 3070 to 3073, 3076, 3077, and 3079) and residues 286 to 290 of VP1 (1286 to 1290) (28, 34, 44). An additional site (N-AgIB) consisting of amino acid residues 1096 to 1104 and 1141 to 1152 in VP1 has been described (43). Based on cross-neutralization studies, Minor (28) has proposed that residues 3076 and 2072 form a functional site distinct from N-AgIII, called N-AgIV.

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Recent studies of rotavirus seem to indicate that mucosal immunization may elicit an immune response to viruses different from the one deriving from parenteral administration of virions (13). The difference may arise either from the specific immune cell repertoire present in mucosa-associated lymphoid tissues or from a possible modification of antigenic determinants on viruses mediated by the particular environment of the gut content, as reported for poliovirus types 2 and 3 (37, 38).

To define the virus epitopes involved in mucosal immunity against poliovirus, we have orally immunized mice with live Sabin type 1 poliovirus and generated a panel of hybridoma cell clones secreting IgA class MAbs. A description of the epitopes recognized by 13 neutralizing IgA MAbs is presented.

MATERIALS AND METHODS

Cells and virus. HEp-2 cells were grown in Eagle's minimal essential medium supplemented with 5% fetal bovine serum at 37°C. The virus used for production of MAbs was the Sabin type 1 poliovirus strain. Wild-type poliovirus serotype 1 (Mahoney strain) and wild-type (MEF 1, P3 Leon) and Sabin type 2 and 3 poliovirus strains were also used to define the neutralization specificity of MAbs.

Virus purification. Poliovirus-infected cell lysates were ultracentrifuged at 35,000 rpm in a Beckman Ti45 rotor for 3 h at 4°C. The pellet was resuspended in phosphate-buffered saline (PBS) and layered onto a 30% sucrose cushion in PBS. After centrifugation in a Beckman SW41 at 35,000 rpm for 2 h at 4°C, the virus pellet was resuspended in 1 to 2 ml of PBS and used for either immunization of mice or coating of enzyme-linked immunosorbent assay (ELISA) plates.

Immunization of mice. Six-week-old female BALB/c mice (supplied by Charles River) were orally administered with 100 μl of Sabin type 1 poliovirus (containing 10^9 PFU) purified as described above in the presence or absence of 10 μg of purified cholera toxin (CT; kindly donated by A. Gallina, Rome, Italy), using 0.5 ml of 7% sodium bicarbonate to neutralize the acidic pH of the stomach. Mice were boosted at 3-week intervals; approximately 10 days after boosting, sera from immunized animals were tested for the presence of neutralizing antibody to poliovirus. Mice immunized in the presence of CT had neutralizing antibody titers ranging from 1:256 to 1:1,412 after the second boost.

Fusion. Mice showing a seroconversion to poliovirus received a final oral boost along with an intravenous injection of virus 4 days before fusion. Each animal was sacrificed, and the small bowel, mesenteric lymph nodes, and spleen were resected. Preparation of spleens for fusion was performed as previously described by Greenberg and coworkers (14). The gut was thoroughly washed with sterile medium (Dulbecco's modified Eagle's medium) supplemented with gentamicin (200 μg/ml) and amphotericin B (Fungizone; 5 μg/ml), and the Peyer's patches were dissected with a fine scalpel and homogenized with a Dounce homogenizer together with mesenteric lymph nodes. The resulting cell suspension was centrifuged through a fetal bovine serum cushion at 1,000 rpm for 8 min. Lymphocytes were counted by using a vital stain, and the concentration of cells was adjusted to about 10^9/ml. Fusion was carried out at the same time for spleen cells and cells from regional lymphoid tissues, using the same procedure (14). Hybridoma supernatants were screened by using a microneutralization test and an ELISA using purified Sabin poliovirus type 1 as the capture antigen and an alkaline phosphatase-conjugated anti-mouse IgA (α chain; Sigma) or IgG (heavy and light chains [H+L]; Sigma) secondary antibody (see below). Selected cultures were cloned by limiting dilution using a mouse thymocyte feeder layer, and MAbs were amplified in mouse ascites fluid. Confirmation of MAb isotype was performed by ELISA using a mouse antibody isotyping kit (Pierce, Rockford, Ill.).

RIPA. ^{35}S-labeled poliovirus-infected HEp-2 cell lysates were prepared as previously described (7). A modified immunoprecipitation assay was developed to overcome the lack of reactivity of IgA MAbs with protein A. Fifty microliters of radiolabeled cell lysate in mild radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 150 mM NaCl, 10 mM Tris-HCl [pH 7.2]) was incubated for 2 h at 4°C with a 1:100 dilution of ascites fluid in mild RIPA buffer. For RIPA under denaturing conditions, SDS in the buffer was made up to 1%. Separately, protein G-conjugated Sepharose beads (Sigma) were incubated with a 1:100 dilution of a goat anti-mouse IgA antibody (Sigma) in PBS for 2 h at room temperature. After extensive washing with RIPA buffer to remove unbound antibodies, beads were added to IgA-antigen reactions and incubation was protracted for 2 h at 4°C. After extensive washing with RIPA buffer, beads were incubated with electrophoresis sample buffer. SDS-polyacrylamide (15%) gel electrophoresis (PAGE) and autoradiography were performed as previously described (23).

ELISA. For analysis of hybridoma supernatants, microtiter ELISA plates were incubated overnight (50 μl/well) with purified Sabin type 1 poliovirus diluted 1:200 in PBS and blocked with either 1% gelatin or 5% FBS in PBS for 1 h at 37°C. Wells were incubated with a 1:10 dilution of hybridoma cell supernatants in PBS containing 1% bovine serum albumin for 2 h at 37°C. After washing, plates were incubated with a 1:2,000 dilution of an anti-mouse IgA (α-chain) antibody conjugated with alkaline phosphatase (Sigma) for 1 h at 37°C. Duplicate plates were examined for IgG and IgM MAbs, and secondary incubation was effected with a 1:2,000 dilution of anti-mouse IgG (H+L) antibody. Reaction was then developed with *p*-nitrophenol phosphate (Sigma 104) in 10 mM diethanolamine [pH 11]. After 1 h of incubation at 37°C, optical densities were measured at a wavelength of 405 nm, using a Bio-Rad ELISA reader.

Western blotting. Purified poliovirus preparations were subjected to SDS-PAGE under denaturing conditions, and viral protein bands were transferred to nitrocellulose paper by using a semidry apparatus (Bio-Rad) according to the manufacturer's instructions. After blocking with 5% skim milk in PBS for 1 h at 4°C, blots were incubated with a 1:100 dilution of ascites fluids in 1% bovine serum albumin in PBS for 3 h at 4°C. Secondary incubation was carried out with anti-mouse IgA (α-chain) or IgG (H+L) antibodies conjugated with alkaline phosphatase. After washing, bands were detected by incubation with bromochloroindolyl phosphate-nitroblue tetrazolium (BCIP-NBT) in 100 mM NaCl–5 mM $MgCl_2$–100 mM Tris (pH 9.5).

Microneutralization assay. Approximately 10^2 PFU of type 1 poliovirus was mixed either with a 1:10 dilution of hybridoma cell supernatants for screening of fusions or with serial dilutions of ascites fluids, and the mixtures were incubated for 60 min at 35°C prior to inoculation onto HEp-2 cell monolayers in 96-well plates. Plates were incubated at 35°C for 30 h, and cell monolayers were scored for cytopathic effect (CPE). Neutralizing titers were expressed as the inverse dilution of cell supernatants or ascites fluids resulting in the absence of CPE.

Production of poliovirus antigenic variants. Neutralization-resistant type 1 poliovirus variants were selected in HEp-2 cell monolayers by direct plaquing of 10^7 to 10^8 PFU of Sabin type 1 poliovirus previously neutralized with each IgA MAb (1:100 dilution of ascites fluid) and including the antibody (1:1,000) in the overlay medium during growth. Single well-developed viral plaques were picked from plates and subjected to at least two further cycles of plaque purification before cell culture expansion in antibody-free medium. Identification of neutralization-escape poliovirus mutants was eventually confirmed by neutralization tests. Virus mutants were designated with the name of the corresponding MAb used for selection preceded by "v."

Nucleotide sequencing. After phenol extraction, genomic RNAs from the neutralization-escape poliovirus variants were ethanol precipitated in the presence of 0.3 M sodium acetate at −70°C. The whole poliovirus capsid protein region was sequenced directly from mRNA by the Sanger method essentially as previously described (10), using a series of negative-strand oligonucleotide primers spanning the region at intervals of 200 to 250 nucleotides. To sequence regions with a high degree of secondary structure, additional plus-stranded primers were used to sequence cDNA synthesized by reverse transcription from viral mRNAs and PCR amplification. Specific DNA regions were sequenced with a DNA sequencing kit (Sequenase version 2.0; U.S. Biochemical Corp., Cleveland, Ohio). RNA from the Sabin type 1 parental poliovirus strain was included as a control.

Structural analysis of poliovirus antigenic sites. All structural analyses were performed by using the INSIGHT II molecular modeling package (MSI, San Diego, Calif.) and coordinates of the highly homologous type 1 poliovirus Mahoney strain crystal structure (16) as present in entry 2PLV from the PDB protein database (1). Icosahedrally related promoters were generated from the matrices present in the 2PLV database entry, using a modified version of the INSIGHT II space group table. Figure 4a was generated with the MOLSCRIPT software (22); for Fig. 4b, display options within the INSIGHT II package were applied.

RESULTS

Development of immunity to poliovirus in mice. The elicitation of neutralizing serum antibody to Sabin type 1 poliovirus was tested 10 days after the second and third administrations of virus via the oral route. No antibody response developed in mice receiving poliovirus alone, whereas increasing serum antibody titers (up to 1:1,412) were detected in mice given poliovirus in combination with CT (Table 1).

Hybridoma screening. A total number of 77 hybridoma cell culture supernatants from fusion of either spleen or Peyer's patches and mesenteric lymph nodes from one mouse were found to react with poliovirus in an ELISA specific for murine IgA antibody (Table 2). Of these, 59 supernatants also showed neutralizing activity in the initial screening. Seventeen more fusion supernatants showed ELISA positivity for both IgA and IgG, and 15 of them were also positive in neutralization assays. Thirteen of 74 neutralizing and 2 of 20 nonneutralizing IgA-positive cultures were randomly chosen for further study and cloned thrice, and MAbs were amplified in mouse ascites fluid. All MAbs were confirmed to be IgA by an ELISA using class- and subclass-specific antisera to mouse immunoglobulins. As-

TABLE 1. Neutralizing serum antibody titers to Sabin type 1 poliovirus in mice orally immunized in the presence or absence of CT

Mouse	Neutralizing titer[a]	
	2 doses	3 doses
Sabin 1 and CT		
1	128	1,412
2	89	256
3	178	1,412
Sabin 1		
1	<8	<8
2	<8	<8
3	<8	<8

[a] Inverse dilution of sera, resulting in the absence of CPE. Details of the assay are described in Materials and Methods.

cites fluids were also analyzed by SDS-PAGE in nonreducing conditions, and the gel was blotted onto nitrocellulose paper. In all cases, staining with an alkaline phosphatase-conjugated antiserum to mouse IgA revealed the presence of a major protein band migrating with an apparent molecular weight (MW) of 300,000 and less evident bands at MWs of 160,000 and 60,000 (not shown). That result indicated that most of antibody in our preparations was in the form of dimers. Two hybridoma cell clones producing nonneutralizing IgG MAbs were also included.

Determination of the specificity of MAbs for poliovirus proteins. To investigate the antigen specificity of the selected MAbs, ascites fluids were tested in a Western blot test against poliovirus proteins separated by electrophoresis. None of the neutralizing MAbs reacted with Sabin type 1 proteins in this assay. Conversely, a viral protein band with an approximate MW of 33,000 was stained by two nonneutralizing IgA (1H9 and 4B9E8) and two IgG MAbs (3H2 and 4B9H8) (Fig. 1).

Ascites fluids were also used in a modified immunoprecipitation test with radiolabeled viral proteins from Sabin type 1 poliovirus-infected cell lysates. Results of these assays are shown in Fig. 2 and 3. The two nonneutralizing IgA MAbs selectively precipitated VP1 from lysates in denaturing conditions (Fig. 2), whereas neutralizing MAbs did not immunoprecipitate any protein in this assay (not shown). All neutralizing MAbs simultaneously immunoprecipitated VP0, VP1, VP2, and VP3 poliovirus proteins when RIPA was performed in nondenaturing conditions (Fig. 3), as did the nonneutralizing IgA MAbs (not shown). Two nonneutralizing IgG MAbs immunoprecipitated VP1 or VP0, VP1, VP2, and VP3, respectively, in denaturing or nondenaturing conditions, in a conventional RIPA using plain protein G-Sepharose beads (Fig. 2).

Isolation of neutralization-escape poliovirus mutants. Neutralization-resistant virus variants were selected by isolation of plaques from poliovirus-infected HEp-2 cell monolayers overlaid with agarose containing each of eight different neutralizing IgA MAbs. These spontaneous antigenic variants were isolated with frequencies ranging from approximately 10^{-3} to 10^{-4}, as previously observed in similar studies (30, 34). All variants could be grown to titers similar to those of parental Sabin poliovirus following replication on cell cultures. After several passages in HEp-2 cells, all mutants maintained the ability to resist neutralization by the corresponding antibodies used for selection.

Neutralization analysis of neutralization-escape poliovirus mutants with IgA MAbs. Sabin type 1 poliovirus variants plaque isolated under the selective pressure of distinct IgA neutralizing MAbs were examined for neutralization by the whole panel of IgA MAbs (Table 3). Based on the results of these assays, the MAbs were divided into four groups, I through IV. Three variants (v1C1, v1D3, and v2F5) could be assigned to a same group of reactivity, all of them being resistant to any of the corresponding MAbs and to MAbs 3B3, 3C10, 4B9F8, 4D9, and 4D11. In addition to the antibodies used for their selection, variants v1F6 and v3G1 also resisted neutralization by MAb 4F4, whereas both poliovirus variants v4F11 and v4G8 were resistant to only MAbs 4F11 and 4G8. Mutant v4F4 proved resistant only to the corresponding antibody, indicating a one-way cross-reactivity between MAb 4F4 and MAbs 1F6 and 3G1. All IgA MAbs studied except 4F11 and 4G8 failed to neutralize the Mahoney strain of poliovirus. No MAb showed cross-neutralization with either wild or Sabin type 2 or 3 poliovirus (data not shown).

Sequence analysis of neutralization-escape mutants. For a more detailed characterization of the virus variants isolated, the nucleotide sequence of the genome portion coding for structural virus proteins (nucleotides 800 through 3500) was analyzed. The gene sequences corresponding to each mutant were compared with the sequence of the parental Sabin type 1 strain. Results of comparisons are shown in Table 4. In all cases, virus variants showed a single base mutation producing a substitution of the amino acid encoded. Virus variants selected by group I MAbs (v1C1, v1D3, and v2F5) had the same amino acid change at position 3059, corresponding to neutralization site N-AgIII. Mutants selected by group II MAbs (v1F6 and v3G1) showed substitution of residue 2164 (N-AgII). Poliovirus variant v4F4 (group III) presented a mutated amino acid in VP2, position 2138, which has not been previously reported. Group IV mutants v4F11 and v4G8 showed substitution of residues 1223 and 1221 (N-AgII), respectively. No antibody selecting for mutations in N-AgI or N-AgIV sites was found.

TABLE 2. Reactivity of hybridoma cell supernatants at the time of fusion screening with Sabin type 1 poliovirus by neutralization and IgA- and IgG-specific ELISA

MAb	No. of supernatants testing positive						Total
	IgA ELISA		IgG ELISA		IgA + IgG ELISA		
	Spleen	PP/MLN[a]	Spleen	PP/MLN	Spleen	PP/MLN	
Neutralizing	15	44	4	3	5[b]	10[b]	81
Nonneutralizing	7	11	2	3	0	2	25
Total	22	55	6	6	5[b]	12[b]	106

[a] PP/MLN, Peyer's patches plus mesenteric lymph nodes.
[b] Mixed clones at the time of fusion screening.

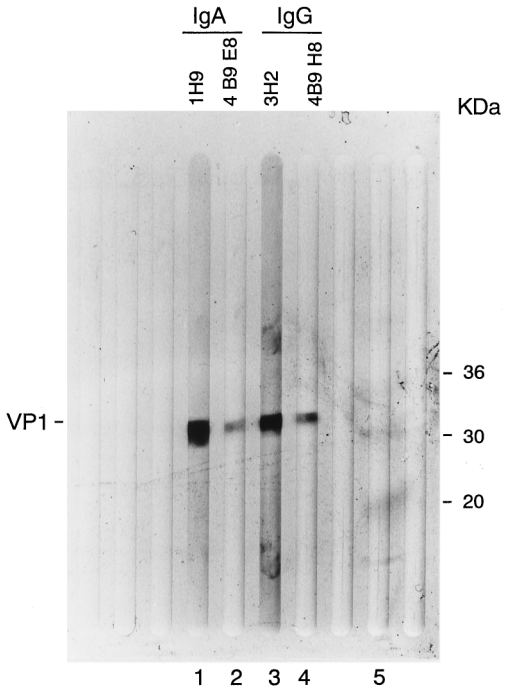

FIG. 1. Western blot staining of Sabin type 1 poliovirus proteins with nonneutralizing IgG and IgA MAbs. Viral proteins from purified Sabin type 1 poliovirus were separated by SDS-PAGE and transferred to nitrocellulose paper. Blots were sequentially incubated with ascites fluids containing IgA (lanes 1 and 2) or IgG (lanes 3 and 4) MAbs, biotin-conjugated anti-IgA or anti-IgG secondary antibodies, and alkaline phosphatase-streptavidin. Immunostained bands were detected with BCIP-NBT as described in detail in Materials and Methods. Lane 5, prestained molecular weight markers.

Predicted structural environment around Thr 2138. Although no direct structural information is available for the Sabin type 1 strain, the crystal structure of the highly homologous Mahoney strain (16) should reflect quite well the structural features of the Sabin strain. For VP2, the two strains differ at only two positions, 2165 (Asp in Sabin and Asn in Mahoney) and 2181 (Phe in Sabin and Leu in Mahoney). In the Mahoney VP2 structure, Thr 2138 is located within a loop region exposed on the viral capsid surface near a loop stabilized by Asp 2164 (Fig. 4), which is part of the neutralization site N-AgII (28). The absence of direct contacts between the side chain of Thr 2138 and the neighboring loop suggests that the substitution of Thr by Ala (as present in the Sabin v4F4 variant) should cause only minor perturbations, leaving both loops essentially unaltered. A rather different situation is instead observed at position 2164, with the aspartate side chain stabilizing the loop conformation through hydrogen bonds with main chain NH groups (Fig. 4). Although the loop would have some intrinsic stability resulting from the presence of two prolines at positions 2163 and 2170, nonconservative substitutions like Tyr and Gly (as present in Sabin escape variants v1F6 and v3G1, respectively) are likely to alter the conformation of the loop, possibly affecting also the segment around Thr 2138. If this is the case, it would explain why the group III MAb 4F4 was unable to neutralize the v1F6 and v3G1 variants whereas variant v4F4 could be recognized by group II MAbs 1F6 and 3G1.

DISCUSSION

For several viruses, resistance to infection seems to correlate with the presence of specific IgA antibody in mucosal secretion (3, 24, 27, 39). Renegar and Small (36) recently demonstrated that immunity to influenza virus could be abrogated by intranasal instillation of antibody to IgA but not anti-IgG or IgM, again consistent with IgA being the major mediator of viral neutralization. The biochemical characteristics of IgA antibodies make them particularly suitable for establishing a first barrier against several viruses at their site of entry into the host. Transport of secretory IgA into the gut lumen is mediated by pIgR, which is largely expressed on mucous membrane epithelial cells. The complex of pIgR and dimeric IgA is endocytosed and transported through vesicular compartments to the apical surface of cells, where proteolysis of pIgR results in release of dimeric IgA bound to the ectoplasmic domain of pIgR (also known as secretory component). It has been recently suggested

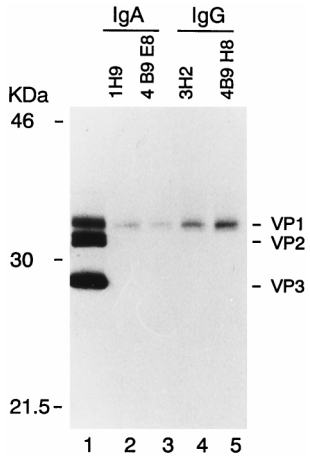

FIG. 2. SDS-PAGE analysis of [^{35}S]methionine-labeled proteins (6 h postinfection) immunoprecipitated by IgA and IgG MAbs from Sabin type 1 poliovirus-infected HEp-2 cell lysates. Immunoprecipitation was performed under denaturing conditions as described in detail in Materials and Methods. Lane 1, labeled capsid proteins from purified Sabin type 1 poliovirus; lanes 2 and 3, nonneutralizing poliovirus IgA MAbs; lanes 4 and 5, nonneutralizing poliovirus IgG MAbs.

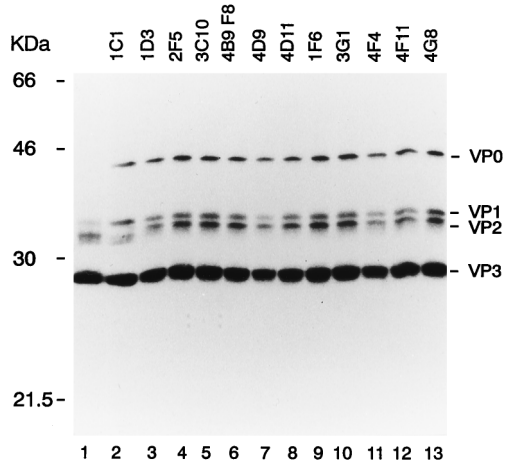

FIG. 3. SDS-PAGE analysis of [^{35}S]methionine-labeled proteins immunoprecipitated by neutralizing IgA MAbs (lanes 2 to 13) from Sabin type 1 poliovirus-infected HEp-2 cell lysates. Immunoprecipitation was performed under nondenaturing conditions as described in detail in Materials and Methods. Labeled capsid proteins from purified Sabin type 1 poliovirus are shown in lane 1.

TABLE 3. Comparison of neutralization titers of IgA MAbs against viral antigenic variants, parental Sabin type 1, and Mahoney type 1 poliovirus strains

MAb	Neutralization titer[a]									
	v1C1	v1D3	v2F5	v1F6	v3G1	v4F4	v4F11	v4G8	Sabin	Mahoney
Group I										
1C1	**<−8**	**<−8**	**<−8**	+1	0	0	0	0	0	**−4**
1D3	**<−8**	**<−8**	**<−8**	0	0	0	0	0	0	**−7**
2F5	**<−7**	**<−7**	**<−7**	0	+1	0	+1	0	0	**<−7**
3B3	**<−11**	**<−11**	**<−11**	−1	0	−1	0	−1	0	**−9**
3C10	**<−9**	**<−9**	**<−9**	−1	−1	−2	−1	−2	0	**−5**
4B9F8	**<−6**	**<−6**	**<−6**	−1	0	+1	+1	0	0	**<−6**
4D9	**<−8**	**<−8**	**<−8**	0	0	−1	0	−1	0	**<−8**
4D11	**<−9**	**<−9**	**<−9**	−1	−1	−1	−1	−1	0	**−7**
Group II										
1F6	−1	0	0	**−6**	**<−7**	0	0	0	0	**−6**
3G1	−1	0	0	**−7**	**<−8**	−1	+1	0	0	**−7**
Group III										
4F4	−2	−1	−1	**<−6**	**−5**	**<−6**	−1	−2	0	**<−6**
Group IV										
4F11	−1	−1	0	−1	0	+1	**<−7**	**<−7**	0	−3
4G8	0	0	0	0	0	0	**−5**	**<−9**	0	−1

[a] Determined for each of the indicated MAbs against the Sabin type 1 neutralization-escape variant and Mahoney type 1 strain and compared with the titers of the same MAb versus parental Sabin type 1. The values represent $\log_2$ titer versus variant − $\log_2$ titer versus Sabin type 1. Differences in titer between variant and parental Sabin type 1 of $>\log_2 3.2$ (10-fold) are in boldface.

that IgA antibodies may also interfere with viral replication by binding to newly synthesized viral proteins within infected cells, thus providing a novel mechanism for neutralization of viruses at the mucosal site (4, 19, 26).

An advantage of the live attenuated oral poliovirus vaccine developed by Sabin compared to killed vaccines is thought to be the elicitation of a strong secretory antibody response at the intestinal mucosa level where primary replication of the virus occurs before invasion and further multiplication in internal target tissues can take place (3, 41). Despite this, most studies aimed to characterize the poliovirus epitopes involved in neutralization have been conducted with IgG or IgM MAbs elicited in mice by immunization via parenteral routes. Although study of secretory antibodies to poliovirus has an undoubted significance, a major difficulty in stimulating a mucosal immune response in mice is the inability of poliovirus to replicate in normal mice. We recently developed an oral immunization strategy that made it possible to isolate a library of murine MAbs of IgA isotype to a rotavirus strain which does not replicate in the gut of BALB/c mice, using CT in combination with a high-titer virus preparation (13). Cholera toxin has been reported to strongly enhance mucosal immune responses to a great variety of soluble and particulate antigens, including viruses (6, 25). The same strategy has been successfully adopted in this study to generate a wide panel of neutralizing IgA MAbs to Sabin type 1 poliovirus.

The suitability of this immunization protocol is supported by our finding that in most mice, a high neutralizing titer to poliovirus was present in sera taken after a single boosting dose of antigen. Conversely, mice orally given poliovirus without CT did not show detectable titers of specific serum antibody after three antigen inoculations. In addition, the majority of cell supernatants from primary fusion cultures showed reactivity with poliovirus antigen in an IgA-specific ELISA, thus suggesting a specific targeting to immune cells involved in secretory antibody response.

As shown by Western blot analysis following electrophoretic separation in nonreducing conditions, all MAbs tested were mostly produced in the form of dimers and may thus be suitable for recognition by the pIgR, which mediates transport of secretory antibodies through mucosal epithelia.

Although the MAbs described in this report represent only a sample of the cultures obtained, it is of interest that 8 of 13 neutralizing antibodies which were randomly selected from the

TABLE 4. Nucleotide and amino acid substitutions in poliovirus type 1 Sabin neutralization-resistant variants

MAb group	Type 1 Sabin variant	Protein	Nucleotide change (position)	Amino acid change (position)	Neutralization site
I	v1C1	VP3	C→A (1941)	Ala→Glu (59)	N-AgIII
	v1D3	VP3	C→A (1941)	Ala→Glu (59)	N-AgIII
	v2F5	VP3	C→A (1941)	Ala→Glu (59)	N-AgIII
II	v1F6	VP2	G→T (1439)	Asp→Tyr (164)	N-AgII
	v3G1	VP2	A→G (1440)	Asp→Gly (164)	N-AgII
III	v4F4	VP2	A→G (1361)	Thr→Ala (138)	New
IV	v4F11	VP1	C→T (3147)	Ala→Val (223)	N-AgII
	v4G8	VP1	C→T (3141)	Ser→Leu (221)	N-AgII

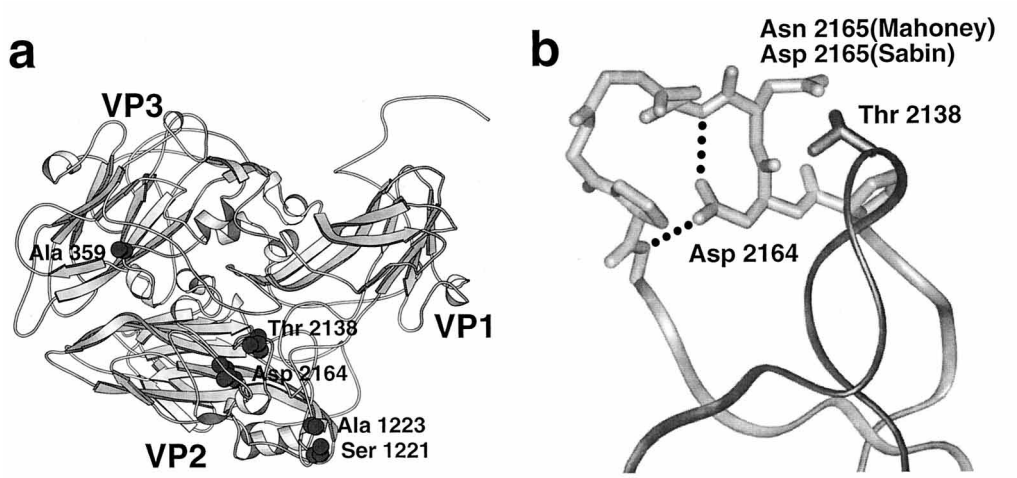

FIG. 4. (a) Schematic MOLSCRIPT drawing of the poliovirus type 1 (Mahoney strain) promoter crystal structure indicating the positions of amino acid substitutions of the escape mutants Ser 1221, Ala 1223, Thr 2138, Asp 2164, and Ala 3059. View is approximately along the fivefold axis. (b) Local structure of Mahoney VP2 around Thr 2138 (dark gray) and Asp 2164 situated in neighboring exposed loops. Although in close proximity, Thr 2138 is not in direct contact with the neighboring loop, and substitution into Ala as in the Sabin group III variant v4F4 is unlikely to affect the local structure of either loop. In contrast, substitution of Asp 2164 by either Tyr or Gly (Sabin group II variant v1F6 or v3G1, respectively) should disrupt the two hydrogen bonds (dotted lines) and impair the structural integrity of the loop, possibly inducing local structural changes affecting also the nearby loop around Thr 2138. This could explain why Sabin variants v1F6 (Asp 2164 to Tyr) and v3G1 (Asp 2164 to Gly) are not recognized by the group III MAb 4F4. The inability of MAbs 1F6 and 3G1 to neutralize the Mahoney strain could instead arise from the change in character of the exposed side chain at position 2165 from Asp (Sabin) to Asn (Mahoney).

whole library appear to be directed at the VP3 epitope at amino acid 59 (N-AgIII). This result indicates that this site of poliovirus type 1 is particularly efficient in eliciting an immune response at the intestinal mucosa level in mice.

Conversely, none of the neutralizing antibodies studied was directed at the N-AgI site of VP1, which is immunodominant for poliovirus types 2 and 3 (11, 30, 31). Our data seem to further confirm that this site (residues 1089 to 1100) in poliovirus type 1 remains inaccessible for recognition by the immune system cells also at the mucosal level, as was indicated earlier by difficulties in obtaining neutralizing MAbs against N-Ag I by parenteral immunization (2, 12, 18). This region is known to be cleaved by trypsin also in poliovirus type 1 (12), and its antigenic reactivity might be lost during passage of the virus through the intestine as has been shown in vitro for type 2 and 3 polioviruses (12, 37, 38). On the other hand, it is to be taken into account that although nontrypsinized poliovirus was used for the screening of fusion in this study, trypsin modification of the N-AgI site may have occurred during the immunization phase. Therefore, we cannot rule out the possibility that neutralizing antibodies recognizing the trypsin-cleaved form of the N-AgI site had indeed been generated but subsequently remained undetected.

A second group of four IgA MAbs (1F6, 3G1, 4F11, and 4G8) are directed at the N-AgII site and selected mutations at amino acid residues 2164 (both MAbs 1F6 and 3G1), 1223 (MAb 4F11), and 1221 (MAb 4G8). Interestingly, IgA MAbs 1F6 and 3G1 were not able to neutralize the Mahoney strain of poliovirus. This is in contrast with previous data related to IgG MAbs selecting for amino acid substitution in the same epitope, all of which were reported to efficiently neutralize wild-type Mahoney virus (34), and likely reflects peculiarities of the antibody binding sites. Comparison of the sequence of type 1 Sabin and Mahoney strains in this area shows for the Mahoney strain different amino acids at residues 2165 (Asp to Asn) and 2181 (Phe to Leu) (20, 32), the former being next to the residue involved in neutralization-escape Sabin variants derived from MAbs 1F6 and 3G1. The former amino acid difference present in the Mahoney strain may thus be critical for the proper recognition of the epitope by IgA MAbs 1F6 and 3G1.

MAb 4F4 selected for an amino acid change in VP2, residue 2138. This mutation has not been reported previously for IgG MAbs; however, a MAb has been reported by Wiegers and coworkers (44) to select a mutation in the nearby residue 2142. Since the same MAb alternatively selected for amino acid changes in the 2165–2168 region of poliovirus type 1, the authors concluded that residue 2142 is also involved in the structure of N-Ag II. Consistently, virus mutants selected by our group II MAbs 1F6 and 3G1 (amino acid 2164) were also resistant to MAb 4F4, thus suggesting this latter MAb also to be directed at N-AgII. However, variant 4F4 was normally neutralized by group II MAbs, suggesting a nonreciprocal effect caused by the 2138 and 2164 mutations. Although we cannot exclude that this observation reflects inhibition of an induced fit on recognition by the MAbs, a possible explanation could also be directly derived from the type 1 poliovirus crystal structure which is available for the Mahoney strain. In Mahoney VP2, Thr 2138 and Asp 2164 are located in two distinct loop regions facing each other on the surface of the molecule. Whereas substitution of the Thr 2138 side chain is unlikely to cause structural alterations in either loop, replacement of Asp 2164 should significantly perturb the local conformation, as previously suggested by Page and coworkers (34), possibly including also the nearby Thr 2138 epitope. Given the nearly identical amino acid sequence of the Mahoney and Sabin strains, it is plausible to assume that also in the latter, the epitope of the group II MAbs is preserved after substitution of the Thr 2138 by Ala. While the replacement of the exposed Asn 2165 (Mahoney) by Asp (Sabin) should have no significant effect on the conformation of the otherwise completely conserved loop, it seems the most likely cause for the inability of our group II MAbs to neutralize the Mahoney strain.

Although both group II (MAbs 1F6 and 3G1) and group IV (MAbs 4F11 and 4G8) MAbs appear to recognize the same N-AgII site, which includes portions of either VP1 and VP2

capsid proteins, neutralization-escape mutants from each group of MAbs were normally neutralized by the other group of antibodies. Similar findings have been reported previously (34), and the existence of the discontinuous epitope relies on the ability of some MAbs to select neutralization-escape variants which bear mutations in one or the other of the regions composing it.

It is noteworthy that only 2 of 13 antibodies characterized in this study are able to neutralize the heterologous wild-type Mahoney poliovirus strain. Although we have not tested the entire panel of MAbs derived in this study in a neutralization test against the wild-type strain, the significance of our results is supported by the fact that the neutralizing MAbs selected for characterization were chosen from the panel randomly. As a consequence, a relevant part of the immune response elicited with the Sabin virus in this study appears to be ineffective against the wild-type poliovirus strain. In this respect, our data confirm and extend previous findings that MAbs directed at the major neutralization determinant of poliovirus type 1, located around residue 3059, generated by using Sabin virus as the immunogen are strain specific and are inactive toward the wild-type Mahoney virus. This has been attributed to the presence of a Thr-to-Lys change at amino acid position 3060 in the Mahoney strain (34). However, the role of the amino acid change from Ala to Glu, which has been observed by Minor and colleagues (30) and in virus variants selected by our group I MAbs, is intriguing. Although representing a rather drastic substitution, it has been concluded that this change is per se antigenically silent for several neutralizing MAbs selecting for mutations in the neighboring residues 3058 and 3060 (34). These findings suggest the existence of subtle differences between our MAbs and the previously described MAbs directed at this region.

The unquestionable efficacy of vaccination with the oral poliovirus vaccine experienced in many years of use in the field suggests that proper stimulation of antibody against the major antigenic determinant of the virus is not an essential prerequisite for establishing protective immunity to the virus in humans. Also, we cannot exclude that human antibodies to this site may be more broadly cross-reactive than demonstrated by mouse MAbs.

Further studies should be aimed at verifying the ability of MAbs raised against the VP3 N-AgIII epitope of Sabin type 1 to recognize this epitope in the presently circulating field strains of wild-type poliovirus type 1.

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