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Poliovirus Neutralization Epitopes: Analysis and Localization with Neutralizing Monoclonal Antibodies

EMILIO A. EMINI,* BRADFORD A. JAMESON, ANITA J. LEWIS, GLENN R. LARSEN,† AND ECKARD WIMMER

Department of Microbiology, School of Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794

Received 5 April 1982/Accepted 18 May 1982

Two hybridomas (H3 and D3) secreting monoclonal neutralizing antibody to intact poliovirus type 1 (Mahoney strain) were established. Each antibody bound to a site qualitatively different from that to which the other antibody bound. The H3 site was located on intact virions and, to a lesser extent, on 80S naturally occurring empty capsids and 14S precursor subunits. The D3 site was found only on virions and empty capsids. Neither site was expressed on 80S heat-treated virions. The antibodies did not react with free denatured or undenatured viral structural proteins. Viral variants which were no longer capable of being neutralized by either one or the other antibody were obtained. Such variants arose during normal cell culture passage of wild-type virus and were present in the progeny viral population on the order of 10^{-4} variant per wild-type virus PFU. Toluene-2,4-diisocyanate, a heterobifunctional covalent cross-linking reagent, was used to irreversibly bind the F(ab) fragments of the two antibodies to their respective binding sites. In this way, VP1 was identified as the structural protein containing both sites.

Since the development more than 20 years ago of the inactivated (37) and attenuated (36) poliovirus vaccines, considerable effort has been expended in attempts to understand the antigenic structure of the virus. Those antigenic structures that are responsible for giving rise to a neutralizing antibody response in host animals are of particular interest, but, in spite of much effort, the location and structure of the poliovirus neutralizing epitopes remain unknown.

The poliovirus virion, a naked, icosahedral particle sedimenting at 155S, is composed of a single-stranded, plus-sense RNA genome and ca. 60 copies of each of four structural polypeptides (VP1, VP2, VP3, and VP4), as well as 1 to 2 copies of VP0, the uncleaved precursor of VP2 and VP4 (15, 35). Cross-linking and surface-labeling studies of intact virions (3, 25, 41) have established that VP1 is the predominantly exposed surface protein. VP2 and VP3 are also located externally, but to a significantly lesser extent than VP1, whereas VP4 appears to be completely internal.

Several morphogenetic precursors to the mature virion have been isolated from infected cells (34). These include 80S naturally occurring empty capsids and 14S precursor subunits (27, 31). The 80S empty capsids contain ca. 60 copies each of VP1, VP3, and the uncleaved VP0 (27). The 14S precursors are pentameric subunits containing five copies each of VP1, VP3, and VP0 (31).

The only poliovirus-specific structures capable of giving rise to a neutralizing antibody response when inoculated into host animals are the intact 155S virion and, to a much smaller extent, the 80S empty capsids (8). Neither the remaining morphogenetic precursors of the virion nor any of the four structural proteins isolated by treatment of the virion with sodium dodecyl sulfate (SDS) have been found to be capable of giving such a response (8, 29, 35). This is in contrast to the work done with two other members of the Picornaviridae family, foot-and-mouth disease virus (1, 20) and coxsackievirus B3 (2), in which certain isolated structural proteins (VP1 and VP2, respectively) were found to be sufficient for eliciting a neutralizing antibody response. It would seem, therefore, that the neutralizing epitope(s) in poliovirus is highly dependent upon the conformational stability rendered to it by the intact virion structure.

This paper reports studies which were aimed at identifying the location and morphogenesis of the poliovirus neutralizing epitope(s). For this purpose, two neutralizing anti-poliovirus monoclonal antibodies were generated and used as specific probes for the neutralizing site. We

† Present address: Genetics Institute, Boston, MA 02115.

found that poliovirions contain more than one neutralizing epitope, that these epitopes are found on viral morphogenetic precursor structures (though to a lesser extent than on intact virions), that the epitopes are subject to a high degree of variation, and that the two neutralizing epitopes uncovered with the monoclonal antibodies are located on the viral structural protein VP1.

MATERIALS AND METHODS

Generation of neutralizing anti-poliovirus monoclonal antibodies. Six 10-week-old male BALB/c mice were inoculated intraperitoneally at 1-week intervals for 4 weeks with 0.5 μg of poliovirus type 1 (Mahoney strain) per inoculum in the presence of complete (first inoculation) or incomplete (subsequent inoculations) Freund adjuvant. At 10 days after the last inoculation, each of the mice was boosted with 0.5 μg of virus inoculated without adjuvant directly into a tail vein. Three days later, the mice were sacrificed, the spleens were removed, and the spleen cells were fused (17, 18) with the nonsecretor mouse myeloma strain P3 × 63 Ag 8.653 (obtained from the Salk Institute, San Diego, Calif.) by using polyethylene glycol 1,000. The cells were distributed to 96-well plates, and successfully fused hybridomas were selected by growing the cells in RPMI (GIBCO Laboratories, Grand Island, N.Y.)–10% NCTC-109 (Microbiological Associates, Bethesda, Md.) with 20% fetal calf serum containing 0.1 mM hypoxanthine, 0.4 μM aminopterin, and 0.01 mM thymidine.

The culture wells were microscopically observed for the presence of hybridoma colonies at 7 to 14 days after the fusion. The cell culture fluid supernatants from those wells containing colonies were individually tested for the presence of neutralizing antibody. This was done by incubating 100 μl of the culture supernatant with 5.0 $\log_{10}$ PFU of virus for 1 h at room temperature and then infecting 2.5×10^5 HeLa monolayer cells. In the absence of neutralizing antibody, 100% cell death was noted after 24 h at 37°C. When neutralizing antibody was present, no cell death was observed at the end of the incubation.

Two stable hybridoma lines, H3 and D3, secreting anti-poliovirus type I neutralizing antibody were isolated in this fashion. The lines were further purified by terminal dilution. Both antibodies were identified as immunoglobulin G, based upon their ability to bind to *Staphylococcus aureus* protein A (12).

Titration of poliovirus in HeLa cells. The preparation of poliovirus to be titrated was diluted in serial 1:10 dilutions in minimal essential medium (GIBCO). A 0.2-ml portion of each dilution was used to infect, in duplicate, 5.0×10^6 HeLa cells in 950-mm^2 wells. After adsorption, the infected cells were overlaid with minimal essential medium–5.0% newborn calf serum–0.9% Noble agar. After 42 h of incubation at 37°C, the cells were stained with 0.01% neutral red, and the subsequently appearing viral plaques were quantitated.

Preparation of poliovirions and 80S naturally occurring empty capsids. A total of 10^8 HeLa S3 cells were infected with poliovirus type I (Mahoney) at a multiplicity of infection (MOI) of 50 PFU per cell in methionine-free medium and were incubated at 37°C. At 2.5 h postinfection, 500 μCi of [^{35}S]methionine was added to the infected culture. At 7.0 h postinfection, the infected cells were transferred to fresh medium containing 10 mM unlabeled methionine. Incubation was continued for 5 min. The cells were then harvested and washed twice with radioisotope-free medium at 4°C. The final cell pellet was suspended in cold reticulocyte standard buffer (RSB) plus Mg^{2+} (10 mM NaCl, 10 mM Tris [pH 7.4], 1.5 mM $MgCl_2$), and the cells were disrupted by Dounce homogenization. The cell nuclei were pelleted. The supernatant was clarified with 1.0% Brij 58 and 1.0% sodium deoxycholate, layered onto a 15 to 30% linear (wt/wt) sucrose-RSB plus Mg^{2+} gradient, and centrifuged in a Beckman SW27.1 rotor at 17,000 rpm and 4°C for 13 h. The gradient was fractionated and assayed for radioactivity. The 155S (virion) and 80S (empty capsid) peaks were separately pooled.

Preparation of 14S precursor subunits. HeLa S3 cell infection and [^{35}S]methionine labeling were carried out exactly as described above.

The final detergent-clarified cell cytoplasmic preparation was layered onto a 5 to 20% linear (wt/wt) sucrose-RSB plus Mg^{2+} gradient and centrifuged in a Beckman SW41 rotor at 24,500 rpm and 4°C for 15 h. The 14S peak was pooled.

Preparation of purified EMC virus. Encephalomyocarditis (EMC) virus was a kind gift of Mark Pallansch. It was labeled with [^{35}S]methionine and purified essentially as described for rhinovirus by Medappa et al. (28).

Immunoprecipitation of virus-specific structures and proteins with monoclonal antibodies. Several thousand counts per minute of the sample (preadsorbed with protein A) to be tested was diluted in TENN buffer (50 mM Tris [pH 7.4], 5.0 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40. Antibody (in excess of antigen) from hybridoma cell culture supernatant was added and allowed to react for 30 min. *S. aureus* protein A (Pansorbin; Calbiochem, La Jolla, Calif.) was then added in excess of antibody. The protein A complex was pelleted, washed three times with SNNTE buffer (5.0% sucrose, 1.0% Nonidet P-40, 500 mM NaCl, 50 mM Tris [pH 7.4], 5.0 mM EDTA), washed once with NTE buffer (50 mM NaCl, 10 mM Tris [pH 7.4], 1.0 mM EDTA), suspended in Laemmli sample buffer (62.5 mM Tris [pH 6.8], 10% glycerol, 2.0% SDS, 5.0% 2-mercaptoethanol, 0.005% bromphenol blue), and heated at 100°C for 3 min. The dissociated protein A was pelleted, and the supernatant was counted in a liquid scintillation counter with Aquasol-2 (New England Nuclear Corp., Boston, Mass.) as the scintillant.

Generation and purification of antibody F(ab) fragments. Before antibody purification, the antibody-containing cell culture supernatant was filtered at 5°C through Whatman no. 4 paper, a glass fiber-containing column, and a Sepharose 6B column (Pharmacia Fine Chemicals, Piscataway, N.J.). The purpose of this extensive filtration was to remove lipids which would otherwise interfere with the subsequent steps.

Antibody was purified from the clarified supernatant by binding to a protein A affinity column (Protein A-Sepharose CL-4B; Pharmacia) in 100 mM Tris (pH 8.0). The antibody was eluted in a single step with 100 mM glycine (pH 3.0). The preparation was then lyophilized, dissolved in 100 mM sodium phosphate (pH 7.2)–10 mM cysteine, and digested with papain (1:100

[wt/wt] enzyme/protein ratio) (Sigma Chemical Co., St. Louis, Mo.) at 37°C for 16 h (32). The F(ab) fragments were purified by Sephadex G-75 (Pharmacia) chromatography (the column buffer was 100 mM Tris [pH 8.0]–150 mM NaCl) followed by protein A affinity chromatography to remove undigested antibody and Fc fragments. The flow-through from the last step was dialyzed against water and lyophilized in 200-μg aliquots. One aliquot was analyzed by electrophoresis on a 10% SDS-polyacrylamide gel (19). A single band with an apparent molecular weight of ca. 40,000, as expected for F(ab) fragments (40), was found by staining.

Covalent cross-linking of antibody F(ab) fragments to virions. Toluene-2,4-diisocyanate (TDI), a heterobifunctional, noncleavable, cross-linking reagent, was obtained from ICN Pharmaceuticals. The procedure used for cross-linking was modified from those used by Boyd and Ottosen (5) and Chan and Boyd (7). (The reactions are outlined in Fig. 3.)

A 1.1-μl portion of fresh TDI was added to a 200-μg aliquot of monoclonal antibody-derived F(ab) fragments in 50 μl of 10 mM sodium phosphate (pH 7.2). The first cross-linking reaction was allowed to proceed at room temperature for 30 min with blending in a Vortex mixer at 3-min intervals. The reaction mixture was then placed at 0°C for 10 min to separate, by solidification, any unreacted TDI. The solid TDI was pelleted, and the supernatant was placed at 0°C for an additional 10 min and then pelleted again.

[^{35}S]methionine-labeled virus was added to the final supernatant [which contained an excess of TDI-linked F(ab) fragments]. The sample was placed at room temperature for 30 min with gentle mixing at 10-min intervals. The pH was then raised to 9.6 by the addition of saturated tribasic sodium phosphate to effect the second cross-linking reaction. After 10 min, the pH was lowered to 7.2 with saturated monobasic sodium phosphate.

The phosphate salt was removed by dialysis against water. The sample was then lyophilized, redissolved in Laemmli sample buffer (see above), heated at 100°C for 2 min, and analyzed on a 12.5% SDS-polyacrylamide gel (19). The radioactively labeled viral proteins were visualized by fluorography (4) with Kodak XAR-5 film.

RESULTS

Characterization of neutralizing monoclonal antibodies. Cell culture supernatant preparations of both the H3 and D3 hybridomas consistently contained antibody capable of neutralizing the infectivity of poliovirus type 1 strain Mahoney. Quantitation, by the plaque-reduction neutralization test, of the neutralizing activities in both supernatants is shown in Table 1.

Identical results (data not shown) were obtained when the poliovirus type 1 Sabin strain, LSc2ab, was substituted for the Mahoney strain. It appears that the binding sites of the two antibodies are equally expressed on the virions of the two viral strains.

Binding of monoclonal antibodies H3 and D3 to viral morphogenetic structures. The only virus-specific structures believed to express neutralizing epitopes are intact virions and, possibly to a small extent, 80S naturally occurring empty capsids. This is based on experiments which show that only virions and (slightly) empty capsids are capable of giving rise to a neutralizing antibody response in host animals (8, 35). We tested the ability of neutralizing monoclonal antibodies to bind specifically to the neutralizing epitope(s) of intact virions, and, if possible, to the epitope(s) of intermediates in the morphogenetic pathway of the virus. Radiolabeled viral structures were separated by velocity gradient sedimentation (Fig. 1) from a 7.0-h, high-multiplicity, infected-cell lysate. Samples from each gradient fraction were tested by immunoprecipitation for binding to either the H3 or the D3 antibodies. Antibody-antigen reactions were seen only with fractions from the virion, 80S empty capsid, and 14S subunit peaks (Fig. 1). The immunoprecipitated antigens were conclusively identified by polyacrylamide gel electrophoresis (data not shown) as either virions containing all four structural proteins or morphogenetic precursors containing predominantly VP0, VP1, and VP3.

TABLE 1. Neutralizing antibody activity in H3 and D3 hybridoma supernatants

Test no.[a]	Virus titer (log_{10} PFU/ml) with:[b]		
	No Ab	H3	D3
1	9.0	4.8	5.3
2	9.1	4.1	5.2
3	9.1	4.8	5.2

[a] Three plaque reduction neutralization tests were conducted at 2-week intervals to check the stability of antibody production by each of the two hybridomas.

[b] Viral titers were determined by titrating a standard virus stock either in the absence of antibody (Ab) or in the presence of an equal volume of cell culture supernatant from the H3 or D3 hybridomas.

The three reactive virus-specific structures were prepared in highly purified forms, and their respective antibody-antigen reactions with H3 or D3 were separately quantitated. The results (Table 2) suggest that each antibody was binding to a site which is qualitatively different from that recognized by the other antibody. H3 bound to a site which was strongly expressed on whole virions, less well expressed on empty capsids, and only slightly expressed on 14S subunits. On the other hand, D3 recognized its binding site strongly on whole virions, to a limited extent on empty capsids (about 50% as well as did H3 with its site), and not at all on 14S subunits. Hence, it appears that poliovirions express at least two neutralizing epitopes. These two epitopes make their appearance at different times and to different extents during the morphogenesis of the virions.

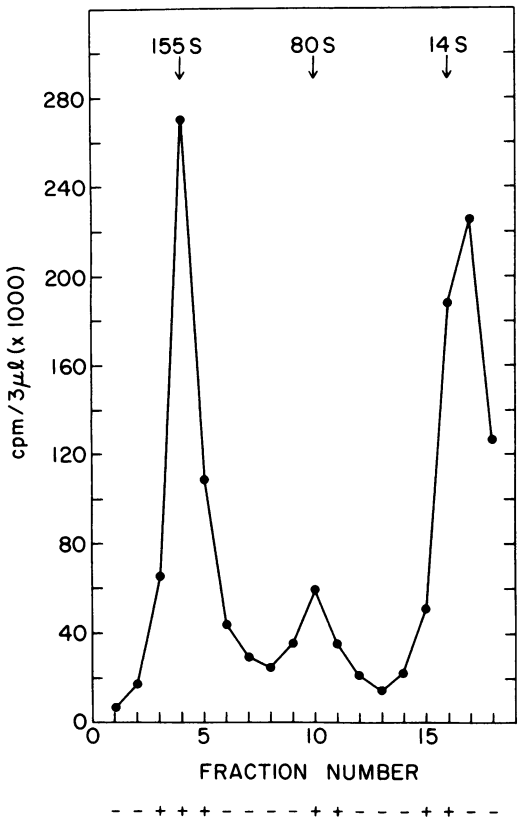

FIG. 1. Immunoprecipitation of virus-specific morphogenetic structures with neutralizing monoclonal antibodies H3 and D3. HeLa S3 cells were infected, virus-specific proteins were labeled with [^{35}S]methionine, and the infected cell cytoplasm was harvested and spun through a velocity sedimentation sucrose gradient as described in the text. Sedimentation was from right to left. The gradient was fractionated into ca.-1.0-ml fractions, of which 50 μl of each was assayed by immunoprecipitation for viral-specific structures capable of binding to either the H3 or the D3 monoclonal antibodies. A mixture of the two antibodies was used. Symbols: +, immunoprecipitation (measured as counts per minute) at least 20-fold greater than that of a control without antibody; −, immunoprecipitation approximately equal to that of a control without antibody.

When the samples of [^{35}S]methionine-labeled virion, 80S empty capsid, and 14S subunit used for these experiments were analyzed by polyacrylamide gel electrophoresis (Fig. 2), all radioactivity was found to be associated with virus-specific proteins before and after immunoprecipitation. A nonimmunoprecipitable minor contaminant (arrow in Fig. 2B) represented only 5.0% of the total label and, therefore, did not significantly interfere with the quantitation of the antibody-antigen reactions. The values of 70 to 73% obtained by immunoprecipitation of whole virus with H3 or D3 are close to the maximum expected with the immunoprecipitation procedures used in these studies. These maximum values were determined by using different antibody dilutions of known antigen-antibody pairs in an identical immunoprecipitation test (data not shown).

The monoclonal antibodies were also tested for their ability to bind to the isolated, purified structural proteins of poliovirus. The virus was radioactively labeled and disrupted by boiling in the presence of SDS, and the structural proteins were purified by preparative polyacrylamide gel electrophoresis as previously described (11, 39). In addition, undenatured VP0, VP1, VP3, and the structural protein precursor P1-1a (21) were obtained from a cell-free RNA translation system (10). None of these viral antigens was found to be capable of being recognized by either antibody in immunoprecipitation tests.

Finally, the reactivity of H3 and D3 with antigenic sites on heat-treated virions was investigated. LeBouvier (23, 24) first noted that poliovirions undergo antigenic changes when subjected to mild heating. These changes are accompanied by the loss of VP4 (6). [^{35}S]methionine-labeled, purified virions in RSB plus Mg^{2+} were exposed to 55°C for 10 min, quickly cooled to 4°C, and analyzed by velocity gradient sedimentation. The heat-treated sample sedimented at 80S, in contrast to the 155S untreated virion control (data not shown). A polyacrylamide gel analysis of the 80S heat-treated virion showed that complete loss of VP4 had occurred, whereas the other structural proteins were present (data not shown). In immunoprecipitation tests the heat-treated virions were completely unreactive with either monoclonal antibody.

Derivation of non-neutralizable viral variants. Three HeLa cell cultures were infected with poliovirus type 1 at an MOI of 10^{-2} PFU per cell. The MOI was low because the stock virus

TABLE 2. Immunoprecipitation of virions, 80S empty capsids, and 14S subunits with monoclonal antibodies H3 or D3[a]

Structure	% Immunoprecipitated (avg ± SD) with:	
	H3	D3
Virions	70.8 ± 5.4	72.8 ± 7.9
80S empty capsids	24.6 ± 3.3	13.4 ± 1.8
14S subunits	13.6 ± 3.0	0.3 ± 0.2

[a] Purified virions, empty capsids, and 14S subunits were immunoprecipitated with either H3 or D3 as described in the text. The percent immunoprecipitated was determined by comparing the precipitated counts per minute with the counts per minute present immediately before the addition of antibody. The values given are averages of four experiments.

used for the inoculation had been diluted to remove existing non-H3- and non-D3-neutralizable variants. The actual inocula were tested for the presence of these variants, and none were found.

The progeny virion populations from these infections were then each passaged once more in the presence of either H3 or D3 antibodies (1.0 μg of antibody per ml of cell culture fluid). The MOIs (1.0 PFU per cell) were chosen so as to insure complete neutralization of the inoculum virus. All of the infected cultures eventually developed 100% cytopathology, indicating the presence of non-H3- and non-D3-neutralizable variants in the inocula. The variant virus-containing culture supernatants were harvested and titrated in the presence and absence of the appropriate antibodies. Each of the six prepara-

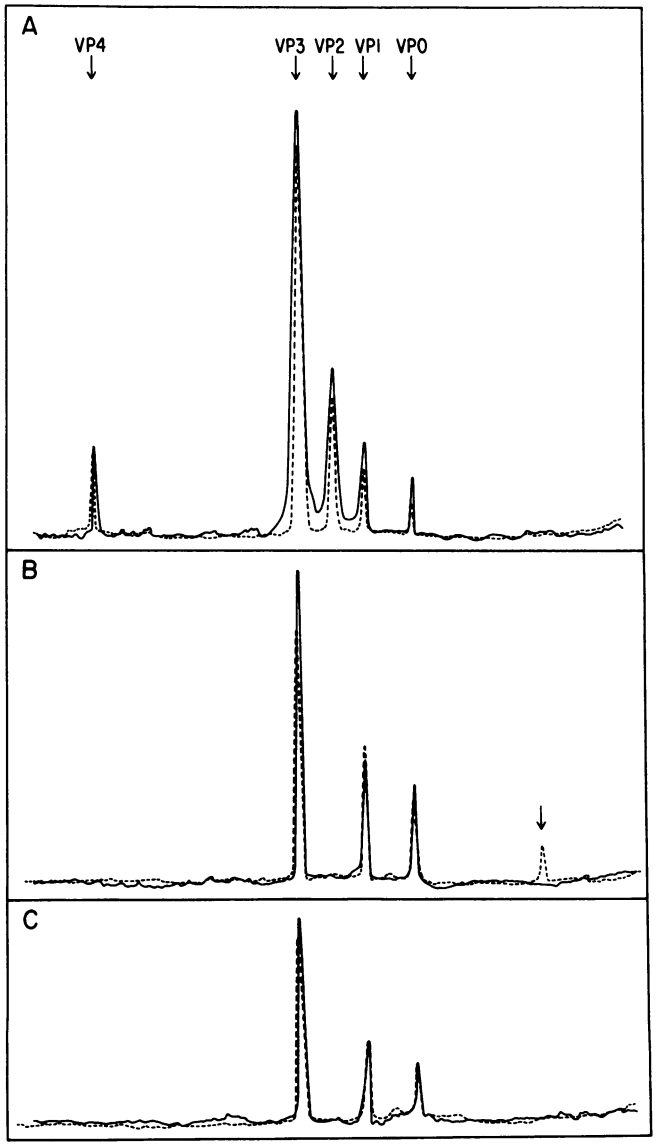

FIG. 2. Densitometer tracings of polyacrylamide gel electrophoresis autoradiographs of samples used in immunoprecipitation studies. (A) Virions; (B) 80S empty capsids; (C) 14S subunits. Symbols: - - -, Sample before immunoprecipitation; —, sample after immunoprecipitation with H3. Direction of migration is from right to left. The arrow in (B) denotes a radioactively labeled contaminant which does not immunoprecipitate with the virus-specific 80S proteins (see text). Identical profiles were obtained with D3 except that the 14S subunits reacted too weakly to be analyzed. Note that peak fractions do not correspond to polypeptide concentrations but to the amount of [^{35}S]methionine incorporated into the protein (e.g., VP3, 12 methionine; VP1, 5 methionine) (16).

tions contained approximately 10.0 $\log_{10}$ PFU/ml of variant virus only.

Finally, each of the three independently generated variant populations that was not neutralized by D3 was tested for its ability to be neutralized by H3 and was found to be as susceptible to H3 neutralization as is the original wild-type population. Similarly, non-H3-neutralizable variants were efficiently neutralized by D3. These data provide strong evidence that H3 and D3 recognize two qualitatively distinct neutralizing epitopes.

Degrees of variation at the neutralization epitopes. The ease with which non-neutralizable variants were obtained is indicative of a fairly high degree of variation at these antigenic sites. The variation was quantitated by infecting cells at a low MOI with wild-type virus. All of the inocula were tested and found to be free of variants. The progeny from these infections were titrated in the presence (under conditions of antibody excess) and absence of the appropriate antibody. The results (Table 3) confirmed a rapid appearance of variants, on the order of 1 variant PFU per 10^4 wild-type PFU, for both antigenic sites.

Localization of the neutralizing epitopes. The easy loss, upon viral disruption, of the epitopes recognized by the H3 and D3 antibodies did not allow for a conventional determination of which virion protein(s) contains these sites (1, 2, 20). Our strategy to solve this problem was to covalently cross-link the respective antibodies to their virion binding sites. The protein bearing the site was then identified by its inability to migrate into a 12.5% SDS-polyacrylamide gel.

TDI was chosen as the cross-linking reagent because of its heterobifunctional nature, rapid reaction kinetics (38), ability to span a small molecular distance (5.0 Å) (30), and successful use in previous studies (5, 7). The cross-linking reactions between antibody F(ab) fragments and intact virions are illustrated in Fig. 3. F(ab) fragments were used instead of intact antibody molecules to avoid any nonspecific cross-linking which might be caused by the Fc "tail" of the intact antibody. Both the H3 and D3 F(ab) fragments were found to be capable of binding to virions (by a modified immunoprecipitation procedure with goat anti-mouse antibodies as the probe) and of neutralizing viral infectivity.

TABLE 3. Variation at the neutralization epitopes[a]

Infection no.	Virus titer ($\log_{10}$ PFU/ml) with:		
	No Ab	H3	D3
1	6.9	3.3	3.2
2	6.9	3.4	3.1
3	6.8	3.4	3.3

[a] Three HeLa cell cultures (5.0×10^6 cells each) were infected with wild-type virus at an MOI of 10^{-2} PFU per cell. The inocula were free of any detectable variants. The progeny virus-containing harvests were titrated in the presence (under conditions of antibody excess) or absence of the monoclonal neutralizing antibodies (Ab).

In aqueous solution, the reactive groups of TDI are converted to carbamic acids (38) and hence lose their reactivity. This property makes it necessary to minimize the length of exposure of TDI to water. This thwarted our attempts to remove excess TDI-linked F(ab) fragments from the reaction mixture before the second cross-linking step. We found that during this purification procedure, the second TDI binding group was rendered inactive.

Figure 4 shows densitometer tracings of 12.5% Laemmli SDS-polyacrylamide gel analyses before and after the cross-linking reactions. After reaction with either the D3 (Fig. 4B) or the H3 (Fig. 4C) F(ab) fragments, a sharp reduction of the VP1 band was observed. This suggests that VP1 had been specifically cross-linked to the F(ab) fragment, a reaction which prevents it from migrating into the gel. The reduction of VP1 was especially noted in the case of D3 (Fig. 4B), in which the gel was exposed to the maximum limit of the film sensitivity, resulting in nearly equal blackening of the bands corresponding to VP2, VP3, and VP4. The lack of complete removal of VP1 may not be surprising, since complete binding of the TDI-linked F(ab) fragments to all 60 VP1 molecules within the virion cannot be expected.

We considered it possible that the removal of VP1 from the electrophoresis profiles was due to a nonspecific cross-linking of excess chemoactivated F(ab) fragments, since VP1 is the predominantly modified virion polypeptide in chemical labeling experiments (3, 25, 41). To test this, we chemoactivated chicken ovalbumin, a protein with a molecular weight (43,000) similar to that of F(ab) fragments, with TDI and reacted it with virions. The ratio of the proteins in this sample (Fig. 4D) was similar to that in the control (Fig. 4A). Some nonspecific loss of VP1 may have occurred. However, the result demonstrate that excessive removal of VP1 through chemoactivated F(ab) fragments by mere chemical tagging does not occur.

A further control was performed by using polio-specific TDI-linked D3 F(ab) fragments and EMC virus. EMC virus, another member of Picornaviridae, contains four structural proteins, of which VP1 is the predominantly exposed surface protein (26). Although some nonspecific cross-linking to the EMC virus VP1 again occurred (Fig. 4E and F), it was by no

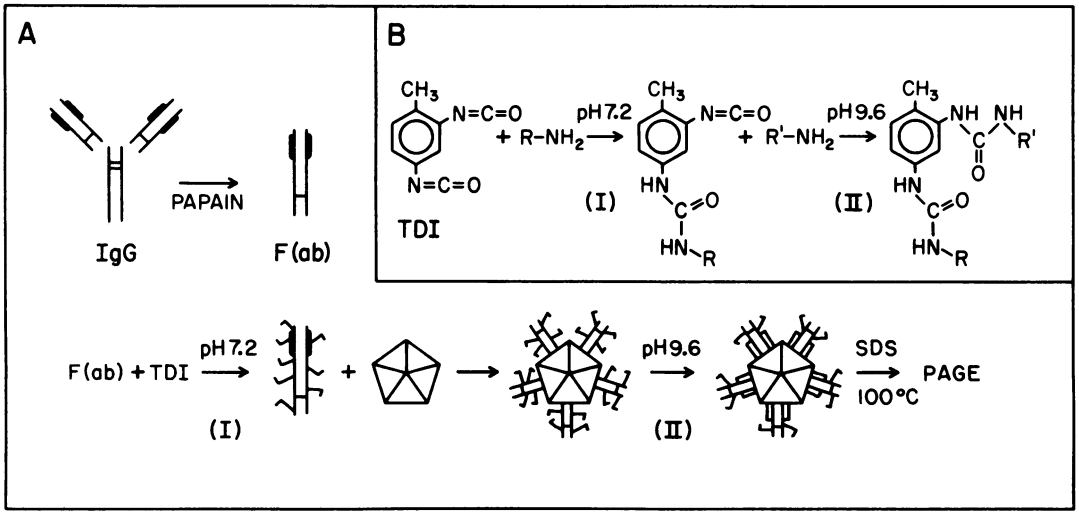

FIG. 3. (A) Schematic representation of the cross-linking reaction. See text for details. The heavy lines on the antibody molecule and F(ab) fragment represent the binding (hypervariable) sites. TDI linked to protein is represented by small bent lines. I and II are the first and second cross-linking reaction steps, respectively. PAGE, Polyacrylamide gel electrophoresis on a 12.5% Laemmli gel. (B) Actual molecular mechanism of the cross-linking reaction. R and R' are lysine residues on F(ab) fragments and virion proteins, respectively.

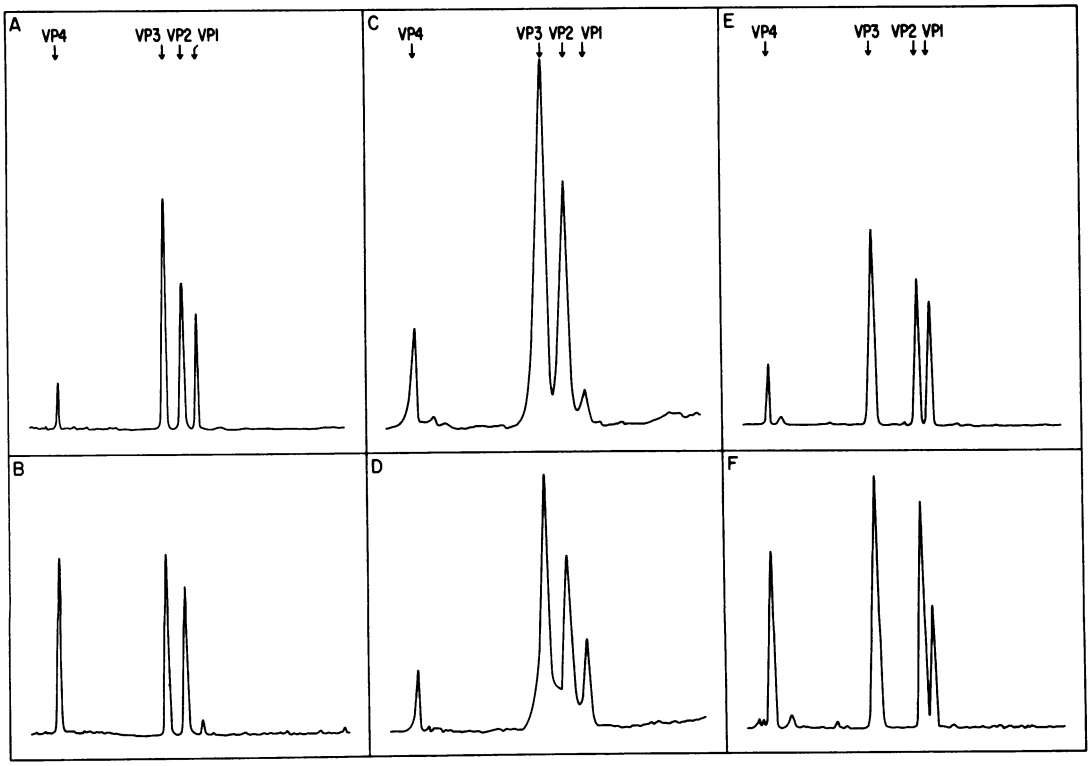

FIG. 4. Densitometer tracings of autoradiograms of 12.5% Laemmli SDS-polyacrylamide gel analyses of the cross-linking reactions. Viral proteins were labeled with [^{35}S]methionine. (A) Unreacted poliovirus control; (B) poliovirus-D3 F(ab) cross-linking; (C) poliovirus-H3 F(ab) cross-linking; (D) poliovirus-chicken ovalbumin cross-linking; (E) unreacted EMC control; (F) EMC-D3 F(ab) cross-linking. See text for details and discussion. Migration of proteins is from right to left. A, B, E, and F represent lanes from one gel; C and D were traced from a second gel.

means to the degree seen when the D3 F(ab) fragment bound specifically to its site (Fig. 4B).

DISCUSSION

Since the introduction of killed and attenuated poliovirus vaccines, investigators have unsuccessfully attempted to identify the viral neutralization epitope(s). The characterization of this site(s) would greatly facilitate the study, on a molecular level, of antibody-dependent virus neutralization as well as be useful in the possible development of new vaccines. Unfortunately, efforts to this end have been thwarted by the instability of the epitope(s), which is generally expressed only on intact virions. We employed a novel strategy that involved developing and using two anti-poliovirus type 1 neutralizing monoclonal antibodies as specific probes for the poliovirus neutralization epitope(s).

The antibodies were first used to study the morphogenesis of their respective binding sites. It became apparent during these studies that the two antibodies are directed against two different neutralizing epitopes, an observation not previously reported for picornaviruses. Moreover, these epitopes appeared at different stages of viral morphogenesis. The H3 site was first evident, to a small extent, on 14S precursor subunits, became more strongly expressed on 80S naturally occurring empty capsids, and was fully expressed on intact virions. The D3 site, on the other hand, was first noted on the 80S empty capsids. Neither site was expressed on any of the denatured, purified viral structural proteins or on undenatured P1-1a, VP0, VP1, or VP3 from a cell-free translation system. These results confirm and extend those recently reported by Icenogle et al. (13), who used a single monoclonal neutralizing antibody raised against the type 1 Sabin strain and found a neutralization epitope which is expressed on the 14S subunits as well as on 80S empty capsids and whole virions.

We are currently developing an entire spectrum of monoclonal neutralizing antibodies to poliovirus. Apart from the possibility of detecting more neutralizing epitopes, we hope to arrive at a more complete understanding of the neutralization phenomenon.

Of particular interest is the observation that variants not neutralizable by either one or the other antibody were obtained very rapidly, even when grown under standard conditions in a host cell for which the virus had been adapted for numerous generations. This reflects a significant degree of variation for poliovirus. Genetic variation of poliovirus upon passage in humans has been reported previously (14). The rate at which variants resistant to neutralization with monoclonal antibodies arise upon passage in HeLa cells, however, was a surprise to us, although similar extents of variation at monoclonal antibody-binding sites have been noted with Sendai, vesicular stomatitis, and influenza A viruses (22, 33, 42). The facility with which variants arise may pose a problem in the future development of synthetic vaccines composed only of a single neutralizing antibody-inducing antigenic site (for example, a single synthetic peptide). These vaccines may have to contain several appropriate, qualitatively different antigenic structures.

Finally, the use of a covalent linkage between a monoclonal antibody probe and its antigenic binding site has proven to be a novel and useful way of identifying the location of unstable antigenic sites. Ongoing experiments are aimed at discerning the exact structures of the two antibody-linked sites on VP1 by an analysis of the primary structure of the viral polypeptide. Complete knowledge of the amino acid sequence of all the capsid polypeptides of poliovirus (9, 16, 21) will greatly facilitate these studies.

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LITERATURE CITED

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