JOURNAL OF VIROLOGY, May 1983, p. 466–474
0022-538X/83/050466-09$02.00/0

Vol. 46, No. 2

Functional Basis of Poliovirus Neutralization Determined with Monospecific Neutralizing Antibodies

EMILIO A. EMINI,* SHAW-YI KAO,[1] ANITA J. LEWIS,[1] RADU CRAINIC,[2] AND ECKARD WIMMER[1]

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

Received 22 November 1982/Accepted 20 January 1983

Antibody-mediated poliovirus neutralization was studied by using a series of 13 monospecific neutralizing antibodies. These antibodies were found to recognize seven individual viral epitopes, several of which functionally overlap one another. Each epitope was capable of undergoing variation so that the variant virus was no longer capable of being neutralized by antibody directed against that epitope. The measured degree of variation for each site varied from -3.1 to -4.2 $\log_{10}$ variant PFU per wild-type PFU. Under nonsaturating but neutralizing conditions, the antibodies, with the exception of those directed to one specific epitope, failed to completely inhibit the virion's binding to the cell. Similarly, none of the neutralizing antibodies completely inhibited viral penetration, but all prevented virus-specific transcription. A strong correlation was established between the binding of each of the neutralizing antibodies, with one exception, to the virion and a significant shift in the virion's pI from 7.0 to ca. 4.0.

The structural and functional bases of poliovirus neutralization by antibody has attracted interest since the development of the poliovirus vaccines some 30 years ago (26, 27). In spite of considerable study and significant progress (21), the specific mechanisms involved in neutralization have not been elucidated. A major difficulty inherent in these studies has been the use of hyperimmune antiserum that contains a mixture of antibodies, not all of which neutralize and which are directed against different epitopes of intact virions. Hence, it is difficult to establish concrete associations between the effects on the virion of the varied antibody populations and the neutralization event.

The recent development of hybridoma technology has allowed for the establishment of cell lines secreting monospecific antibody (13, 14). Application of this technology to the poliovirus sytem has led to the production of a number of anti-poliovirus neutralizing monoclonal antibodies (2, 5–7, 9, 10, 24). Thus far, some of these antibodies have been used to show that at least several poliovirus neutralization epitopes are located in VP1, one of the virion's four structural proteins (5, 24). This result was supported by serological studies with polyclonal antibody raised against isolated VP1 (1). In addition, a monospecific neutralizing activity was recently detected in rabbit antiserum prepared against purified VP4 of poliovirus type 1 (4a). This activity appears to be directed to a single site on VP3.

The experiments described in this report made use of 12 anti-poliovirus type 1 neutralizing monoclonal antibodies along with the neutralizing anti-VP4 serum to study the effects of such monospecific antibodies on the early events of poliovirus replication. A genetic study identified seven individual epitopes that are recognized by different neutralizing monospecific antibodies. Several of these epitopes, which we call neutralization epitopes, functionally overlap with one another to different degrees. Each neutralization epitope was independently capable of undergoing variation so that the resulting variant virus was no longer neutralized by the respective monospecific antibody. We have also studied the effect of the binding of monospecific neutralizing antibodies to the different epitopes with respect to the virion's interaction with the cell surface, viral penetration, and viral macromolecular synthesis. In addition, we report antibody-induced changes in the pI of the virions.

MATERIALS AND METHODS

Establishment of hybridoma cell lines secreting neutralizing monoclonal antibody. Hybridomas secreting A13, A145, A229, and A611 were prepared from mice immunized with poliovirus type 1 (Mahoney strain) exactly as described by Emini et al. (5). Hybridomas secreting I CJ 27, I H 8-4, I H 8-25, I CJ 12-9, and I CJ 31-10 were prepared from mice immunized with the

Mahoney strain as described by Crainic et al. (2) and Couillin et al. (1a). Antibody 1 BM 55-6 was prepared by the latter methodology from a mouse immunized with the Sabin strain of poliovirus type 1.

Viral plaque titration. Virus was biologically quantitated by plaque titration in HeLa cells as described by Emini et al. (5).

Labeling and purification of poliovirions. Virions were labeled with [^{35}S]methionine or [^{3}H]uridine and purified as previously described (5).

Preparation of rabbit antisera. Rabbit antisera directed against the purified structural protein VP1 or VP3 were prepared essentially as described in Emini et al. (4a) for anti-VP4. Rabbit anti-poliovirus type 1 hyperimmune serum was obtained from M. A. Bioproducts (Walkersville, Md.).

Column isoelectric focusing. Isoelectric focusing was carried out in a 110-ml LKB focusing column. The column was composed of a 102.4-ml 0 to 50% (wt/vol) stepwise sucrose gradient containing 0.1% Triton N-101 and 1.0% ampholytes (pH 3 to 10; Pharmalytes, Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). The cathode (top electrode) buffer was 1.6% ethylenediamine. The anode (bottom electrode) buffer was 60% (wt/vol) sucrose–1.5% H_2SO_4. The column was run overnight at a maximum voltage of 1,800 V, a maximum power of 15 W, and a maximum current of 15 mA. At the completion of focusing the gradient was fractionated into 1.5-ml fractions. The pH of each fraction was determined and the fractions were assayed for the focused sample.

RESULTS

We have studied 12 monoclonal antibodies (2, 5) (Table 1) that effectively neutralize poliovirus type 1 (Mahoney strain). One additional neutralizing activity was rabbit antiserum prepared against the purified structural protein VP4 of type 1 (Mahoney) and which was found to contain neutralizing antibodies directed against a single site on the VP3 structural protein (4a).

Identification of distinct functional neutralization epitopes. The number and relationships of the neutralization epitopes on the poliovirion have never been determined. Previous studies have shown that monoclonal antibodies D3 and H3 are directed toward different sites on the virion's VP1 protein (5) and that the neutralizing activity of the anti-VP4 serum is directed toward a single site on VP3 (4a). It was of interest to determine whether some different antigenic sites could be recognized with the other 10 monoclonal antibodies.

We prepared viral variants that could no longer be neutralized by their respective antibody for all of the neutralizing activities. The procedure used was the same as that reported by Emini et al. (5). These variants were then used in cross-neutralization tests against each of the neutralizing activities. The results are presented in Table 2. For analyzing the data, we arbitrarily assumed that two neutralizing antibodies are directed against the same epitope if one antibody reduced the plaque titer of the other antibody's non-neutralizable variant by 1.0 $\log_{10}$ PFU/ml or less. On this basis we identified seven functionally distinct neutralization epitopes. The neutralizing activities directed against each epitope are listed in Table 3.

TABLE 1. Neutralizing monospecific activities directed against poliovirus type 1 (Mahoney)

Stony Brook[a]	Pasteur Institute[b]
D3	I CJ 27
H3	I H 8-4
A13	I H 8-25
A145	I CJ 12-9
A229	I CJ 31-10
A611	1 BM 55-6
Anti-VP4	

[a] These activities were prepared and isolated at the State University of New York at Stony Brook. The first six are monoclonal antibodies that were prepared as described for D3 and H3 by Emini et al. (5). The last is serum prepared against purified structural protein (4a).

[b] These activities were prepared and isolated at the Pasteur Institute, Medical Virology Department, Paris, France. All are monoclonal antibodies.

Analysis of the cross-neutralization data also showed that a certain degree of overlap exists among several of the epitopes. Epitopes A and B (Table 3) are somewhat related as judged by the inability of antibodies D3, A145, A229, and A611 to neutralize the H3 variant as well as each neutralized the wild-type virus. Similarly, antibody H3 failed to neutralize the D3 variant as well as it neutralized the wild-type virus. Epitope E is closely related to epitope B since the antibodies directed against these two sites were inefficient neutralizers of each other's variants, yet efficient enough to be considered two different functional sites.

Degree of variation at the neutralization epitopes. It was previously determined that the epitopes which bind the D3 and H3 antibodies vary on the order of ca. -4.0 $\log_{10}$ variant PFU per wild-type PFU (5). The determination of this degree of variation was based on the number of non-neutralizable variants which appeared after a single passage in cells that had been infected with a variant-free inoculum. In a similar fashion, the degree of variation for the anti-VP4 activity was estimated to be -4.5 to -4.8 $\log_{10}$ variant PFU per wild-type PFU (4a). It was of interest to determine the degree of variation exhibited by the remaining neutralization epitopes. The variation was measured, as it had been for the D3 and H3 epitopes, exactly as

TABLE 2. Neutralization of non-neutralizable variants with monospecific neutralizing activities[a]

Monospecific antibody	Neutralization of Non-neutralizable variants (log_{10} PFU/ml reduction of plaque titer)[b]										
	wt	D3	H3	A13	Anti-VP4	I CJ 27	I H 8-4	I H 8-25	I CJ 12-9	I CJ 31-10	1 BM 55-6
D3	4.6	—[c]	3.7	4.4	5.0	4.5	4.4	4.6	4.7	4.1	4.5
H3	5.1	3.1	—	4.9	5.1	4.9	5.2	4.9	5.0	1.1	4.9
A13	3.6	3.5	3.9	—	3.5	4.5	3.7	3.7	3.9	3.7	3.9
Anti-VP4	1.7	1.6	1.9	1.8	—	1.7	1.5	1.7	1.6	2.0	1.9
I CJ 27	3.0	2.6	3.1	3.7	3.1	—	1.0	0.1	0.2	2.9	2.9
I H 8-4	2.7	2.7	2.4	3.3	3.2	0.2	—	0.2	0.2	2.8	2.6
I H 8-25	3.0	2.9	3.2	3.8	3.7	0.5	0.4	—	0.4	2.9	2.6
I CJ 12-9	5.3	4.6	5.0	4.5	5.1	0.5	0.3	0.1	—	5.1	5.4
I CJ 31-10	5.2	5.1	1.3	4.5	4.5	5.3	5.2	5.1	5.0	—	4.8
1 BM 55-6	5.1	5.4	4.9	5.4	5.0	4.3	5.0	4.9	4.7	4.6	—
A145	5.3	0.3	4.0		4.8						
A229	5.1	0.6	4.0		5.1						
A611	5.3	0.3	3.6		4.9						

[a] Each variant was tested against each neutralizing activity in a standard plaque reduction neutralization test, using undiluted antibody preparations.

[b] Variants are listed by the antibody against which they are resistant. wt, Wild-type virus.

[c] —, Plaque reduction was <0.5 PFU/ml.

described by Emini et al. (5). Results (Table 4) show that the measured degrees of variation were similar for all of the epitopes and ranged from −3.1 to −4.2 log_{10} variant PFU per wild-type PFU.

Effect of monospecific neutralizing antibodies on virus-cell association. The groups of monospecific antibodies (Table 3) all have in common the ability to neutralize the infectivity of poliovirus. Since some of these antibodies appear to attach to different sites on the surface of the virion, we tested whether each group exerts similar or different effects on the early events of poliovirus infection. First, we studied the adsorption of virions to susceptible cells and the events immediately after adsorption. [^{35}S]methionine-labeled virions were treated with either buffer or neutralizing antibody. The treated virions were then allowed to attach to HeLa cells. L cells, which do not have poliovirus-specific receptors (3), were used as controls. The assay was carried out such that a standard amount of virions (known by the specific radioactivity of incorporated [^{35}S] methionine) was treated with increasing amounts of antibody. The number of antibody molecules was estimated by calculating the amount of immunoglobulin found after protein A-Sepharose chromatography of a portion of each sample containing the neutralizing activity. (The specific extinction coefficient of mouse immunoglobulin G, $A_{280}^{1\%} = 15.0$, was used for these calculations.) All samples were adjusted to approximately equal antibody concentrations. At a virus sample/antibody sample ratio of 1:1, we estimated that four antibody molecules would bind to the virion. The 1:10 and 1:20 ratios should yield complete saturation of virion

TABLE 3. Grouping of neutralizing activities by functional epitopes[a]

A	B	C	D	E	F	G
D3	H3	A13	I CJ 27	I CJ 31-10	1 BM 55-6	Anti-VP4
A145			I H 8-4			
A229			I H 8-25			
A611			I CJ 12-9			

[a] Antibodies were grouped together on the basis of their ability to neutralize the non-neutralizable variants of the other antibodies (see text).

TABLE 4. Degree of variation at the neutralization epitopes[a]

Infection no.	Virus titer (log_{10} PFU/ml)				
	No Ab	+A13	+I CJ 27	+I CJ 31-10	+1 BM 55-6
1	8.2	4.4			
2	8.3	4.1			
3	8.3	5.2			
1	8.9		5.5		5.3
2	8.7		5.1		5.2
1	9.3			5.5	
2	8.8			5.4	

[a] Two or three HeLa cell cultures (5.0×10^6 cells each) were independently infected with wild-type virus at a multiplicity of infection of 10^{-2} PFU per cell. The inocula were free of 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).

TABLE 5. Effect of monospecific neutralizing activities on virion-cell association

Neutralizing activity	% Cell-associated virus-specific radioactive counts[a]							
	HeLa cell				L cell			
	1:20[b]	1:10	1:5	1:1	1:20	1:10	1:5	1:1
Hyperimmune serum	21.0	20.0	31.0	260.0	13.0	22.0	23.0	220.0
D3 (A)[c]	17.0	22.0	50.0	69.0	8.0	6.0	8.0	6.5
H3 (B)	12.0	10.0	30.0	54.0	8.0	6.0	9.0	6.0
A13 (C)	86.0	97.0	257.0	286.0	97.0	194.0	228.0	280.0
I CJ 27 (D)		9.0		20.0		6.0		12.0
I CJ 31-10 (E)		11.0		43.0		8.0		31.0
1 BM 55-6 (F)		14.0		262.0		10.0		252.0
Anti-VP4 (G)	27.0	26.0	33.0	47.0	7.0	5.0	6.3	4.5
Anti-VP3[d]	106.0	109.0			9.0	9.0		
Anti-VP1[d]	118.0				16.0			

[a] A total of 5.0×10^6 HeLa S3 or L suspension cells were washed several times with phosphate-buffered saline. The cells were resuspended in spinner minimal Eagle medium (GIBCO, Grand Island, N.Y.) and infected with [^{35}S]methionine-labeled virus at a calculated multiplicity of infection of 5.0 PFU per cell. The cells were incubated for 30 min at 37°C and then washed twice with phosphate-buffered saline. Finally, the cells were resuspended in phosphate-buffered saline containing 2.0% sodium deoxycholate (Sigma Chemical Co., St. Louis, MO.) and solubilized in scintillation fluid (Aquasol-2; New England Nuclear, Boston, Mass.) for determination of radioactive counts. Approximately 40% of the added radioactive counts attached to the HeLa cell in the antibody-free control sample. The latter value was taken as 100% binding, and the values listed represent percent binding with respect to this control. The maximum amount of virion–L-cell association observed in the absence of antibody was 19%.

[b] Virus/antibody ratio. In each case the volume of virus added remained constant and the amount of antibody used varied. The antibody concentration of each antibody preparation was adjusted to approximately equal levels.

[c] Epitope grouping to which the listed antibody belongs is given in parentheses (see Table 3).

[d] Anti-VP3 and anti-VP1 are non-neutralizing sera containing antibodies which bind to the virions (see text).

by antibody, since it can be assumed that there are 30 antibody binding sites per virion (on the basis of 60 identical structural subunits per virion; 25). In all cases, the 1:1 ratio of antibody/virion was sufficient to neutralize 99% of viral infectivity (data not shown).

Table 5 presents the data of virus-host cell interaction. The data are expressed as percentage of virus-antibody complex bound compared with binding of untreated virions. It can be seen that monoclonal antibodies of groups C and F enhanced the binding of virions under nonsaturating conditions to the extent of hyperimmune serum, an effect observed previously for hyperimmune neutralizing serum by Mandel (17, 18). This effect, however, cannot involve specific interactions between poliovirus-specific host-cell receptor and the virion surface since it was also seen with L cells. In the case of the other groups of neutralizing activity (A, B, D, E, and G) binding was inhibited to different extents, at a ratio of 1:1. Inhibition was enhanced when the concentration of antibody was increased. A small amount of nonspecific binding appeared to be mediated by I CJ 31-10 at the 1:1 ratio. Monoclonal antibody I CJ 27 appeared to completely inhibit binding even under nonsaturating conditions. This is an effect of binding to a specific epitope and not a property of I CJ 27 since each of the other antibodies of group D (I H 8-4, I H 8-25, and I CJ 12-9) had the same effect on virion binding (data not shown).

All of the effects on virus-cell association seen in these experiments were mediated solely by neutralizing antibodies. When antibodies were analyzed that bind to the surface of the virion but do not neutralize, the result was different. Anti-VP3 and anti-VP1, two antisera prepared against the respective purified viral capsid proteins, were both incapable of inhibiting binding even under highly saturating conditions (Table 5). Both sera fail to neutralize viral infectivity, but both contain antibodies which bind to virions in immunoprecipitation tests (data not shown).

Finally, it should be noted that these adsorption studies were carried out at 37°C instead of 0°C since preliminary experiments indicated that viral adsorption at the lower temperature was somewhat inefficient. Elution of virus from cells after binding (16) was found not to have contributed significantly to the results. Release of labeled virus into the medium did not exceed 3.0% of total bound virus during the 30-min course of the experiment.

Effect of monospecific neutralizing antibodies on virus penetration. Since most of the monospecific neutralizing antibodies did not totally pre-

vent virus from binding to the cell under neutralizing but nonsaturating conditions, the effects of the antibodies on viral penetration were investigated. The process of penetration is poorly understood. In this study we assayed for "penetration" by an elution procedure. Radioactivity of [^{3}H]uridine-labeled virus remaining associated with HeLa cells after elution with 8.0 M urea at pH 7.5 (8) was considered to indicate penetrated virus. Specifically, radioactively labeled virions were allowed to bind to HeLa cells at 5°C in the presence or absence of antibody. The amount of antibody used was equivalent to the 1:1 ratio of Table 5. The temperature was then shifted to 37°C, and at 0, 15, and 30 min after the shift, virus still exposed on the surface of the cell was removed with 8.0 M urea in phosphate-buffered saline.

The results in Table 6 show that by 30 min after the shift to 37°C only ca. 30% of the untreated virus-specific counts could be recovered from the cell surface. This value did not decrease further up to 120 mins post-shift (data not shown). Hyperimmune antiserum effectively inhibited viral penetration, a result in agreement with that of Holland and Hoyer (8). However, none of the monospecific neutralizing antibodies completely inhibited penetration. Instead, penetration was inhibited by only slightly more than half of the antibody-free control. No additional penetration was seen after 30 min (data not shown). Non-neutralizing antibody (anti-VP1) also inhibited penetration somewhat. Since the latter serum does not decrease the plaque titer of the viral preparation (data not shown), it may be concluded that the levels of inhibition of penetration exhibited by the neutralizing antibodies are not significant with respect to the neutralization event.

As shown before (Table 5), I CJ 27, a group D neutralizing monoclonal antibody, prevented the specific attachment of virions to the host cell. I CJ 31-10, on the other hand, slightly enhanced the unspecific binding (Table 5). When virions were treated with a mixture of these antibodies, penetration was observed to the same extent as with I CJ 31-10 alone. Thus, the blocking of specific attachment did not prevent extensive penetration as assayed by 8.0 M urea elution. Finally, a mixture of five antibodies, each belonging to a different group (A, B, C, E, and F), was also not capable of inhibiting penetration in a fashion similar to the hyperimmune serum.

That the penetration in the presence of neutralizing monoclonal antibodies was due to a distinct interaction with the host cell and was not the result of an unspecific aggregation with the cell surface was shown as follows. Virus was treated with antibody A13 and attached to L cells (see Table 5). Virus was then eluted and it was found that the attached virus-specific counts were completely recovered up to 60 min after the temperature shift, indicating that poliovirus penetration cannot be mediated by this nonpermissive cell (data not shown).

Effect of monospecific neutralizing antibodies on viral transcription. Since penetration was not completely inhibited by the monospecific neutralizing antibodies, the effects of these antibodies on a later stage of viral replication were examined. Untreated and antibody-treated virions were used to infect HeLa cells. At 1 h

TABLE 6. Effect of monospecific neutralizing activities on virus penetration

Neutralizing activity	% Recoverable virus-specific counts[a] at time after shift to 37°C:		
	0 min	15 min	30 min
No antibody	100	70.0	31.8
Hyperimmune serum	100	95.0	100
D3 (A)[b]	100	86.3	69.1
H3 (B)	100	76.1	68.4
A13 (C)	100	75.7	67.3
I CJ 27[c] (D)			
I CJ 31-10 (E)	100	90.4	75.5
1 BM 55-6 (F)	100	85.2	77.2
Anti-VP4 (G)	100	79.4	76.4
Anti-VP1[d]	100	76.9	65.0
I CJ 31-10 + I CJ 27	100	84.5	62.7
Mixture[e]	100	77.2	75.0

[a] A total of 5.0×10^6 HeLa monolayer cells were washed twice with phosphate-buffered saline. The cells were then infected with [^{3}H]uridine-labeled virus at a calculated multiplicity of infection of 5.0 PFU per cell. Virus was allowed to adsorb for 15 min at 5°C in the presence or absence of antibody. Approximately 10 to 20% of the added radioactive counts attached to the cells. After adsorption, the cells were overlaid with minimal Eagle medium (GIBCO) and placed at 37°C. At 0, 15, and 30 min after the temperature shift, the cells were washed once with phosphate-buffered saline and twice with 8.0 M urea in phosphate-buffered saline. (Microscopic examination of the monolayers after the urea washes did not reveal any damage to the cells.) The latter two washes were pooled, and radioactive counts were determined with Aquasol-2 (New England Nuclear) as scintillation fluid. Counts recovered at 0 min were considered to represent 100% recovery. Counts at 15 and 30 min were compared with these values for calculation of percent recovery.

[b] Epitope grouping to which the listed antibody belongs is given in parentheses (see Table 3).

[c] I CJ 27 prevented viral adsorption (see Table 5).

[d] Anti-VP1 is non-neutralizing antiserum which contains antibodies that bind to the virion (see text).

[e] The antibody mixture contained the following neutralizing monospecific antibodies: D3, H3, A13, I CJ 31-10, and Anti-VP4. The final antibody concentration was calculated to be nonsaturating for the virion.

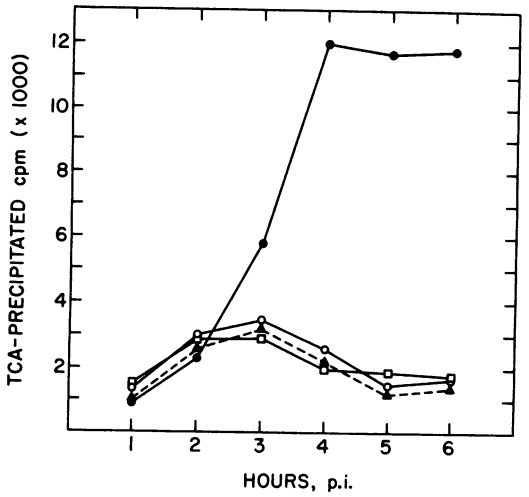

FIG. 1. Effect of neutralizing antibodies on virus-specific transcription in HeLa cells. A total of 2.5×10^7 HeLa S3 cells were infected at a multiplicity of infection of 20 PFU per cell with untreated or antibody-treated (1:1 virus/antibody ratio of Table 5) poliovirus. After 1.0 h at 37°C, 5.0 μg of actinomycin D (Calbiochem, La Jolla, Calif.) per ml was added. The cells were placed at 5°C for 1.0 h and then transferred to 37°C to continue the infection. A 20-μCi amount of [^{3}H]uridine was added upon transfer to 37°C. Samples, 100 μl, were removed from the cultures every hour for 6 h, postinfection (p.i.), and added to 1.0 ml of cold 10% trichloroacetic acid (TCA). Precipitates were filtered through Whatman GF/A filters. Radioactive counts on the dried filters were determined with 2,5-diphenyloxazole (PPO) and 1,4-bis-(5-phenyloxazolyl) benzene (POPOP) in toluene as scintillant. Symbols: (●) untreated virus; (○) virus neutralized with A13; (□) virus neutralized with anti-VP4 serum; (▲) uninfected cells. Virus neutralized with D3, H3, I CJ 27, I CJ 31-10, and 1 BM 55-6 gave results similar to the A13-treated virus (data not shown).

postinfection, the cells were treated with actinomycin D and subsequently incubated in the presence of [^{3}H]uridine. Samples of the cell cultures were taken every hour and mixed with trichloroacetic acid. Incorporation of radioactive material into trichloroacetic acid-precipitable material was then determined. The results in Fig. 1 show that treatment by the monospecific neutralizing antibodies successfully inhibited virus-specific transcription from occurring.

Effect of monospecific neutralizing antibodies on the virion's pI. An attempt was made to determine whether a common effect(s) of the neutralizing antibodies on the poliovirion could be found and whether this effect(s) could be associated with the neutralization event. Mandel (19) reported that hyperimmune antiserum alters the pI of type 1 poliovirus from 7.0 to 4.5. It was felt that this was reflective of an antibody-induced conformational change by the virion. Following this strategy we determined the pI of virions before and after binding to the monospecific neutralizing antibodies (under the nonsaturating 1:1 virus/antibody ratio conditions of Table 5) by column isoelectric focusing.

The results are presented in Table 7. Hyperimmune antiserum was found to alter the virus pI from 7.0 to 4.5, in agreement with previous results (19). In addition, all of the monospecific neutralizing antibodies, with the sole exception of the anti-VP4 activity, lowered the pI of poliovirions from 7.0 to 3.6 to 4.1. The non-neutralizing anti-VP1 serum (see above) did not cause a change in the pI.

Antibody 1 BA 12-1 is a monoclonal antibody that was prepared against the Sabin strain of type 1 poliovirus (2). This antibody was capable, in immunoprecipitation tests, of binding to both the Mahoney and Sabin strains (data not shown). However, it is capable of effectively neutralizing only the Sabin strain (2), whereas the Mahoney strain is neutralized extremely poorly. The effects of this antibody on the virion's pI (Table 7) show that a distinct correlation exists between the neutralization event and the alteration of the pI. Antibody 1 BA 12-1 did not change the Mahoney strain's pI, but did lower the Sabin strain's pI value to 3.7.

Finally, the structural integrity of the neutral-

TABLE 7. Effect of monospecific neutralizing activities on the virion's pI

Virus strain	pI[a]
Mahoney	
No antibody	7.0
+Hyperimmune serum	4.5
+Anti-VP1	7.0
+D3 (A)[b]	3.6
+H3 (B)	3.6
+A13 (C)	3.8
+I CJ 27 (D)	3.8
+I CJ 31-10 (E)	4.1
+1 BM 55-6 (F)	3.6
+Anti-VP4 (G)	7.0
+1 BA 12-1	7.0
Sabin	
No antibody	7.0
+1 BA 12-1	3.7

[a] The pIs were determined by column isoelectric focusing (see text). Antibody-treated or antibody-free [^{35}S]methionine- or [^{3}H]uridine-labeled virus samples were layered into the column at approximately one-third the column length from the cathode. At the completion of focusing, 100 μl of each fraction was assayed for radioactive counts. In each case, over 90% of the virus-specific counts focused at the listed pI.

[b] Epitope grouping to which the listed antibody belongs is given in parentheses (see Table 3).

ized virions was investigated. All neutralized virions whose pI had been changed still contained the viral RNA as shown by the retention of a [^{3}H]uridine label in the virus (data not shown). In addition, none of the neutralizing antibodies significantly aggregated the virus under the nonsaturating condition. Both [^{35}S]methionine- and [^{3}H]uridine-labeled virus migrated through velocity sedimentation gradients in identical fashion in both the presence and the absence of antibody. Significant aggregation was only noted when antibody was present in saturating or greater amounts (data not shown).

DISCUSSION

The availability of monospecific neutralizing antibodies to poliovirus type 1 has provided a unique opportunity to study antibody-mediated poliovirus neutralization. Such studies, in the past, have been difficult since hyperimmune antiserum contains many different antibodies directed against the surface of the virion, not all of which neutralize. Even the non-neutralizing anti-VP1 and anti-VP3 sera used in these studies contained antibodies which recognized epitopes on the intact virion. Hence, it has not been possible to draw correlations between the effects of the hyperimmune antiserum on the virion and the neutralization process itself. Major unresolved questions include the number of viral sites available for antibody-dependent neutralization of infectivity, the structure of these sites, and the mode by which their interaction with immunoglobulin renders the virion inactive.

Using 13 antibody activities, we have detected seven functionally distinct neutralization epitopes on the virion. Whether more neutralization epitopes can be detected by our strategy remains to be seen. A certain degree of functional overlap was noted among several of the seven epitopes. This overlap may be reflective of some structural overlap among the epitopes or may be due to conformational change induced in one site by antibody binding to a structurally distinct site. Ongoing experiments involving competitive binding of the individual antibodies to purified virions may provide information in this regard.

Each epitope was found capable of undergoing variation so that the resulting variant virus was no longer neutralized by the respective antibody. Such variation to non-neutralizability occurred on the order of -3.1 to -4.8 $\log_{10}$ variant per wild-type virus PFU (see also 4a and 5). Such a degree of variation appears high for poliovirus, which exists in three highly stable serotypes. However, recent studies of poliovirus isolated from infected individuals have shown that the viral genome is capable of undergoing considerable mutation upon passage in humans (11, 23, 28). The apparent serological stability of the virus type, which is based on its ability to be neutralized by the appropriate reference sera, seems to be more a reflection of the number of functionally distinct neutralization epitopes than a reflection of the phenotypic stability of any individual antigenic site.

Mandel (17, 18), in studying the effects of hyperimmune serum, notes that, paradoxically, under nonsaturating but neutralizing conditions the serum enhances adsorption of neutralized virus to susceptible cells. This enhancement now appears to be due to a set of antibodies which bind to specific epitopes. Surprisingly, the enhancement also seems to be nonspecific with respect to cell type as it was observed with L cells that lack the poliovirus receptor. It is not known whether such enhancement is a result of a conformational change in the virion induced by these antibodies or whether it is mediated by the antibodies themselves. There also exists a set of neutralizing antibodies which somewhat, but not completely, inhibit adsorption. Finally, antibodies directed against a specific epitope (epitope D of Table 3) all inhibit adsorption even under nonsaturating conditions. It would be particularly intriguing if this epitope is responsible for viral attachment to the cellular receptor.

None of the monospecific neutralizing antibodies was found to completely inhibit viral penetration once the virus bound to the cell. The degree of penetration was reduced, but this appears to be more a function of antibody being bound to the virion than a specific function of the neutralizing antibodies. Hyperimmune antiserum did, however, inhibit penetration. This observation leads to the speculation that adsorption and penetration are mediated by two different sites on the virion and possibly by two different cellular receptors. It is interesting to note that Medrano and Green (22) isolated cell hybrids to which a number of picornaviruses adsorb, but which fail to eclipse the attached virus.

The decrease or increase in adsorption and decrease in penetration caused by the monospecific neutralizing antibodies is not sufficient to account for the 99% reduction in infectivity seen under nonsaturating conditions. Yet, these antibodies appear to have an effect early in viral replication since neutralized virus did not undergo virus-specific transcription and fails to shut off host-cell macromolecular synthesis (unpublished data). A close association was established between the antibody-caused neutralization event and alteration of the virion's pI by neutralizing antibody. The pI change was not caused by mere antibody attachment and is probably reflective of a virion conformational change induced by antibody binding specifically to a neu-

tralization epitope. Such a conformational change may prevent the virus from undergoing an important early replicative step once it has penetrated the cell. This step may be the uncoating and release of the viral RNA as postulated by Mandel (20). Experiments directed towards the fate of the antibody-virion complex after adsorption, such as attempts to recover infectious virus or assays for release of dyes, are necessary to answer these questions. Of particular interest is the neutralizing activity of the anti-VP4 serum, the only exception to the correlation between possible conformational change and neutralization. These neutralizing antibodies are directed to a single site on the VP3 protein of the intact virion (4a) and apparently neutralize by a mechanism distinct from pI change.

It is of interest, in ongoing studies, to determine the exact structures of each of the neutralization epitopes and to correlate these structures with the functions reported here. Since the nucleotide sequence of the type 1 (Mahoney strain) polioviral RNA and the amino acid sequences of the structural proteins are known (4, 12, 15), direct comparisons between the wild-type virus and each of the non-neutralizable variants can be made. These comparisons are being carried out both by enzymatic analysis of the proteins and by direct sequencing of the appropriate regions of the viral RNA. It should be kept in mind, however, that a mutation to resistance to neutralization may reside either in the epitope itself or in the surrounding polypeptide domains, thereby influencing the secondary structure of the epitope.

ACKNOWLEDGMENTS

We are grateful to Bradford Jameson, Andrew Dorner, and Bert Semler for helpful discussions, to Sally Lynch for assistance in preparation of antisera, to Marlies Schmidt for assistance in the establishment of the hybridoma cell lines, and to Susan Studier for preparation of the manuscript.

This work was supported by Public Health Service grants AI-15122 and CA-28146 from the National Institutes of Health. E.A.E. is the recipient of Public Health Service Postdoctoral Fellowship AI-06485 from the National Institutes of Health.

LITERATURE CITED

1. **Blondel, B., R. Crainic, and F. Horodniceanu.** 1982. Le polypeptide structural VP1 du poliovirus type 1 induit des anticorps neutralisants. C. R. Acad. Sci. **294**:91–94.
1a. **Couillin, P., R. Crainic, N. Cabau, F. Horodniceanu, and A. Boue.** 1982. Strain specific type 1 poliovirus-neutralizing monoclonal antibodies. Ann. Virol. (Inst. Pasteur) **133E**:315–323.
2. **Crainic, R., P. Coullin, N. Cabau, A. Boue, and F. Horodniceanu.** 1981. Determination of type 1 poliovirus subtype classes with neutralizing monoclonal antibodies. Dev. Biol. Stand. **50**:229–234.
3. **Crowell, R. L., B. J. Landau, and J. S. Siak.** 1981. Picornavirus receptors in pathogenesis, p. 169–184. *In* K. Lonberg-Holm and L. Philipson (ed.), Virus receptors, part 2. Receptors and recognition, Ser. B, vol. 8. Chapman and Hall, London.
4. **Dorner, A. J., L. F. Dorner, G. R. Larsen, E. Wimmer, and C. W. Anderson.** 1982. Identification of the initiation site of poliovirus polyprotein synthesis. J. Virol. **42**:1017–1028.
4a. **Emini, E. A., A. J. Dorner, L. F. Dorner, B. A. Jameson, and E. Wimmer.** 1983. Identification of a poliovirus neutralization epitope through use of neutralizing antiserum raised against a purified viral structural protein. Virology **124**:144–151.
5. **Emini, E. A., B. A. Jameson, A. J. Lewis, G. R. Larsen, and E. Wimmer.** 1982. Poliovirus neutralization epitopes: analysis and localization with neutralizing monoclonal antibodies. J. Virol. **43**:997–1005.
6. **Ferguson, M., Y.-H. Qi, P. D. Minor, D. I. Magrath, M. Spitz, and G. C. Schild.** 1982. Monoclonal antibodies specific for the Sabin vaccine strain of poliovirus 3. Lancet **ii**:122–124.
7. **Ferguson, M., G. C. Schild, P. D. Minor, P. J. Yates, and M. Spitz.** 1981. A hybridoma cell line secreting antibody to poliovirus type 3 D antigen: detection in virus harvest of two D antigen populations. J. Gen. Virol. **54**:437–442.
8. **Holland, J. J., and B. H. Hoyer.** 1962. Early stages of enterovirus infection. Cold Spring Harbor Symp. Quant. Biol. **27**:101–112.
9. **Humphrey, D. D., O. M. Kew, and P. M. Feorino.** 1982. Monoclonal antibodies of four different specificities for neutralization of type 1 polioviruses. Infect. Immun. **36**:841–843.
10. **Icenogle, J., S. F. Gilbert, J. Grieves, J. Anderegg, and R. Rueckert.** 1981. A neutralizing monoclonal antibody against poliovirus and its reaction with related antigens. Virology **115**:211–215.
11. **Kew, O. M., B. K. Nottay, M. H. Hatch, J. H. Nakano, and J. F. Obijeski.** 1981. Multiple genetic changes can occur in the oral polio vaccines upon replication in humans. J. Gen. Virol. **56**:337–347.
12. **Kitamura, N., B. L. Semler, P. G. Rothberg, G. R. Larsen, C. J. Adler, A. J. Dorner, E. A. Emini, R. Hanecak, J. J. Lee, S. van der Werf, C. W. Anderson, and E. Wimmer.** 1981. Primary structure, gene organization, and polypeptide expression of poliovirus RNA. Nature (London) **291**:547–553.
13. **Kohler, G., and C. Milstein.** 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature (London) **256**:495–497.
14. **Kohler, G., and C. Milstein.** 1976. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. Eur. J. Immunol. **6**:511–519.
15. **Larsen, G. R., C. W. Anderson, A. J. Dorner, B. L. Semler, and E. Wimmer.** 1982. Cleavage sites within the poliovirus capsid protein precursors. J. Virol. **41**:340–344.
16. **Lonberg-Holm, K., L. B. Gosser, and J. C. Kaver.** 1975. Early alteration of poliovirus in infected cells and its specific inhibition. J. Gen. Virol. **27**:329–342.
17. **Mandel, B.** 1958. Studies on the interactions of poliomyelitis virus, antibody, and host cells in a tissue culture system. Virology **6**:424–447.
18. **Mandel, B.** 1967. The interaction of neutralized poliovirus with HeLa cells. I. Adsorption. Virology **31**:238–247.
19. **Mandel, B.** 1971. Characterization of Type 1 poliovirus by electrophoretic analysis. Virology **44**:554–568.
20. **Mandel, B.** 1976. Neutralization of poliovirus: a hypothesis to explain the mechanism and the one-hit character of the neutralization reaction. Virology **69**:500–510.
21. **Mandel, B.** 1979. Interaction of viruses with neutralizing antibodies, p. 37–121. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 15. Plenum Publishing Corp., New York.
22. **Medrano, L., and H. Green.** 1973. Picornavirus receptors and picornavirus multiplication in human-mouse hybrid cell lines. Virology **54**:515–524.
23. **Minor, P. D., G. C. Schild, M. Ferguson, A. Mackay, D. I. Magrath, A. John, P. J. Yates, and M. Spitz.** 1982. Genetic and antigenic variation in type 3 polioviruses: charac-

terization of strains by monoclonal antibodies and T_1 oligonucleotide mapping. J. Gen. Virol. **61**:167–176.

24. **Osterhaus, A. D. M. E., A. L. van Wezel, G. van Steenis, A. G. Hazendonk, and G. Drost.** 1981. Production and potential use of monoclonal antibodies against polio viruses. Dev. Biol. Stand. **50**:221–228.
25. **Rueckert, R. R.** 1976. On the structure and morphogenesis of picornaviruses, p. 131–213. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 6. Plenum Publishing Corp., New York.
26. **Sabin, A. B.** 1957. Properties of attenuated polioviruses and their behavior in human beings. Spec. Publ. N.Y. Acad. Sci. **5**:113–127.
27. **Salk, J. E.** 1953. Principles of immunization as applied to poliomyelitis and influenza. J. Public Health **43**:1384–1398.
28. **Yoneyama, T., A. Hagiwara, M. Hara, and H. Shimojo.** 1982. Alteration in oligonucleotide fingerprint patterns of the viral genome in poliovirus type 2 isolated from paralytic patients. Infect. Immun. **37**:46–53.