

Synthetic peptides from four separate regions of the poliovirus type 1 capsid protein VP1 induce neutralizing antibodies

(poliovirus type 1 Mahoney strain/biotin immunoblots/host immune response)

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ABSTRACT Peptides from different regions of the poliovirus type 1 capsid protein VP1 were synthesized. Antibodies raised against these peptides in rabbits and rats recognized the cognate peptides and denatured VP1. Peptides from four regions of VP1 generated antisera with neutralizing titers specifically against poliovirus type 1. Antisera against all other regions of VP1 failed to neutralize virus infectivity, although some of the antisera clearly bound to native virions. Thus, the neutralizing determinants on VP1 reside in specific noncontiguous regions of the protein and can be defined by specific peptides from these regions.

An animal's immunologic response against a viral antigen is a composite of many antibodies, of which those that neutralize infectivity comprise a specific subset. Because the neutralizing response plays a major protective role, the characterization of the antibodies, their specificities, and their functional role in the neutralization process is crucial to an understanding of the pathobiology of a virus. In addition, the definition of neutralizing epitopes should help in the understanding of initial virus-cell interactions.

It previously has been shown that major neutralizing determinants are found on the poliovirus type 1 capsid protein designated VP1. The isolated, denatured VP1 is capable of inducing virion-neutralizing antibodies in rats (1) and rabbits (2). VP1 is immunoprecipitated by a neutralizing anti-virion monoclonal antibody, C-3 (3). In addition, using this monoclonal antibody, Wychowski *et al.* (4) have shown that a neutralization epitope was located within the region of amino acid residues 90-104 of VP1. Analysis of virion mutants that are resistant to neutralizing anti-virion monoclonal antibodies showed that a major neutralizing determinant for poliovirus type 3 was localized within VP1 in the region of amino acid residues 96-106 (5, 6). Characterization of a large set of neutralizing anti-virion monoclonal antibodies against poliovirus type 1 also indicated that the amino acid region 94-104 of VP1 contained a neutralizing determinant (7). In addition, an additional amino acid region (residues 70-80) was recognized by some monoclonal antibodies (7). Thus, it appears that VP1 potentially possesses several neutralizing determinants.

To further characterize the neutralization response against VP1 and to structurally characterize the neutralization epitopes, we derived a partial antigenic map of VP1, using antisera made against synthetic peptides from different regions throughout the VP1 protein. We report here that synthetic peptides spanning four separate regions of the VP1 sequence will induce neutralizing antibodies. These serotype-specific antibodies recognize VP1 and the intact mature poliovirus virion. The ability of these four regions to generate neutraliz-

ing antibodies is independent of the choice of immunizing host.

METHODS

Synthesis and Coupling of Peptides. The peptides were synthesized by R. Houghten, with solid-phase methods using a Beckman model 990B peptide synthesizer (8, 9). The peptides were cleaved from the resin and passed through a Sephadex G-25 sizing column. The void volume was collected, and the amino acid composition of the peptide sample was determined. In the rare case when the amino acid composition varied more than 5% from the theoretically predicted composition, the peptide sample was further purified by using high-pressure chromatographic techniques. In general, the Sephadex G-25-purified peptides were used directly for further studies.

Keyhole limpet hemacyanin (KLH; Calbiochem-Behring) was used as the carrier protein in all coupling reactions. Two peptides were not sufficiently soluble in any of the reaction buffers tried and were injected into the animal as free, uncoupled peptide. Two methods of coupling were used. Peptides that contained cysteine were coupled by using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) as described by Liu *et al.* (10). Peptides that did not contain a cysteine residue were coupled by using glutaraldehyde as described by Baron and Baltimore (11).

Immunization of Animals. New Zealand White rabbits and Lewis rats, 8 wk old (Charles River Breeding Laboratories), were immunized with either KLH-coupled or free peptides. The animals were injected intraperitoneally and intradermally with emulsions containing 100 μ g of the coupled carrier protein or of the free peptides. To equalize variability among the individual animals, each peptide was injected into at least two (usually three) rats and two rabbits. The emulsions were made by mixing equal volumes of the protein sample and complete Freund's adjuvant. After 3 wk, the initial injection was followed by three or four booster injections 2 wk apart. Each booster injection contained 100 μ g of protein in a 50% emulsion with incomplete Freund's adjuvant for rats or with alum for rabbits. Animals were bled 8-10 days after each booster injection, and antiserum was isolated. Antisera were heated to 65°C for 30 min before storage at -20°C.

Slot-Blot Assays. The specificity of the antisera was examined by using a slot-blot assay. Peptide or protein samples (0.5 μ g/100 μ l) were bound to nitrocellulose filter strips presoaked in phosphate-buffered saline by using a vacuum manifold (Schleicher & Schuell). The air-dried filter was blocked with blocking buffer [phosphate-buffered saline containing 3% bovine serum albumin (fraction V, Pentex, Kankakee, IL)] at 37°C for 90 min with shaking. At the end of the incubation, the filter was incubated with the serum samples,

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Abbreviations: KLH, keyhole limpet hemacyanin; pfu, plaque-forming units.

which had been diluted in blocking buffer. Peptide competition of serum samples was done by incubating the filter with serum samples in the presence of peptide at 100 $\mu\text{g}/\text{ml}$. The incubations were at 37°C for 30 min. The filters were rinsed briefly with blocking buffer and then washed three times with a large excess of the same buffer at room temperature for 15 min with shaking. For the rabbit antisera, the washed filters were incubated with ^{125}I -labeled *Staphylococcus aureus* protein A (1 $\mu\text{Ci}/\text{ml}$, New England Nuclear; 1 Ci = 37 GBq) in blocking buffer for 60 min at 37°C with shaking. The filters were washed three times with Triton buffer (blocking buffer containing 1.0% Triton X-100) at room temperature for 15–20 min each and then were air-dried. The filters were autoradiographed (Kodak, XAR film) at -70°C with intensifying screens (Lightning Plus, Dupont). For the rat antisera, the filters were incubated with a second antibody, biotinylated-rabbit anti-rat IgG. The antibody complexes were visualized by using an avidin-biotinylated alkaline phosphatase complex by the methods of Costello *et al.* (12) and Leary *et al.* (13).

Neutralization Titers. The titer of neutralizing antibody present in the anti-peptide antisera was measured by a plaque-reduction assay and a cytopathic-effect end-point inhibition assay. The plaque-reduction assay, with an input of 200 plaque-forming units (pfu) was as described (1). Titers were given as the antiserum dilution that would diminish the apparent titers by 50%. The ability of the antisera to completely neutralize virus infectivity and consequently its lytic cytopathic effect was measured by incubating serial dilutions (1:2) of antiserum in phosphate-buffered saline with 1000 pfu of poliovirus in a final volume of 50 μl for 2 hr at 25°C and then overnight at 4°C. The virus-antiserum sample was allowed to adsorb to CV-1 cells plated in microtiter plates (10^4 cells per well) for 30 min at 37°C. The inoculum was removed, and the wells were overlaid with fresh medium (Dulbecco's modified Eagle's medium containing 5% fetal calf serum and 0.01 M Hepes, pH 7.2) and incubated at 37°C for 24 hr (polio type 1, Mahoney) or for 48 hr (polio type 2, Lansing). All assays were carried out in duplicate and were visualized by staining with crystal violet. Titers were given as the first dilution of antiserum where the cells were completely lysed by the virus.

All other methods were as previously described (1).

RESULTS

The capsid protein of poliovirus type 1 (Mahoney strain), VP1, is 302 amino acids long, and its complete amino acid sequence is known from its nucleotide sequence (14, 15). A series of peptides were selected to represent regions that map throughout the entire VP1 sequence (Fig. 1). Among the peptides selected were ones with high hydrophilicity indices or with significant sequence variability between strains. In addition, after comparison of the chemical properties of the VP1 peptide regions for all known picornavirus strains—including the known VP1 sequences for different strains of foot and mouth disease virus (FMDV) (unpublished data)—several peptides were selected because they might span regions that were analogous to peptide sequences of FMDV

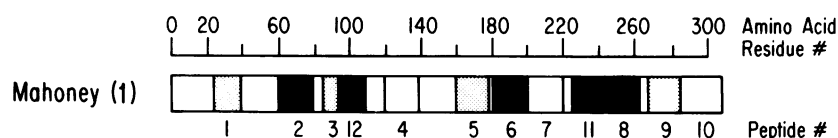


FIG. 1. Location of the synthetic peptides within the VP1 sequence. The peptide numbers denote the locations of the synthetic peptides along the linear VP1 amino acid sequence for the type 1, Mahoney strain; the amino acid sequence was determined from the nucleotide sequence (14, 15). The amino acid residues are numbered with the NH_2 -terminal glycine at position 1 and residue 302 at the COOH terminus. The exact amino acid residues spanning each peptide is specified in Table 1. ■, Peptides that induce neutralizing responses; ▨, peptides whose antisera will recognize and bind virions but do not neutralize (see text).

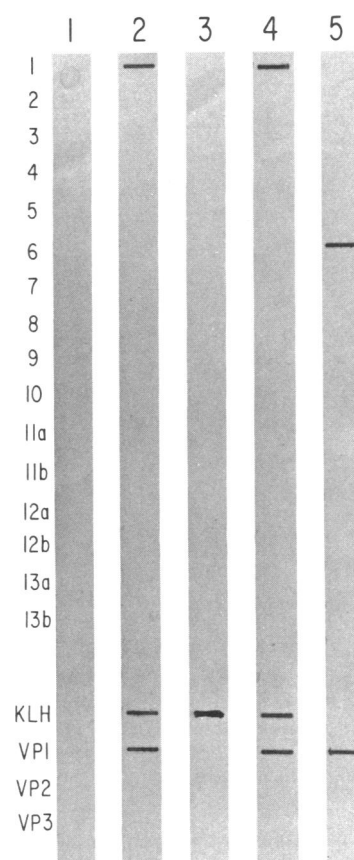


FIG. 2. Antigen specificities of the anti-peptide antisera. The antigenic analysis of one anti-peptide antisera (anti-peptide 1) is shown. Peptides 1–13b, KLH, VP1, VP2, and VP3 were bound to a nitrocellulose filter and subsequently incubated with rat antisera. The antibody complexes were visualized by using a biotinylated rabbit anti-rat IgG-avidin-biotinylated alkaline phosphatase sandwich. The filters were incubated with normal rat serum (lane 1), anti-peptide 1 antiserum (lane 2), anti-peptide 1 antiserum in the presence of peptide 1 (lane 3), anti-peptide 1 antiserum in the presence of peptide 6 (lane 4), and anti-peptide 6 antiserum (lane 5). The anti-peptide 1 antiserum was raised against peptide 1 coupled to KLH. The anti-peptide 6 antiserum was raised against uncoupled peptide 6.

that induced neutralizing antibodies (16, 17). These chemically synthesized peptides were coupled to KLH and injected into rats and rabbits. Antisera were obtained from the immunized animals 1 week after the last booster.

Characterization of Anti-Peptide Antisera. The antigen specificities of the various anti-peptide antisera were qualitatively assayed by using slot blots (Figs. 2 and 3). Every antiserum reacted with its cognate peptide and, in cases when the peptides were coupled to the carrier protein, with KLH (Fig. 2, lanes 2 and 5; Fig. 3 A and B; Table 1). The antisera were quite specific. With the exception of the antisera to peptides 3, 12a, 12b, and 8, each antiserum recognized solely its cognate peptide. The recognition by each antiserum was specifically blocked in competition by the cognate peptide

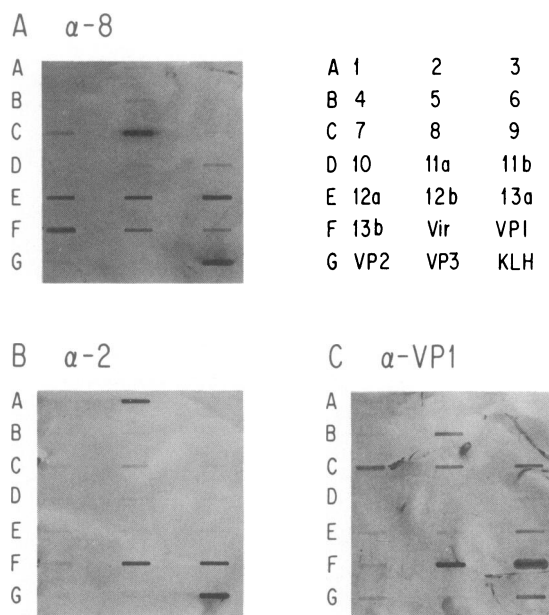


FIG. 3. Peptide recognition by anti-peptide and anti-VP1 antisera. The crossreactivity of anti-VP1 and anti-peptide 8 antisera with the different VP1 peptides was tested by using slot blots. The peptides were spotted according to the key given in the upper right-hand corner of this figure. The filters were incubated with anti-peptide 8 (A), anti-peptide 2 (B), and anti-VP1 (C) rat antisera. The antibody complexes were visualized as described.

and not by any of the other VP1 peptides (Fig. 2, lanes 3 and 4). As would be expected, antiserum to peptide 3 recognized not only its cognate peptide, but it also recognized other peptides that overlap the peptide 3 amino acid sequence, peptides 12a, 12b, 13a, and 13b (Table 1). Similarly, antisera obtained against 12a and 12b strongly recognized peptides 12a and 12b and to a lesser extent peptides 3, 13a, and 13b. Although antiserum to peptide 8 showed high reactivity against 8, it appeared to bind weakly to peptides 3, 12a, 12b, 13a, 13b, and 7 (Fig. 3A). Incubation of the antisera with an excess of peptide 8 in solution effectively blocked recognition of all of the peptides except peptide 7 (data not shown). This indicates that the reactivity of the antiserum of anti-peptide 8 with peptides 3, 12a, 12b, 13a, and 13b is specific and that

the recognition of peptide 7 is probably nonspecific. This spectrum of recognition by anti-peptide 8 antiserum with peptides 3, 12a, 12b, 13a, and 13b was seen with both the rat and the rabbit antisera, suggesting that the cross-reactivity was characteristic of the amino acid sequence represented in peptide 8.

All of the anti-peptide antisera reacted with isolated denatured VP1. Sera directed against peptides 7, 8, and 2 consistently showed much weaker reactivity against the denatured VP1 when compared with the other anti-peptide antisera. None of the anti-peptide antisera recognized isolated, denatured VP2 or VP3, as might be expected from their sequences.

The ability of sera raised against VP1 to react with peptides was also assessed (Fig. 3C). Peptides 5, 7, 8, and 9 were specifically recognized, and these reactions were competitively blocked by the addition of isolated VP1.

Neutralization Activity of Anti-Peptide Antisera. The ability of the anti-peptide antisera to neutralize poliovirus infectivity was assayed by (i) 50% reduction of titer upon exposure of 200 poliovirus pfu to the serum or (ii) total inhibition of the viral cytopathic effect and cell lysis induced by 1000 pfu by using various dilutions of sera in an end-point titration in liquid culture (Table 2). Antisera against several peptides (2, 6, 12a, 12b, 8, 11a, and 11b) showed similar neutralizing titers in both the cytopathic-effect and plaque-reduction assays. These peptides map to four separate regions of VP1, amino acids 61–80, 95–109, 182–201, and 222–264. The neutralizing titers varied for the different peptides, and they were uniformly low compared to the titers of anti-virion antisera.

Several of the anti-peptide antisera (against peptides 4, 7, and 10) showed no neutralizing activity in either assay (Table 2). These antisera were directed against peptides from three different regions of VP1. Thus, peptides from specific regions in VP1 will induce antibodies that recognize the peptide as well as denatured VP1 but fail to neutralize the infectivity of the virus. Consistent with these observations, each of these anti-peptide antisera failed to precipitate labeled virions from solution.

Four anti-peptide antisera (against peptide 1, 3, 5, and 9) consistently showed no neutralizing titers in the end-point titration assay, even at undiluted serum concentrations, but showed measurable titers in the plaque-reduction assay. These sera were raised against peptides from amino acid re-

Table 1. Preparation and specificity of antisera

Peptide no.	Antiserum preparation		Cognate peptide reactivity	Other peptide reactivities	Antiserum specificity				
	Amino acid no.	Coupling method/carrier protein			Reaction with:				
					KLH	VP1	VP2	VP3	Virions
1	24–40	MBS/KLH	+		+	+	–	–	+
2	61–80	MBS/KLH	+		+	+	–	–	+
3	86–103	MBS/KLH	+	12a,b; 13a,b	+	+	–	–	+
4	121–141	Free	+		–	+	–	–	–
5	161–181	Gluter/KLH	+		+	+	–	–	+
6	182–201	Free	+		–	+	–	–	+
7	202–221	MBS/KLH	+		+	+	–	–	–
8	244–264	MBS/KLH	+	12a,b; 13a,b; 3; 7	+	+	–	–	+
9	270–287	Free	+		–	+	–	–	+
10	286–302	MBS/KLH	+		+	+	–	–	–
11a	222–241	Gluter/KLH	+		+	+	–	–	+
11b	222–241	MBS/KLH	+		+	+	–	–	+
12a	100–109	MBS/KLH	+	3; 12b; 13a,b	+	+	–	–	+
12b	91–109	MBS/KLH	+	3; 12a; 13a,b	+	+	–	–	+
13a	100–109								
13b	91–109								

Peptides 1–12b correspond to the sequence of poliovirus type 1 capsid protein, Mahoney strain; peptides 13a and 13b are derived from the VP1 sequence of type 1 Sabin strain. MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester.

Table 2. Neutralizing titers of anti-peptide antisera

Antisera against peptide no.	Dilution giving 50% PR against type 1 virus (rabbit)	Dilution preventing CE		
		Type 1 virus		Type 2 virus
		Rabbit	Rat	Rabbit
1	1:100	—	—	ND
2	1:150	1:200	1:200	1:10
3	1:280	—	—	—
4	—	—	—	ND
5	1:4	—	—	ND
6	1:150	1:200	1:150	1:5
7	—	—	—	ND
8	1:10	1:20	1:25	—
9	1:10	—	—	ND
10	—	—	—	ND
11a	1:100*	ND	1:80	1:5*
11b	1:100*	ND	1:140	1:5*
12a	1:150*	ND	1:100	1:10*
12b	1:50*	ND	1:80	1:5*

ND, not determined; CE, cytopathic effect; PR, plaque reduction.
*Titers were determined using rat antisera.

gions 24–40, 86–103, 161–181, and 286–302 in VP1. This group of antisera also will precipitate virions. These results are best explained by aggregation of the virus particles by the antibodies. The fact that specific regions on the virion surface generate antibodies that bind to, but do not neutralize, the virus indicates that the neutralization determinants are specific sites on the virus and not a general, nonspecific surface interference with virus infectivity.

Similar neutralizing titers were observed in antisera from immunized rats as in rabbits (Table 2). The peptides that elicited neutralizing antibodies in rats also elicited neutralizing antibodies in rabbits. The relative titers for each peptide were also similar between the two animal systems. Thus, for these sequences, the ability of a peptide to induce the production of neutralizing antibody is apparently a property of the peptide sequence and is not a reflection of unusual immunological characteristics peculiar to the animal host system chosen. Although the observed titers for the anti-peptide antisera in both animal systems were extremely low compared with those obtained for antisera against poliovirus virions, these anti-peptide titers were comparable to those measured for antiserum made against isolated, denatured VP1 protein (1). In addition, similar to the characteristics of the anti-VP1 antiserum, antisera made against peptides from the type 1 Mahoney strain VP1 sequence appear to neutralize type 1 poliovirus (Mahoney strain) more effectively than type 2 poliovirus (Lansing strain). Thus, the observed neutralizing activity for both anti-VP1 and anti-peptide antisera is apparently serotype specific, mimicking the characteristics of the neutralizing anti-virion antisera.

DISCUSSION

We have demonstrated that peptides from four separate regions of the poliovirus capsid protein VP1 can induce neutralizing antibodies in two mammalian species. These antibodies recognize and will bind to the cognate peptide, denatured VP1, and native virions. Although the titers are extremely low compared to the neutralizing titers of anti-virion antisera, the anti-peptide titers are comparable to those observed with antisera to denatured VP1 (1) and are serotype specific. Peptides from other regions of VP1 will induce antibodies that bind to virions but fail to completely block the infectivity of virus. Finally, several peptides induce antibodies that bind to the cognate peptide and denatured VP1 but fail both to bind to the native virus and to neutralize the infectivity of poliovirus.

Comparison with Earlier Results. Our results agree with

and extend previous analyses of antigenic sites on the poliovirion. By analyzing mutants that escape neutralization by monoclonal antibodies, Minor *et al.* (5) suggested that amino acids 96–106 of the type 3 VP1 protein should be a neutralization site. (The analogous region from the type 1 VP1 is contained in our peptide 12b.) Emini *et al.* (7) showed that neutralizing monoclonal anti-type 1 antibodies would react with regions defined by our peptides 12b and 2. They also showed that a peptide consisting of amino acids 93–103 would elicit neutralizing antibodies. Using a series of deletions, Wychowski *et al.* (4) also showed that recognition of VP1 expressed in bacterial cells by the neutralizing anti-virion monoclonal antibody C-3 resided within the region of amino acid residues 90–104.

Our observation of neutralizing regions separate from those defined previously was presaged by Kew and Nottay (18), who indicated that other undefined neutralizing determinants were present in VP1. They found that in Sabin type 1 isolates that had antigenically drifted after immunization of healthy infants, there was no consistent correlation with nucleotide sequence changes corresponding to amino acids 1–125. Thus, these data implied the presence of neutralizing determinants in the COOH-terminal half of the VP1 protein. Our definition of two peptide regions from the COOH-terminal half of VP1 that will elicit neutralizing antibodies is consistent with these observations. Further analysis of the viral epitopes will be necessary to determine whether these neutralizing regions represent the complete set of neutralizing determinants present in VP1 or whether additional sites exist.

A curious aspect of our results was that peptide 3 (amino acids 86–103) generated antibodies that bound to virions but did not neutralize the infectivity of the virus. The COOH-terminus of this peptide overlapped into a region that was demonstrated to be a neutralizing determinant (peptides 12a and 12b; see Table 1), and Emini *et al.* (7) had shown that only the amino acids from positions 93–103 were needed to elicit a response. The lack of neutralizing activity by peptide 3 is therefore puzzling, but possibly the extra six residues at the NH₂ terminus of peptide 3 may conformationally mask the COOH-terminus, the NH₂-terminal region may be immunodominant, or antibodies directed to the NH₂-terminal region may interfere with neutralization by antibodies recognizing the COOH-terminal sequences.

Nature of Neutralization Sites. The mechanism of neutralization of infectivity by antibody remains obscure. Our data and much previous evidence (19–22) implies that some antibodies bind to virions but do not neutralize infectivity. In our case, certain peptides specifically induced such antibodies, which were recognized by their ability to quite significantly reduce viral titers without ever abolishing infectivity, a behavior we interpret as due to aggregation of virions. Why binding of antibody to some parts of the virion elicits neutralization and binding to others does not is a puzzle that might be clarified if the three-dimensional structure of the virion were known. Neutralization appears to require binding of multiple bivalent antibody molecules to the virion surface (23, 24), suggesting a crosslinking stabilization effect, so that the physical relationship of the 60 identical sites on the virion surface may be an important parameter of neutralization. The four VP1 sites that induce neutralizing antibodies appear to be separated along the one-dimensional structure of the polypeptide sequence but could come together to form a single site when the polypeptide is folded up in the virion. X-ray crystallography is most likely to illuminate this question (25).

The anti-peptide antisera do allow us to identify regions of VP1 that are accessible to antibody and, therefore, operationally at the surface of the virion. The blackened and stippled areas in Fig. 1 define surface regions, showing, as would be expected, that the polypeptide is folded in a com-

plicated configuration. A large percentage of the tested peptides are at least partially exposed at the surface, consistent with reports that VP1 is the most exposed of the three major virion proteins (26, 27).

That two different species of animals both produced neutralizing antibodies to the same set of determinants suggests that it is the structure of the virion that determines the biological phenotype of the antisera, not the immune response genetics of the inoculated animal. However, it may be significant that both species are insusceptible to poliovirus infection by virtue of lacking cell-surface receptors (28–30). Perhaps primates or humans, in which poliovirus grows productively, might respond differently.

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