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A poliovirus type 1 neutralization epitope is located within amino acid residues 93 to 104 of viral capsid polypeptide VP1

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Using nuclease *Bal*31, deletions were generated within the poliovirus type 1 cDNA sequences, coding for capsid polypeptide VP1, within plasmid pCW119. The fusion proteins expressed in *Escherichia coli* by the deleted plasmids reacted with rabbit immune sera directed against poliovirus capsid polypeptide VP1 (αVP1 antibodies). They also reacted with a poliovirus type 1 neutralizing monoclonal antibody C3, but reactivity was lost when the deletion extended up to VP1 amino acids 90–104. Computer analysis of the protein revealed a high local density of hydrophilic amino acid residues in the region of VP1 amino acids 93–103. A peptide representing the sequence of this region was chemically synthesized. Once coupled to keyhole limpet hemocyanin, this peptide was specifically immunoprecipitated by C3 antibodies. The peptide also inhibited the neutralization of poliovirus type 1 by C3 antibodies. We thus conclude that the neutralization epitope recognized by C3 is located within the region of amino acids 93–104 of capsid polypeptide VP1.
*Key words: Bal*31 deletions/monoclonal antibodies/ neutralization epitope/poliovirus/synthetic peptide.

Introduction

Since the development of the inactivated (Salk, 1953) and attenuated (Sabin, 1957) poliomyelitis vaccines, considerable efforts have been made to understand the antigenic structure of poliovirus. The virion is composed of a single-stranded RNA molecule of plus strand polarity, and of 60 copies each of four structural polypeptides, VP1, VP2, VP3 and VP4 (Rueckert, 1976). Cross-linking and surface labeling studies of intact virions (Beneke *et al.*, 1977; Lonberg-Holm and Butterworth, 1976; Wetz and Habermehl, 1979) have established that VP1 is the predominantly exposed surface protein of the virion. VP2 and VP3 are also, but to a lesser extent, located externally on the particle, whereas VP4 appears to be completely internal.

A neutralizing antibody response against poliovirus can be obtained by immunizing animals with intact virions (D-particles). A lower level neutralizing antibody response was also obtained with heat-inactivated virions (C-particles) (Dernick, 1981), or with purified capsid polypeptide VP1, but not with purified polypeptides VP2 or VP3 (Blondel *et al.*, 1982; Chow and Baltimore, 1982). Polyclonal as well as monoclonal antibodies raised against D-particles fail, however, to react with C-particles or with the isolated capsid polypeptides (Icenogle *et al.*, 1981; Blondel and Crainic, 1981; Emini *et al.*, 1982), suggesting that the epitopes they recognize result, at least in part, from the three-dimensional arrangement of the proteins in the virion.

However, a neutralizing monoclonal antibody, C3, which was obtained by immunizing mice with purified C-particles, was found to react not only with native and heat-inactivated virions but also with isolated VP1 (Blondel *et al.*, 1983), suggesting that the epitope it recognizes depends directly on the amino acid sequence of the protein.

To localize the C3 neutralization epitope, we analysed the antigenicity of a VP1-β-lactamase fusion protein synthesized in *Escherichia coli* transformed by a recombination plasmid, pCW119, which carries the poliovirus cDNA sequences representing nucleotides 2445–3417 of the viral RNA inserted in phase in the β-lactamase gene of pBR327. The VP1-β-lactamase fusion protein, p49, with an apparent mol. wt. of 49 kd, was made of nine amino acids from the β-lactamase N-terminal end, and a histidine residue resulting from the fusion, followed by 11 amino acids from the C-terminal end of VP3, the 302 amino acids of VP1, 12 amino acids from the N-terminal end of NCVP3b, and the 104 amino acids from the remaining β-lactamase C-terminal half (van der Werf *et al.*, 1983a, 1983b). It was specifically immunoprecipitated by immune sera from rabbits immunized against purified poliovirus capsid polypeptide VP1 (αVP1 sera) and by the neutralizing monoclonal antibody C3 (van der Werf *et al.*, 1983b).

By generating deletions inside the VP1 sequence, we were able to locate the C3 neutralization epitope in the domain between amino acids 91 and 104 of VP1. The C3 neutralization epitope was further characterized by examining the reactivity of a synthetic peptide corresponding to VP1 amino acids 93–104, with the neutralizing antibody.

Results

Localization of the C3 neutralization epitope

We previously showed that deleting the VP1 sequence from pCW119 from nucleotides 2546–2861 (VP1 amino acids 24–128) resulted in the expression of a truncated fusion protein which could still be specifically immunoprecipitated by αVP1 antibodies but no longer by C3 (van der Werf *et al.*, 1983b).

To localize the C3 epitope within the 24–128 amino acid domain of VP1, deletions of variable length were generated within the VP1 sequence of pCW119. For that purpose, the plasmid DNA was first cleaved at the unique *Kpn*I site (poliovirus nucleotide 3064; Figure 1). The sequences at both ends of the linearized molecules were then digested away by treatment with nuclease *Bal*31. The proteins expressed by each of the deleted plasmids were analysed by SDS-polyacrylamide gel electrophoresis (PAGE) after [^{35}S]methionine labeling and immunoprecipitation with either αVP1 immune sera or C3 antibodies. In parallel, the plasmids' DNAs were analysed by restriction endonuclease analysis. As already reported (van der Werf *et al.*, 1983b), preliminary characterization of the *Bal*31 deleted plasmids allowed us to show that plasmids with internal deletions extending up to the

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*Alu*I site located at nucleotide 2787 (VP1 amino acid 103) expressed truncated fusion proteins that were still immunoprecipitated by C3. On the other hand, plasmids with deletions extending further upstream on the DNA than the *Alu*I site located at poliovirus nucleotide 2764 (VP1 amino acid 95), expressed fusion proteins that were not immunoprecipitated by C3, although they were still immunoprecipitated by αVP1 immune sera.

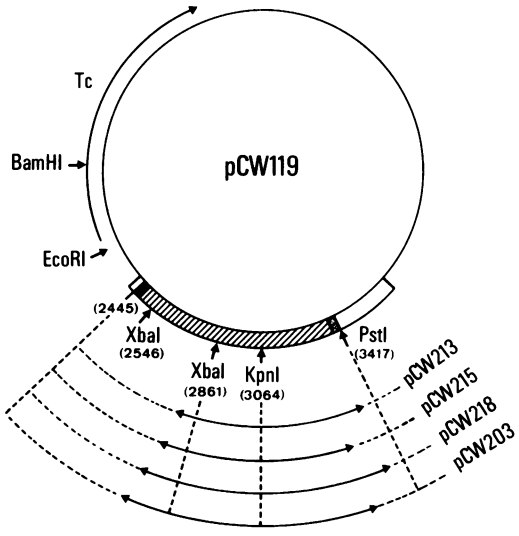

Fig. 1. Structure of plasmid pCW119 and of its *Bal*31 deleted derivatives. A 972-bp fragment of poliovirus cDNA encoding part of VP3 (black box), VP1 (hatched box) and part of NCVP 3b (dotted box) was fused in-phase within the β-lactamase sequence of pBR327 (open boxes) as described (see text, and van der Werf *et al.*, 1983a, 1983b). Numbers refer to the poliovirus nucleotide sequence (Dorner *et al.*, 1982). For the construction of *Bal*31 deleted derivatives, plasmid pCW119 DNA was linearized at the *Kpn*1 site, digested with nuclease *Bal*31 and recircularized using T4 DNA ligase (see text and Materials and methods). The deleted sequences are indicated by the arrows.

To map the deletions precisely, the DNA nucleotide sequence of representative plasmids was determined by the dideoxynucleotide terminators method, after subcloning into bacteriophage M13mp8, or directly from the plasmid DNAs, using the method of Guo and Wu (1982). As shown in Figure 2, the deletions in plasmids pCW203, 218 and 215 extended upstream up to poliovirus nucleotides 2738, 2751 and 2793, respectively, and downstream to nucleotides 3328, 3385 and 3319, respectively. In the case of pCW213, the sequence data did not allow us to determine whether the deletion was from nucleotides 2807–3383 or 2809–3385. It was expected that the deletions generated by nuclease *Bal*31 would extend to similar distances upstream and downstream from the *Kpn*I site at nucleotide 3064 on the plasmids DNAs. This was indeed the case for plasmids pCW218 and 215, whereas plasmids pCW213 and 203 showed a 62 nucleotide disymmetry in the extents of the deletion on either side of the *Kpn*I site.

The proteins expressed by the *Bal*31 deleted plasmids in strain GC26 were labeled with [^{35}S]methionine, immunoprecipitated with αVP1 serum and analysed by SDS-PAGE as described (van der Werf *et al.*, 1983a). The apparent mol. wt. of the immunoprecipitated proteins was found to vary over a wide range, independent of the extent of the deletions (Table I). This was due to whether the sequences on each side of the deletion were fused in phase with each other or not. Thus, whereas the mol. wt. of the protein expressed by pCW203, 30 kd, was that expected from the size of the deletion, the mol. wts. of the proteins expressed by pCW213, 215

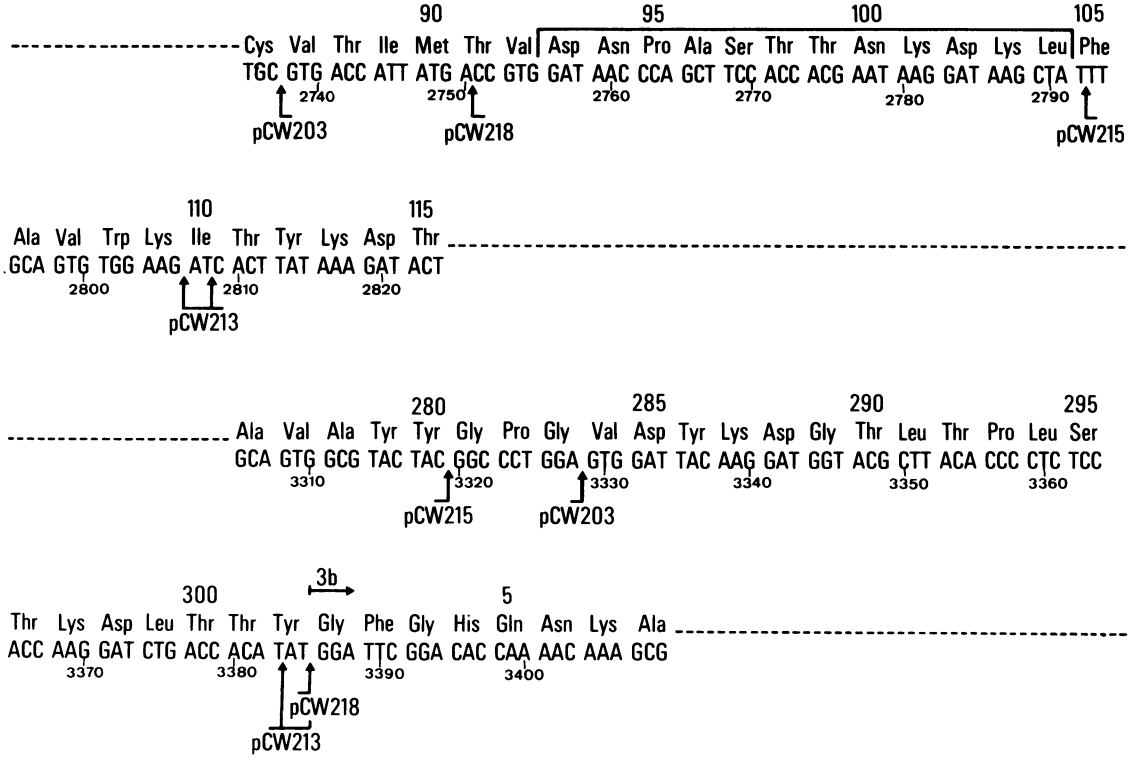

Fig. 2. Location of deletions in the *Bal*31 deleted plasmids. Nucleotide and amino acid sequence of capsid polypeptide VP1 was after Kitamura *et al.* (1981) and Racaniello and Baltimore (1981). The arrows indicate the positions of the deletions in the *Bal*31 deleted plasmids as determined by sequencing of the plasmids' DNA. The amino acid sequence of the chemically synthesized peptide is indicated by the horizontal bar.

and 218, were much smaller than expected, because the sequences downstream from the deletions were read in a different reading frame from the (normal) upstream sequences (the sequences in pCW215 and 218 were read in the second reading frame and those in pCW213 in the third reading frame).

As shown in Table I, the truncated fusion proteins expressed by plasmids pCW213 and pCW215 were immunoprecipitated by the neutralizing monoclonal antibody, C3, while the proteins expressed by the other plasmids did not react with C3. Therefore, VP1 could be deleted from its C-terminal end (pCW213) up to amino acid residue 105 (pCW215) without losing its reactivity with C3, whereas its deletion up to amino acid 90 (pCW218), or further, resulted in total loss of reactivity with C3. These results show that the C3 neutralization epitope is most probably located in the region of VP1 amino acids 90 and 104.

Table I. Properties of *Bal*31 deleted plasmids

Plasmid	Extent of deletion (nucleotides)[a]	Fusion protein	
		Apparent mol. wt.[b] (kd)	Reactivity with C_3[c]
pCW213	576	16.5	+
pCW215	526	23.5	+
pCW218	634	17.0	−
pCW203	590	30.0	−

[a]As determined from DNA sequencing (see Figure 2).
[b]As analysed in GC26 bacteria transformed by the indicated plasmids. [^{35}S]Methionine-labeled bacterial extracts were prepared and immunoprecipitated with αVP1 serum as described (van der Werf *et al.*, 1983a). The immune precipitates were analysed on a 20% SDS-polyacrylamide gel, and the apparent mol. wts. of the fusion proteins were determined using ^{14}C-labeled myosin (200 kd), phosphorylase B (92.5 kd), bovine serum albumin (69 kd), ovalbumin (46 kd), carbonic anhydrase (30 kd) and lysozyme (14.3 kd) as mol. wt. standards.
[c]As determined by immunoprecipitation.

Reactivity of a synthetic peptide with C3

The search for potential antigenic determinants of a protein can be facilitated by a computer search for regions of the protein with a high local hydrophilicity (Hopp and Woods, 1981). This method has proved most useful to identify potential antigenic determinants in some proteins. For the N-terminal half of VP1, the 'antigenicity plot' according to Hopp and Woods (Figure 3) showed five main hydrophilic regions, located at amino acid residues 12–41, 69–76, 93–103, 111–122 and 165–171, respectively. The hydrophilic domain between amino acid residues 93 and 103 is located in the very region of VP1 identified as the C3 neutralization epitope.

To ascertain that this domain of VP1 indeed contained the C3 neutralization epitope, a peptide with the amino acid sequence of VP1 residues 93–104 (see Figure 2) was synthesized chemically and its reactivity with C3 was studied. The synthetic peptide was coupled with glutaraldehyde to keyhole limpet hemocyanin (KLH) as a carrier protein (see Materials and methods), after which the peptide conjugate was labeled with ^{125}I using chloramine T. As the peptide contains no tyrosine residue, labeling will occur only on the carrier protein, preventing alteration of the antigenic properties of the peptide. As shown in Figure 4A, the peptide conjugate was immunoprecipitated by C3 antibodies, and not by another monoclonal antibody, C18, which reacts with capsid polypeptide VP2 but not with VP1 nor with intact virions, and which does not neutralize virus infectivity (Blondel *et al.*, 1983). Figure 4B shows that another, more reactive, preparation of C3 antibodies immunoprecipitated the peptide-KLH

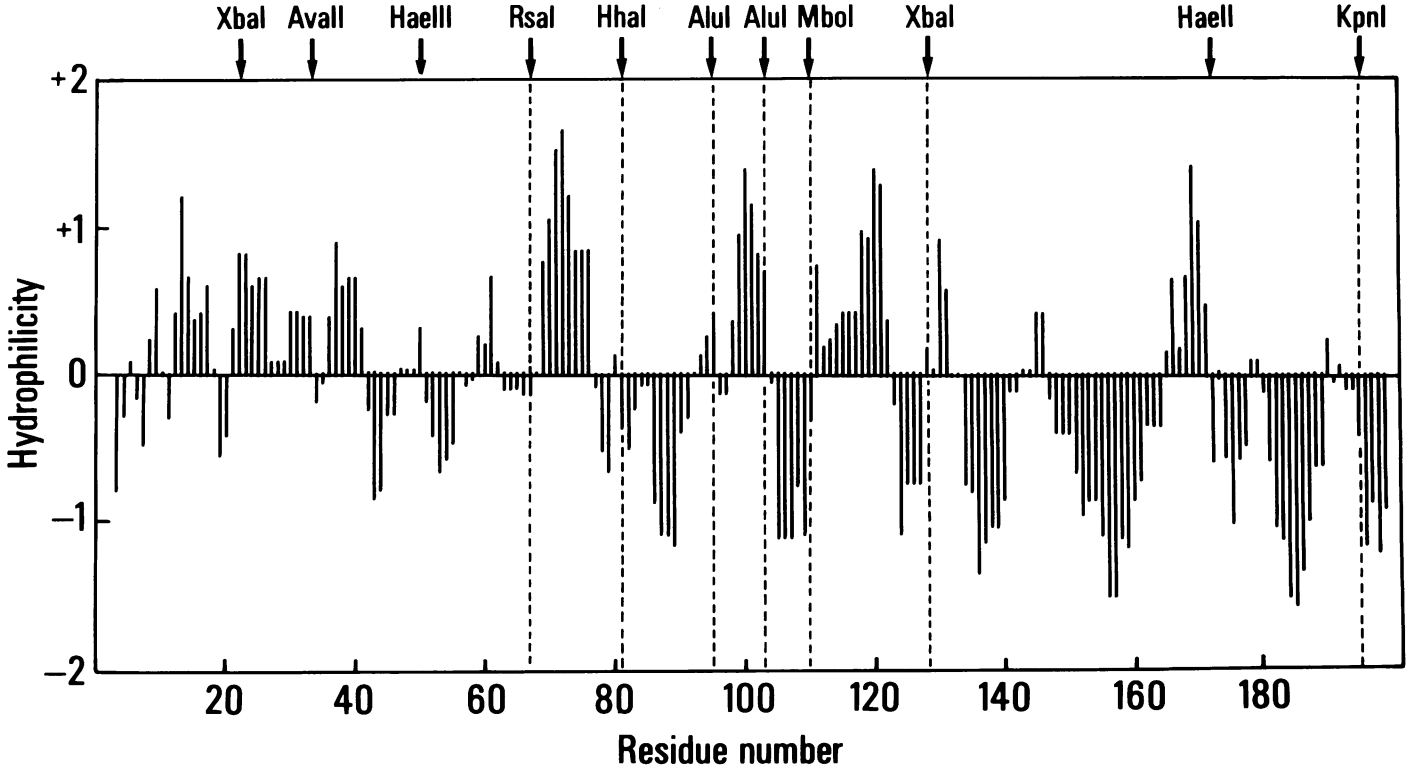

Fig. 3. 'Antigenicity plot' of poliovirus capsid polypeptide VP1. Computer search for hydrophilic domains in the VP1 amino acid sequence was according to Hopp and Woods (1981). Positions of restriction sites used to analyse the *Bal*31 deleted plasmids are indicated on top.

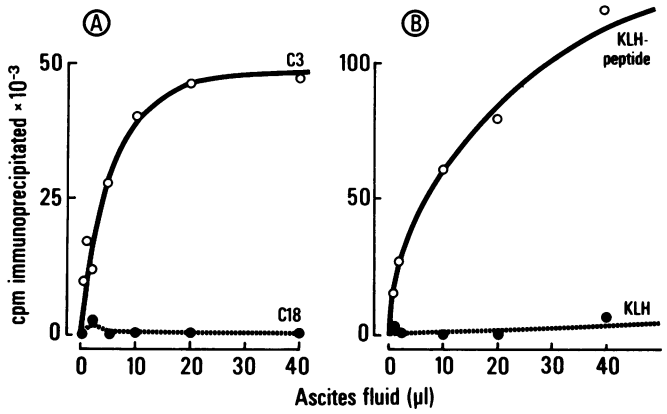

Fig. 4. Reactivity of the peptide conjugate with monoclonal antibodies. The KLH-peptide conjugate or KLH alone were labeled with ^{125}I using chloramine T and immunoprecipitated as indicated. **Panel A**: 200 µl (1.56 x 10^6 c.p.m.) of peptide-coupled KLH at 5 µg/ml (8.5 µg/ml of KLH) were immunoprecipitated using increasing amounts of the neutralizing monoclonal antibody C3 (open circles), or of the non-neutralizing monoclonal antibody, C18 (closed circles). **Panel B**: 200 µl (1.56 x 10^6 c.p.m.) of KLH at 8.5 µg/ml (closed circles) or of peptide conjugate (open circles) were immunoprecipitated using increasing amounts of the neutralizing monoclonal antibody C3. Immune precipitates were collected on protein A-Sepharose and assayed for radioactivity as described (Bruneau *et al.*, 1983). Background values (1.76 x 10^4 c.p.m.) obtained with protein A-Sepharose alone were subtracted. The C3 ascites fluids used in these experiments were derived from two different subclones of the same hybridoma cell line.

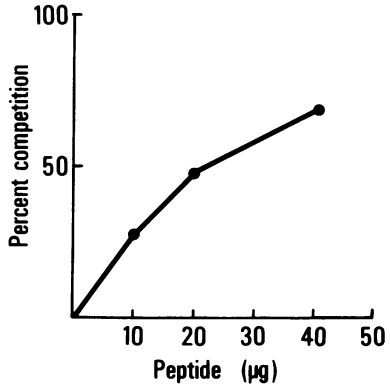

Fig. 5. Neutralization inhibition test. The amount of monoclonal antibody (a 4^{-6} dilution of C3 ascites fluid) just sufficient to neutralize 150 p.f.u./ml of poliovirus was mixed in a total volume of 0.25 ml with the indicated amounts of peptide. After 2 h incubation at 37°C, each tube received 0.25 ml of virus, and incubation was continued for an additional 2 h. The infectious virus remaining in each sample was titrated by plaque assay, and the titer compared with that found after incubation in the absence of monoclonal antibody (166 p.f.u./ml) or in the absence of peptide (25 p.f.u./ml). Figures were computed as percent inhibition of neutralization.

conjugate but failed to immunoprecipitate the carrier protein alone, indicating that the reaction was indeed specific of the peptide moiety. No reactivity of the peptide-KLH conjugate could be detected with polyclonal neutralizing antibodies prepared against the intact virion (results not shown).

The peptide conjugate was also found to react with C3 antibodies, but not with C18 antibodies, in an indirect enzyme linked immunosorbent assay (ELISA) (data not shown). However, even at 25 µg per well, free uncoupled peptide failed to react with C3 in similar ELISA tests. This might be due to different affinities of the free peptide and its high mol. wt. conjugate for the plastic surface of the microtiter plates used in the assay, or for the antibody, or to possible modification of the conformation of the peptide when bound to the plastic.

Free uncoupled peptide that had been labeled with the Bolton-Hunter reagent, could not be immunoprecipitated by C3 antibodies, suggesting that the free peptide in solution has only a limited probability of assuming the correct antigenic configuration needed for interaction with the antibody. However, C3 antibodies mixed with a large excess of free peptide in solution lost the ability to neutralize poliovirus type 1 in a seroneutralization test (Figure 5). This shows that, at the proper molar ratio, the free peptide in solution can be recognized by, and bound to, the C3 antibodies.

Discussion

Antigenic determinants in a protein can be mimicked by short carrier-linked peptides. Antibodies raised against these peptides recognize the corresponding sequential run of amino acids within the native or denatured protein (Sela *et al.*, 1967; Langbeheim *et al.*, 1976; Audibert *et al.*, 1981; Green *et al.*, 1982). This approach has been recently applied to viruses by synthesizing chemical peptides that mimic those antigenic determinants which induce a virus neutralizing antibody response in the animal (Lerner, 1982). Antibodies against such peptides neutralize virus infectivity and can protect against the disease. In the case of foot and mouth disease virus (FMDV), for example, a synthetic peptide representing the sequence of a neutralization epitope of the virion has been shown to induce high titers of neutralizing antibodies in animals (Bittle *et al.*, 1982; Pfaff *et al.*, 1982). However, in the case of poliovirus, localization and identification of neutralization epitopes are still lacking and remain a prerequisite to the development of synthetic peptides as possible 'subunit vaccines'.

As illustrated in this report, the antigenic properties of poliovirus capsid polypeptide VP1, isolated in a native state, could be approached through molecular cloning of poliovirus cDNA sequences and expression in *E. coli*. This was done by fusing the VP1 sequence in-phase into the β-lactamase gene of plasmid pBR327. The resulting recombinant plasmid, pCW119, expressed a VP1-β-lactamase fusion protein that was specifically immunoprecipitated by αVP1 serum and by the neutralizing monoclonal antibody C3 (van der Werf *et al.*, 1983a, 1983b). To identify the location of the C3 neutralization epitope, we generated a series of deletions within the VP1 sequence of pCW119, using nuclease *Bal*31. The fusion proteins expressed by the deleted plasmids were analysed for reactivity with C3 (van der Werf *et al.*, 1983b). The whole C-terminal part of VP1 could be deleted without affecting the immunological reactivity of the protein. Reactivity with C3 was maintained as long as the deletion of the plasmid did not extend further upstream than poliovirus nucleotide 2792 (VP1 amino acid 105). Reactivity with C3 was lost, however, as soon as the deletion reached poliovirus nucleotide 2750 (VP1 amino acid residue 91), or extended further upstream. It follows that the C-terminal end of the stretch of amino acids constituting the C3 neutralization epitope must be located between VP1 amino acids Thr(91) and Leu(104). The alternative hypothesis, namely that the epitope could be located in a more N-terminal part of the protein, but would be conformationally dependent on the presence of the 91 – 104 amino acids region, can be ruled out in view of the antigenic properties of the synthetic peptide with the sequence Asp(93) –

Leu(104), as discussed below.

Accurate mapping of the N-terminal end of the C3 neutralization epitope on poliovirus capsid polypeptide VP1 was not feasible in this type of study, due to the lack of deletions extending in the opposite direction from the N-terminal end of VP1 towards the region of the C3 neutralization epitope. The lack of conveniently located restriction enzyme cleavage sites in the 5'-terminal part of the VP1 cDNA sequence precluded the generation of N-terminal deletions using nuclease *Bal*31, as had been done for the C-terminal deletions. We therefore used another type of approach to localize the C3 neutralizaton epitope.

A computer analysis of the VP1 sequence using the method of Hopp and Woods (1981), revealed a marked hydrophilicity peak between amino acid residues 93 and 103, i.e., in the very region identified above (see Figure 3). As hydrophilic regions are more likely to be exposed at the protein surface and therefore to be antigenically relevant, it was tempting to predict that the neutralization epitope recognized by C3 lay in this region of VP1. That this was indeed the case, was demonstrated by the fact that a chemically synthesized peptide, representing VP1 amino acids Asp(93) to Leu(104), specifically reacted with C3 antibodies, while it did not react with other poliovirus-specific monoclonal, or polyclonal antibodies raised against D- or C-particles.

Reactivity of the peptide with the antibody was demonstrated by ELISA, by immunoprecipitation and by a neutralization inhibition test. In the latter test, >40 μg of peptide (i.e., ~30 nmol) were required to efficiently bind the amount of monoclonal antibody just able to neutralize the infectivity of 42 p.f.u. of virus. As there are 60 copies of the capsid polypeptide VP1 per virion, and ~100 virions per p.f.u., the amount of VP1 in the test was $\sim 4 \times 10^{-10}$ nmol. It follows that the binding of the peptide in solution to the C3 antibody was $\sim 7.5 \times 10^{10}$-fold less efficient, on a strictly molar basis, than that of VP1 in the virion. These figures illustrate well the very low antigenicity of the free peptide in solution.

This very low antigenicity might account for the fact that no reactivity of the peptide conjugate could be detected by immunoprecipitation with polyclonal antibodies raised against native virions, heat-denatured virions or purified capsid polypeptide VP1. Furthermore, antibodies that harbor the same specificity as the C3 neutralizing monoclonal antibody most likely represent a minor fraction of the antibodies present in these polyclonal sera.

Comparison of the VP1 nucleotide sequence of poliovirus type 3 (Minor *et al.*, 1982) and of the attenuated Sabin strain of poliovirus type 1 (Nomoto *et al.*, 1982) with that of the wild-type 1 Mahoney strain (Kitamura *et al.*, 1981; Racaniello and Baltimore, 1981) reveals that the C3 neutralization epitope lies in a highly variable region of VP1. This can be correlated to the fact that the C3 antibodies exhibited a much lower neutralization efficiency for Sabin type 1 virus as compared with Mahoney type 1 virus (van der Werf *et al.*, 1983b). A similar hypervariable region has been noted in the close vicinity of the major FMDV neutralization epitope (Pfaff *et al.*, 1982; Makoff *et al.*, 1982).

Identification of a poliovirus neutralization epitope in the region of VP1 amino acid residues 93 – 104 opens the way towards the use of synthetic peptides as possible poliomyelitis 'subunit vaccines'. It will be of major interest to study the immunogenicity of such peptides in animals.

Materials and methods

Bacterial strains and plasmids

E. coli strain 1106 (803 $r_k^- m_k^-$) (Murray *et al.*, 1976) was used for selection and propagation of recombinant plasmids. The minicell-producing strain GC26 (Bl^-, thr^-, leu^-) was provided by G.Cesareni. Strain 7118 (Messing *et al.*, 1977) was used as a host for phage M13. Plasmid pCW119 has been described (van der Werf *et al.*, 1983b).

Construction of plasmids and recombinant M13 phages

Plasmid DNA or M13 RF DNA molecules were cleaved by restriction endonucleases following the conditions specified by the manufacturers. Digestion of plasmid pCW119 with nuclease *Bal*31 was for 15 min at 30°C in 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.6 M NaCl, 12 mM $CaCl_2$, 12 mM $MgCl_2$, using an enzyme to DNA ratio of 0.12 U per μg. Ligation reactions (Maniatis *et al.*, 1982) were performed using 1 U of T4 DNA ligase per μg of DNA. For sequence determination, plasmid DNAs were digested with *Eco*RI and *Pst*I and ligated to M13mp8 RF DNA that had been digested with *Eco*RI and *Pst*I and treated for 30 min at 68°C with bacterial alkaline phosphatase (0.02 U per μg DNA). Recombinant phages were selected by dot blot hybridization of phage DNA using as a probe a *Pst*I-*Pst*I fragment (poliovirus nucleotides 2243 – 3417) ^{32}P-labeled by nick-translation.

DNA sequence analysis

DNA sequence determination on plasmid pCW203 was according to Guo and Wu (1982), starting with 1.3 pmol of DNA. The DNA was linearized with *Xba*I, treated with exonuclease III (40 U/pmol DNA) for 29 min at 23°C, then digested with *Pst*I before use as a template in the four sequencing reactions with dideoxynucleotide terminators. Other sequence determinations were done on M13mp8 recombinant phage DNA according to Sanger *et al.* (1977).

Synthesis of the peptide

The peptide was synthesized using the solid phase method (Marglin and Merrifield, 1970). The free peptide was purified by gel filtration on Biogel P4, then by ion-exchange chromatography on CM32 cellulose, followed by h.p.l.c., and its amino acid composition was determined.

Coupling of the peptide to carrier protein

50 μg of peptide were labeled with 25 μCi of Bolton-Hunter reagent (Amersham, 2000 Ci/mmol) in 0.1 M borate buffer, pH 8.5, according to Bolton and Hunter (1973). After 20 min incubation at 4°C, the reaction was stopped by addition of 0.2 M glycine in 0.1 M borate buffer, and the peptide was purified by gel filtration on a Sephadex G10 column. A mixture of 2 mg of unlabeled peptide and of 3 μg of labeled peptide was added with 2.25 mg of KLH (Calbiochem). Coupling with glutaraldehyde was as described by Boquet *et al.* (1982). Coupling efficiency was 65%, as judged from recovery of radioactivity after extensive dialysis of the mixture against phosphate buffered saline, pH 7.0.

Labeling of the peptide conjugate

A sample of 44 μg of peptide-coupled KLH was labeled with 1 mCi of ^{125}I (Amersham, 100 mCi/ml) using chloramine T according to Hunter and Greenwood (1962), then purified by gel filtration on a Sephadex G75 column in 25 mM Tris-HCl, pH 8.6, 0.7 mM $Na_2H\,PO_4$, 137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (TBS). The labeled conjugate was adjusted with TBS to a concentration of 5 μg/ml of peptide prior to immunoprecipitation.

Immunoprecipitation

Conditions for immunoprecipitation using protein A-Sepharose were as described previously (van der Werf *et al.*, 1983a) using either a non-immune rabbit serum, anti-VP_1 (αVP_1) rabbit immune serum (Blondel *et al.*, 1982), or anti-poliovirus monoclonal antibodies C3 or C18 (Blondel *et al.*, 1983) under the form of ascites fluids. The immune precipitates were either directly assayed for radioactivity, or analysed by SDS-PAGE and autoradiography as described (Bruneau *et al.*, 1983).

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