

Structural Factors Modulate the Activity of Antigenic Poliovirus Sequences Expressed on Hybrid Hepatitis B Surface Antigen Particles

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We have studied the functional expression of antigenic poliovirus fragments carried by various hybrid hepatitis B surface antigen (HBsAg) particles. Several constructions were made by using two different insertion sites in the HBsAg molecule (amino acid positions 50 and 113) and two different sequences, one derived from poliovirus type 1 (PV-1) and the other from PV-2. The inserted fragments each encompassed residues 93 to 103 of the capsid protein VP1, a segment which includes the linear part of the neutralization antigenic site 1 of the poliovirus. The antigenicity and immunogenicity of the hybrid particles were evaluated and compared in terms of poliovirus neutralization. A high level of antigenic and immunogenic activity of the PV-1 fragment was obtained by insertion at position 113 but not at position 50 of HBsAg. However, a cooperative effect was observed when two PV-1 fragments were inserted at both positions of the same HBsAg molecule. Antibodies elicited by the PV-2 fragment inserted at amino acid position 113 did not bind or neutralize the corresponding poliovirus strain. They did, however, bind a chimeric poliovirus in which the homologous antigenic fragment of PV-1 had been replaced by that of PV-2. The only virions that were neutralized by these antibodies were certain mutants carrying amino acid substitutions within the PV-2 fragment. These results show that position, constraints from the carrier protein, and nature of the inserted sequences are critically important in favoring or limiting the expression of antigenic fragments as viral neutralization immunogens.

Peptide fragments used as antigens and immunogens are valuable tools for analyzing the antigenic structures of proteins and microorganisms and have raised new possibilities for creating vaccines (50). These antigenic fragments can be obtained as chemically synthesized peptides (free or carrier bound) (43, 52) or can be produced in bacteria as recombinant, globular, hybrid proteins (54). When injected into animals, such immunogens usually induce anti-peptide or anti-protein antibodies but rarely elicit antibodies highly active against the target microorganism, partly because the configurations that peptide fragments may adopt differ from that in the original microorganism (5, 30).

Recently, recombinant DNA methods have made it possible to present antigenic peptide fragments at the surface of virus or virus-like particles. This approach can be used to stabilize concentrated antigens on highly immunogenic particulate structures (for a review, see reference 26). It has been successfully used to express antigenic fragments at the surface of noninfectious virus-like yeast particles (2), hepatitis B surface (HBsAg) and core (HBcAg) antigen subviral particles (9, 14, 32, 41, 45, 48, 51), and empty tobacco mosaic virus capsids (23). Moreover, antigenic, chimeric, infectious particles of bacteriophage ϕ 1 (44) and of poliovirus (10, 19, 29, 35, 36) can express foreign epitopes. Antigenic sequences exposed on hybrid particles have been found that induce neutralizing antibodies in animals against poliovirus (14, 23), human immunodeficiency virus (19, 32), and *Plasmodium falciparum* (41). Immunization with hybrid HBcAg particles carrying foot-and-mouth disease virus antigens protects guinea pigs against virus challenge (9). These systems are of value for a wide range of immunological purposes; in particular, they allow the antigenic and immuno-

genic activities of viral peptide fragments to be studied in the context of heterologous, viral, antigenic structures.

Mammalian cells transformed by plasmid expression vectors carrying the S gene of the hepatitis B virus (HBV) synthesize and secrete multimeric lipoprotein particles. These HBsAg particles resemble the empty HBV envelopes found in sera of infected patients (46). We have previously shown that in-phase insertions in one or both of the S-gene regions encoding the two hydrophilic domains of the major HBsAg protein did not prevent particle synthesis and secretion (13). One of the inserted sequences encoded an antigenic poliovirus fragment. The poliovirus occurs as three independent serotypes (PV-1, PV-2, and PV-3), which display roughly the same antigenic structure (33, 35, 37, 53). N-AgI, one of the four neutralization-antigenic sites of the poliovirus, mainly consists of a peptide fragment of the VP1 capsid protein; this sequence is located on a prominent loop of the viral shell (24). We have shown that when inserted in one of the hydrophilic regions of the HBsAg molecule, a PV-1 fragment, which included the linear part of N-AgI, induced poliovirus-neutralizing antibodies in animals (14-16).

In this work we have analyzed how structural factors influence the antigenic and immunogenic activity of the N-AgI fragment when the fragment is borne by various HBsAg poliovirus particles. Several constructions were made with two different insertion sites in the HBsAg molecule (one in each of the two major hydrophilic regions) and two homologous fragments, one derived from PV-1 and the other from PV-2. The antigenicities and immunogenicities of these hybrid particles were evaluated and compared in terms of poliovirus neutralization. The results not only revealed critical aspects of the structural relationships among carrier protein, inserted sequences, and target virus, but also pro-

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vided a basis for manipulating the activities of antigenic fragments presented on hybrid particulate antigens.

MATERIALS AND METHODS

Recombinant plasmids. To construct the plasmids pPAP and pPAL, synthetic *Bam*HI fragments were ligated to *Bam*HI-cleaved pLAS (Fig. 1A). For plasmids pBAP and pDAP a synthetic DNA fragment was ligated to *Bal*I-treated pLAS and pPAP, respectively. Transformants were screened for recombinant plasmids in situ hybridization using ³²P-labeled oligonucleotides and by polyacrylamide gel electrophoresis (PAGE) after *Bst*NI digestion. The inserted region of recombinant plasmids was sequenced according to the method of Sanger et al. (42).

Cell culture, detection, and purification of hybrid proteins. Mouse L cells were grown in Dulbecco's modified minimum essential medium supplemented with 10% newborn calf serum and 50 µg of gentamicin per ml. Each recombinant plasmid was cotransfected with the plasmid pW, encoding G418 resistance (11), by using the phosphate calcium technique as previously described (16). Colonies appearing after transfection were grown to confluence, and supernatants were tested for HBsAg with the Enzygnost HBsAg micro-immunoassay (Behring). For the purification of antigens, the supernatants of HBsAg-producing cellular clones were clarified, and a saturated solution of ammonium sulfate, pH 7.5, was added to a final concentration of 50%. The precipitate was collected by centrifugation, dissolved in TNE buffer (10 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 1 mM EDTA), and dialyzed against the same buffer. CsCl was added to a concentration of 0.3 g/ml, and centrifugation was carried out at 4°C for 18 h at 50,000 rpm in a Beckman VTi50 rotor. Fractions containing HBsAg were collected, pooled, and recentrifuged in two successive four-step CsCl gradients from 1.3 to 1.1 g/cm³ with a Beckman SW41 rotor at 40,000 rpm for 16 h at 4°C. Finally, HBsAg-positive fractions were dialyzed against TNE buffer. The protein concentrations were measured by the Lowry micromethod and by sodium dodecyl sulfate (SDS)-PAGE, with human serum albumin and HBsAg as references. Particles were visualized by electron microscopy after being stained with 2% phosphotungstic acid.

Trypsin treatment. Purified antigen (1.3 µg) was digested in 20 µl of phosphate-buffered saline (PBS) by 2 µg of trypsin (Sigma) for 2 h at 37°C in the presence or absence of 1% β-mercaptoethanol. The polypeptides from treated antigens were analyzed by SDS-PAGE and silver staining.

Cell labeling and immunoprecipitation. Antigen-producing cells were labeled with [³⁵S]methionine as previously described (13). In immunoprecipitation experiments, rabbit antiserum to human HBsAg (1:100 dilution), rabbit antiserum to poliovirus (1:25), or ascites fluid (1:25) containing the monoclonal antibody (MAB) C3 was added to clarified supernatants of cells and the mixture was kept at 4°C for 16 h. Immune complexes were precipitated with protein A-Sepharose CL4B (Pharmacia), washed with RIPA buffer (10 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate), and run on SDS-PAGE, as previously described (16). Alternatively, the first incubation was performed in the presence of 1% Triton X-100–0.1% SDS–1% sodium deoxycholate.

Enzyme-linked immunosorbent assay. Microtitration plates were coated with 100 µl of a 1-µg/ml antigen or peptide solution in PBS for 16 h at 4°C and washed with 0.5% Tween 20 in PBS. The plates were then saturated with the same

solution containing 3% bovine serum albumin (BSA) and incubated with various dilutions of MABs or rabbit sera in PBS–Tween 20–1% BSA for 2 h at 37°C. Peroxidase-labeled goat anti-mouse or anti-rabbit immunoglobulin G was used to detect bound antibodies. Alternatively, a first-step reaction was performed by using peroxidase-labeled rabbit anti-human HBsAg (Behring). The two antibody sandwich immunoassays were performed by using the MAB C3-containing ascites fluid (1:500 dilution) for coating. Fixed antigens were revealed by using the peroxidase anti-HBsAg antibodies.

Immunization. New Zealand White rabbits were immunized with 20 µg of antigen by a 1-ml subcutaneous injection of a 50% emulsion with Freund complete adjuvant and again 2 weeks later with Freund incomplete adjuvant (20 µg of antigen which, with one insertion per molecule, contains roughly 1 µg of heterologous peptide). Serum samples were collected every 2 weeks until 56 days after the first injection. On day 84, animals received an intravenous injection of 10 µg of antigen in PBS and were bled 12 days later.

Eight-week-old BALB/c mice were immunized with 5 µg of purified antigen in 200 µl of a 50% emulsion of complete Freund adjuvant, and the injection was repeated on days 14 and 70 with incomplete Freund adjuvant. The antigen solution was injected in the hind footpad (50 µl) and intraperitoneally (150 µl).

Poliovirus immunoprecipitation. Poliovirus was labeled with [³⁵S]methionine and purified as previously described (4). Poliovirus (5,000 cpm) was incubated with 10 µl of rabbit or mouse serum in 100 µl of PBS–2% BSA. After 2 h at room temperature the immune complexes were precipitated with 50 µl of a 10% solution of inactivated *Staphylococcus aureus* (Cowan 1 strain), and incubation was continued further for 30 min on a rotary shaker. After centrifugation at 12,000 × g for 10 min, the supernatants were collected and counted. The percent values of immunoprecipitated radioactivity were calculated by using the immunoprecipitation values of the [³⁵S]methionine-labeled poliovirus with MAB C3 as 100%.

Alternatively, immunoprecipitated proteins were analyzed by SDS-PAGE. Infectious [³⁵S]methionine-labeled poliovirus was diluted in PBS–3% BSA or denatured in 5% SDS–2% β-mercaptoethanol for 10 min at 95°C and then diluted 20-fold in RIPA buffer. Immunoprecipitation was performed with rabbit sera at a 1:50 dilution and protein A-Sepharose, as described above for the HBsAg product.

Poliovirus neutralization. The titer of neutralizing antibodies in each serum sample was measured by a standard plaque reduction assay on Vero cells, with an input of 100 PFU of poliovirus. The reciprocals of the dilutions of serum samples giving 50% plaque reduction were computed from the regression curves of mean values obtained in at least two independent experiments. Alternatively, an infectivity inhibition assay was performed by using 10 poliovirus 50% tissue culture infectious doses.

Anti-HBsAg antibodies. Anti-HBsAg titers were determined with the AUSAB radioimmunoassay (Abbott Laboratories) according to the semiquantitative method recommended by the manufacturer.

RESULTS

Construction of recombinant plasmids. In the pLAS plasmid, the S gene, encoding the 226-amino-acid major envelope protein of HBsAg, is expressed from the simian virus 40 early promoter. Two different 39-bp oligonucleotides have been inserted in the unique *Bam*HI site of pLAS, yielding

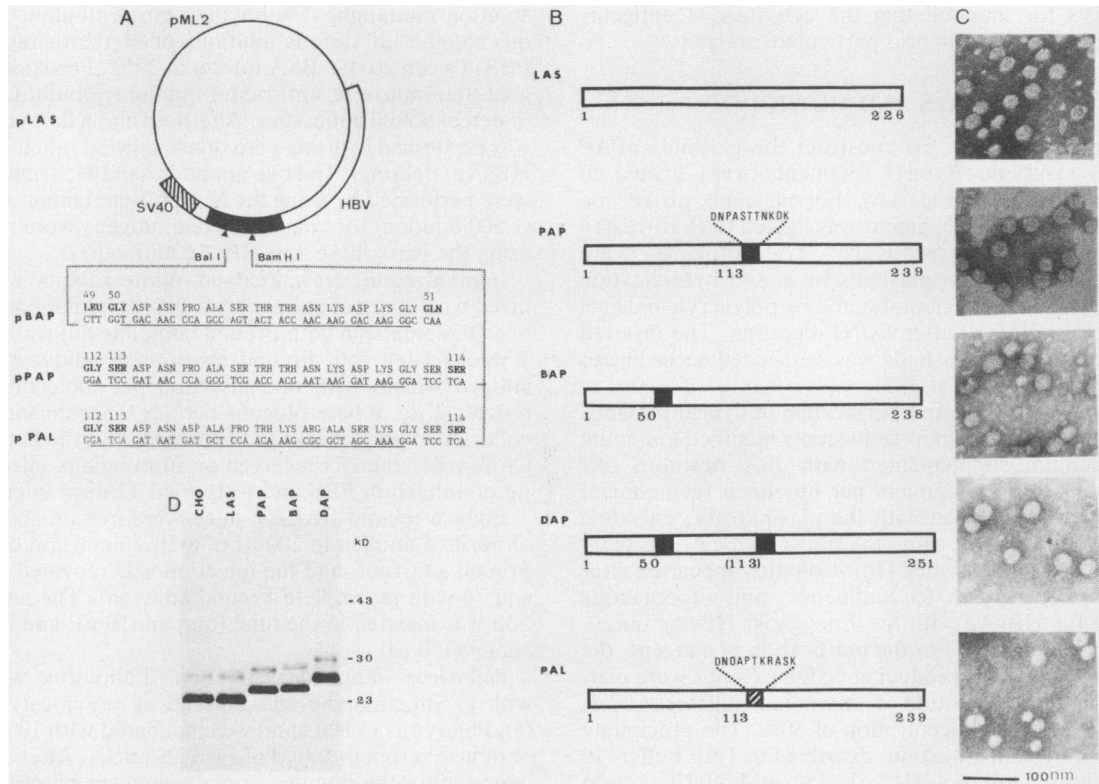


FIG. 1. Characterization of hybrid HBspolioAg particles. (A) Plasmid pLAS carries the simian virus 40 early promoter (hatched box) and the fragment of HBV genome (open box) including the S gene (solid box), as previously described (16). The *Bal*I or *Bam*HI restriction site in the S gene was used to insert different synthetic DNA fragments (underlined) in the indicated plasmids. Nucleotide and corresponding amino acid sequences inserted in the S gene are given. Numbers refer to amino acid residues in unmodified HBsAg. (B) Schematic representation of HBspolioAg polypeptides encoded by the corresponding plasmids, showing the position and number of poliovirus fragments in the HBsAg molecule. Solid and hatched boxes refer to serotype 1 and 2 sequences, respectively. (C) Electron microscopy of purified HBsAg and various HBspolioAg particles represented in panel B. (D) SDS-PAGE of proteins from purified particles, revealed by silver staining. CHO refers to HBsAg particles purified from CHO cells and used in the GenHevac B vaccine (Pasteur Vaccins). Prestained molecular size standards in kilodaltons (kD) are indicated (GIBCO; Bethesda Research Laboratories).

the plasmids pPAP (14) and pPAL. This led to duplication of amino acids 112 and 113 of HBsAg and insertion of an 11-amino-acid sequence between the duplicated residues (Fig. 1A). The sequences inserted into pPAP and pPAL encode amino acid residues 93 to 103 of capsid protein VP1 of PV-1 Mahoney strain and PV-2 Lansing strain, respectively (Fig. 1B). These sequences, which differ from each other at 6 of the 11 residues, are part of poliovirus N-AgI. The PV-1 fragment carries a neutralization epitope (C3) recognized by MAb C3 (4, 49). The PV-2 sequence reacts with two MABs, I10 (12) and HO2 (a generous gift from A. Osterhaus, described as 10C9E6 in reference 47), when exposed at the surface of the Lansing strain or when carried by a type 1/type 2 chimeric poliovirus in which the N-AgI fragment of PV-1 was substituted by that of the PV-2 virus (29).

The plasmids pBAP and pDAP were constructed by inserting a 36-bp synthetic DNA fragment at the *Bal*I site of the S gene carried by the plasmids pLAS and pPAP, respectively (Fig. 1A). The insertion encodes the 11-amino-acid fragment which carries the C3 epitope of PV-1 between the duplicated amino acid residue 50 (Gly) of HBsAg. Therefore, the plasmid pDAP encodes a hybrid protein which bears two copies of the PV-1 fragment, one at amino acid position 50 and the other at amino acid position 113 of HBsAg (Fig. 1B).

Synthesis and characterization of hybrid HBsAg particles.

Each plasmid was mixed with plasmid pW (11) encoding G418 resistance and cotransfected into mouse L cells as described above. G418-resistant transfectants were screened for HBsAg expression by enzyme immunoassay, and positive clones were isolated. Those clones producing the highest levels of antigen were selected for antigen characterization and purification. The HBsAg-related products were purified on successive CsCl density gradients. All hybrid particles banded between 1.20 and 1.22 g/cm³, a density similar to that of HBsAg particles from pLAS and from sera of HBV-infected humans. Direct visualization by using electron microscopy showed that the hybrid HBsAg molecules were assembled into particles roughly similar to HBsAg from human serum (Fig. 1C). When polypeptides of purified antigens were subjected to PAGE, two major bands appeared in the 23- to 30-kDa range, corresponding to the nonglycosylated and glycosylated forms of the protein, respectively (Fig. 1D). The differences among the apparent molecular sizes of the various HBsAg proteins lacking or carrying insertions are in good agreement with the lengths of the amino acid sequences encoded by the inserts. Moreover, extensive silver staining of polyacrylamide gels showed, for each construct, two minor, higher bands with apparent molecular masses of around 40 and 50 kDa. These may be unreduced dimers of glycosylated and nonglycosylated poly-

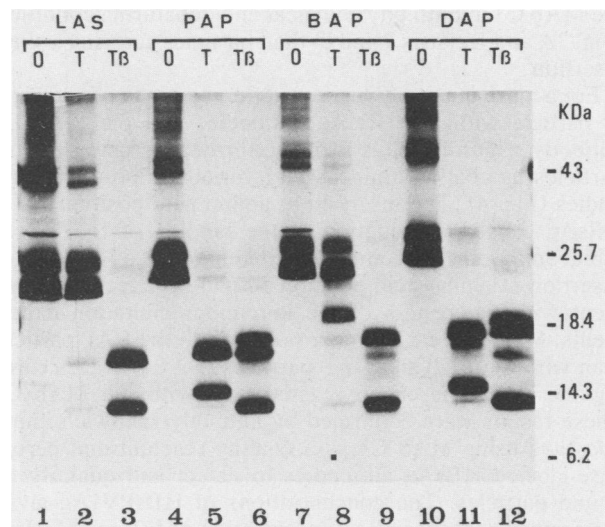


FIG. 2. Effects of trypsin treatment on polypeptides in HBsAg and HBsPV1Ag particles. SDS-PAGE of proteins from purified HBsAg and HBsPV1Ag before and after treatment with trypsin. 0, Nontreated controls; T and T β , trypsin treatment under nonreducing and reducing conditions, respectively. Prestained molecular size standards (GIBCO; BRL) are indicated on the right. The dot indicates the position of a minor, 6.4-kDa band appearing after trypsin digestion of BAP product.

peptides, as described by Mishiro et al. (34) (Fig. 2, lanes 1, 4, 7, and 10).

Trypsin sensitivity of HBsAg particles carrying the PV-1 fragment. In an attempt to establish whether the inserted poliovirus sequence was exposed at the surface of the particles and to examine whether it had created conformational changes in the envelope protein, we studied the protease sensitivities of HBsAg particles carrying the PV-1 fragment (HBsPV1Ag) (Fig. 2). Unmodified HBsAg particles are highly resistant to trypsin under nonreducing conditions (Fig. 2, lane 2). In the presence of a reducing agent, HBsAg is cleaved exclusively at Arg-122 (39) (Fig. 2, lane 3).

When HBsPV1Ag PAP particles (insertion at amino acid position 113 of HBsAg) and DAP particles (insertions at amino acid positions 50 and 113) were treated with trypsin under nonreducing conditions, almost all molecules were cleaved at a unique site (or several closely spaced sites) (Fig. 2, lanes 5 and 11). The sizes of the fragments are compatible with a cleavage within the sequence inserted at amino acid position 113 of HBsAg, as previously described for PAP particles (14). Under the same conditions, the HBsPV1Ag BAP particles (insertion at amino acid position 50 of HBsAg) were more resistant to trypsin; nevertheless, BAP particles were partially cleaved into fragments of 19.5 and 6.4 kDa (Fig. 2, lane 8). This suggests that the trypsin site(s) is at the Lys residue(s) in PV-1 rather than within the adjacent Arg residues at amino acid position 24 or 73 of the HBsAg molecule. Therefore, the sensitive Lys residue(s) of the poliovirus might be exposed at the surface of these hybrid particles. However, cleavage at Lys-160 or Arg-169 of HBsAg, which could yield fragments of the same size, cannot be excluded, although it is less likely. Moreover, that a proportion of molecules was sensitive to trypsin may have resulted from unintended particle disruption.

HBsPV1Ag was treated with trypsin in the presence of a reducing agent. Two cleavage products were produced. The polypeptides carried by HBsPV1Ag particles were com-

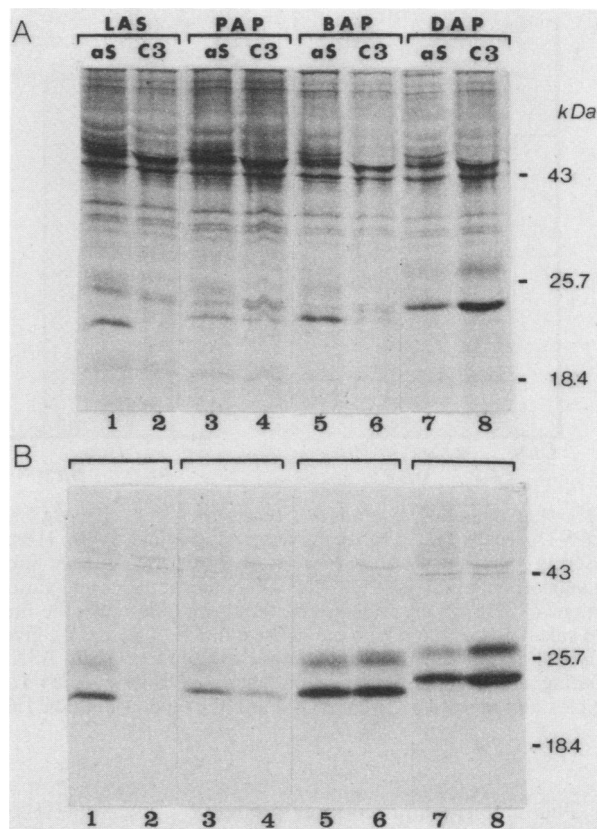


FIG. 3. Immunoprecipitation of HBsAg and HBsPV1Ag particles. [35 S]methionine-labeled proteins from cell culture supernatants were immunoprecipitated with antiserum to HBsAg (α S) or the anti-poliovirus MAb C3 and analyzed by SDS-PAGE. The autoradiograms of two different gels are shown; the antigen-antibody incubation was performed in physiological conditions (A) or in the presence of detergents (B). Prestained molecular size standards (GIBCO; BRL) are indicated on the right.

pletely cleaved, probably at residue 122 of the HBsAg molecule (Fig. 2, lanes 6, 9, and 12), as were the unmodified HBsAg particles (Fig. 2, lane 3). Patterns compatible with a partial cleavage at poliovirus Lys residues inserted at amino acid position 113 of HBsAg molecule (Fig. 2, lanes 6 and 12) were also detected. No cleavage was detected at Lys residues of the PV-1 fragment inserted at amino acid position 50 of HBsAg in BAP or DAP particles (Fig. 2, lanes 9 and 12). It might be that cleavage at HBsAg Arg-122 induces a rearrangement in the polypeptide structure which prevents trypsin from reaching poliovirus residues inserted at position 50 of HBsAg or that simultaneous cleavage leads to less-stable and therefore undetectable fragments.

None of the other potential cleavage sites of HBsAg were accessible to trypsin in the hybrid particles, suggesting that introduction of the PV-1 peptides does not significantly alter the overall conformation or stability of HBsAg, despite increasing the length of the polypeptide chain by 10%.

Antigenicity of HBsAg particles carrying the PV-1 fragment. The reactivity of the hybrid particles with anti-HBsAg antibodies and the anti-poliovirus MAb C3 was tested by SDS-PAGE analysis of proteins labeled *in vivo* and immunoprecipitated from the cell culture supernatants of the producing clones (Fig. 3). All particles reacted with a rabbit anti-HBsAg serum. However, only the HBsPV1Ag particles were immunoprecipitated by the poliovirus-specific MAb

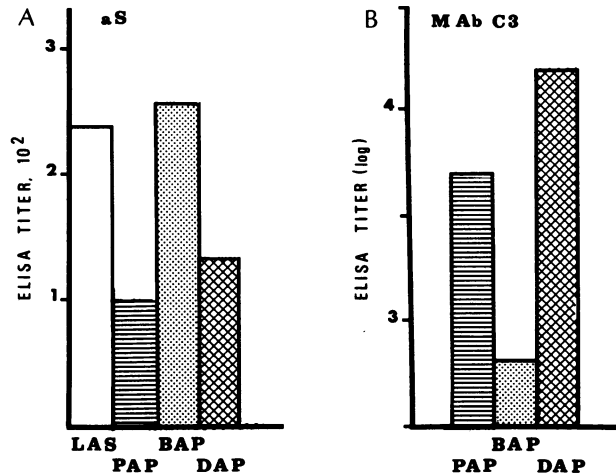


FIG. 4. Comparative antigen reactivities of HBsAg and HBsPV1Ag particles. The reactivities of particles with HBsAg antiserum (aS) and MAb C3 were analyzed by enzyme-linked immunosorbent assay with particles as coating antigens and various dilutions of sera, as described in Materials and Methods; the titers were calculated as the reciprocal of the dilution (\log_{10} for B), giving an optical density equal to three times the basic level (with BSA as a coating antigen) (A) and twice the optical density given by LAS particles and the MAb C3-containing ascites fluid at dilution 1:640 (B).

C3. The material immunoprecipitated by the anti-HBsAg and the MAb C3 was compared for each type of particle. When the antigen-antibody incubation was performed in physiological conditions (cell culture medium), the poliovirus epitope inserted at amino acid position 50 seemed to be poorly recognized by the MAb C3 (Fig. 3A, lanes 5 and 6). Denaturing conditions (RIPA detergents) strongly increased the binding (Fig. 3B, lanes 5 and 6). DAP particles carrying the double insertion bound significantly greater amounts of

the MAb C3 in both physiological and denaturing conditions (Fig. 3A and B, lanes 7 and 8) than particles carrying a single insertion.

For a more quantitative view of the reactivity of each kind of particle with the HBsAg antibodies and the MAb C3, antibody capture assays were performed by using purified particles as coating antigens and various dilutions of antibodies (Fig. 4). The insertion at amino acid position 113 of HBsAg caused a reduction in the binding of the HBsAg rabbit antibodies, as compared with unmodified HBsAg (14). Insertion at amino acid position 50 had no effect. In agreement with the results of the immunoprecipitation experiments, MAb C3 reacted more poorly with the BAP particles than with the PAP and DAP particles. DAP particles carrying the double insertion reacted best with the MAb C3. These results were confirmed by antibody sandwich immunoassays using MAb C3 as a coating reactant and peroxidase-labeled HBsAg antibodies to detect and quantify the bound particles. The concentrations of HBsPV1Ag giving the same absorbance as a concentration of 5.2 μg of HBsAg LAS particles per ml were 0.15, 2.00, and 0.06 $\mu\text{g}/\text{ml}$ for PAP, BAP, and DAP particles, respectively. The reactivity of DAP particles with the MAb C3 is higher than could be expected from the reactivities of PAP and BAP particles added together, indicating a synergism between the two insertions leading to better recognition of the MAb C3.

Immunogenicity of particles carrying the PV-1 fragment. To evaluate and compare the immunogenicities of each particle, four rabbits and six BALB/c mice were immunized with highly purified preparations of each type of antigen.

High levels of HBsAg antibodies were detected in all immunized rabbits (Fig. 5); the titers were stable as a function of time, and no major differences were detected among the serum samples of individual rabbits (not shown). Despite the lower reactivities of PAP and DAP particles with HBsAg antibodies when compared with LAS and BAP particles, there was no significant difference in their abilities to elicit antibodies in these animals. This appears to contra-

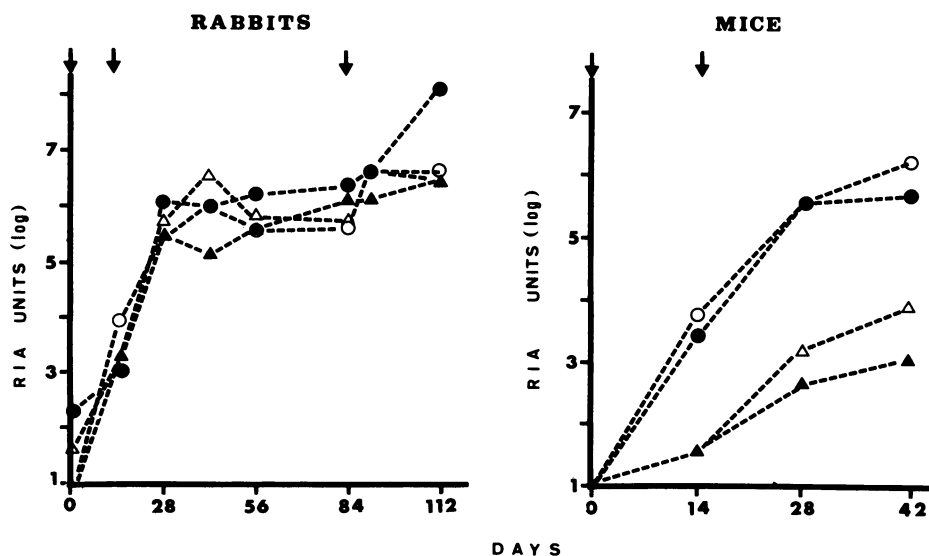


FIG. 5. Anti-HBsAg response of mice and rabbits to HBsPV1Ag particles. Rabbits and mice were immunized at the indicated times (arrows) with the indicated types of particle. Pools of sera from each group of animals were analyzed for HBsAg antibodies with a radioimmunoassay (RIA) (AUSAB; Abbott Laboratories). Titers are expressed as \log_{10} . Symbols: O, LAS particles; ●, BAP particles; Δ, PAP particles; ▲, DAP particles.

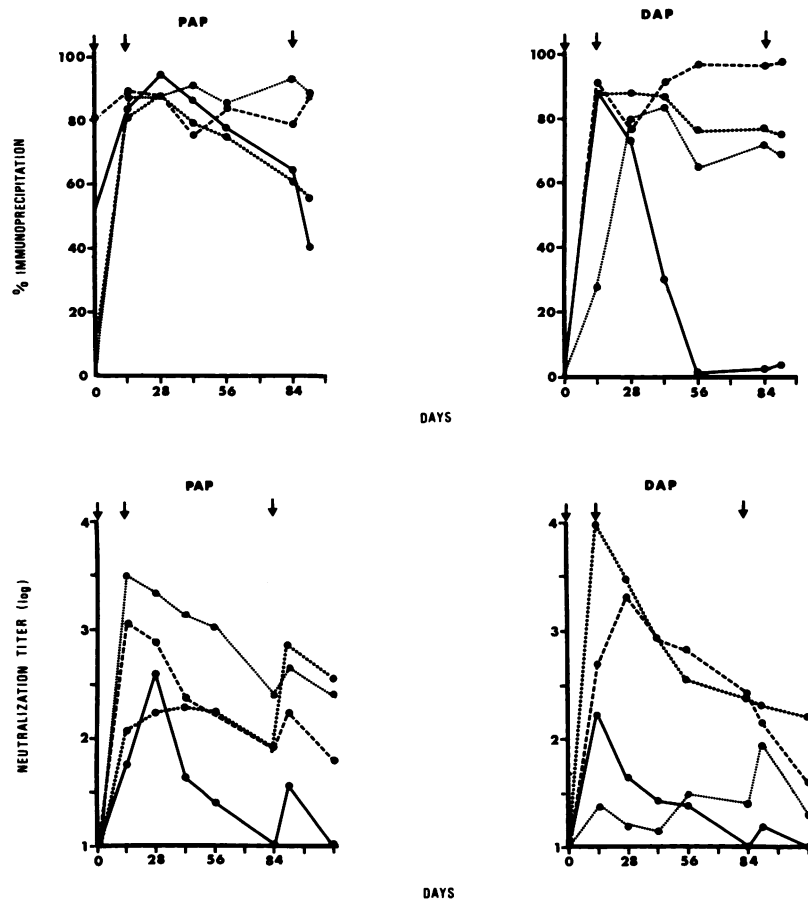


FIG. 6. Anti-PV-1 response of rabbits to HBsPV1Ag particles. Four rabbits were immunized with both HBsAg and HBsPV1Ag particles at the indicated times (arrows). Individual serum samples were collected and analyzed for poliovirus antibodies. Antibody levels were recorded as percentages of immunoprecipitated [35 S]methionine-labeled poliovirus. The neutralizing-antibody titer was measured by a standard plaque reduction assay and expressed as the reciprocal of the serum dilution (\log_{10}) giving 50% plaque reduction. Each type of line corresponds to the same rabbit in immunoprecipitation and neutralization experiments.

dict our previous results indicating a low anti-HBsAg response of rabbits immunized with PAP particles (16). The high degree of particle purity, due to the new isolation procedure used in this study, might explain both this discrepancy and the unexpected fact that five of eight rabbits showed a steep anti-poliovirus response just after the first injection (Fig. 6). However, consistent with the antigenicity of hybrid particles, the anti-HBsAg response was lower in mice immunized with PAP or DAP particles than in mice immunized with LAS or BAP particles (Fig. 5). That the immune response of mice and rabbits was not the same may be due to the differences in species or in the injection routes.

Despite the high level of response to HBsAg in both species (Fig. 5), the BAP particles carrying the C3 epitope at position 50 of HBsAg did not induce detectable levels of poliovirus antibodies (Fig. 7, data not shown for rabbits). The corresponding immune sera did not immunoprecipitate the infectious Mahoney strain of PV-1 and did not neutralize the virion. Moreover, these sera were unable to immunoprecipitate denatured poliovirus capsid protein VP1, and they reacted poorly with a synthetic peptide carrying a trimer of the amino acid 93 to 103 fragment of VP1 in antigen capture assays (Fig. 7).

In contrast, PAP and DAP particles induced poliovirus antibodies in both rabbits and mice. All sera from the rabbits immunized with these particles contained immunoprecipitat-

ing and neutralizing poliovirus antibodies (Fig. 6). The highest level of response was obtained 2 weeks after the first or the second injection. The level of neutralizing antibodies subsequently decreased with time. Following the third intravenous injection, a slight but consistent anamnestic response was observed in six of the eight animals. The neutralizing-antibody titers were heterogeneous and in some cases were very high; two of four rabbits in each group showed a neutralizing titer of more than 3 \log_{10} units (Fig. 6). Despite the fact that the serum of one rabbit immunized with DAP particles showed the highest neutralizing titer, the heterogeneity of the response did not allow meaningful comparison of the poliovirus-specific immunogenicity of PAP and DAP particles. Pooled sera from groups of BALB/c mice immunized with PAP and DAP particles also showed immunoprecipitating and neutralizing poliovirus antibodies (Fig. 7) but at a lower level than rabbit sera. Poor responses to poliovirus-specific antigens have already been described for mice which received PAP particles (14). However, the poliovirus-neutralizing titer in mice immunized with DAP particles was twice as high as in those immunized with PAP particles.

Although BAP particles did not induce poliovirus antibodies, these particles could prime animals for a subsequent challenge with an active poliovirus epitope. All groups of mice were given a third injection of poliovirus-immunogenic

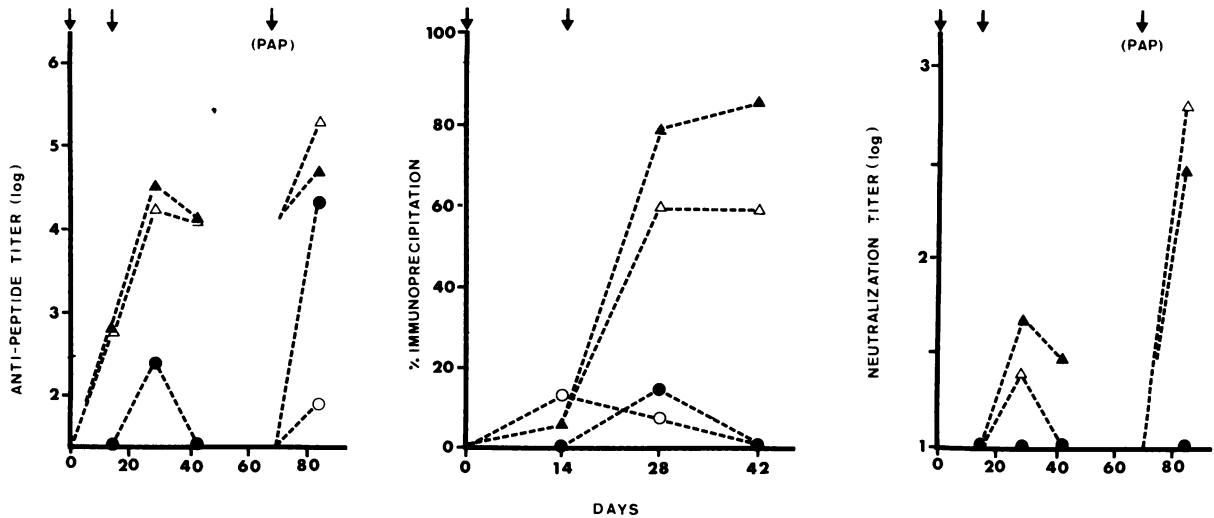


FIG. 7. Anti-PV-1 response of mice to HBSPV1Ag particles. Six mice were immunized twice at the indicated times (arrows) with the indicated type of particles. All mice received a third injection of PAP HBSPV1Ag particles. Serum samples were collected, pooled, and analyzed for anti-poliovirus immunoprecipitating and neutralizing antibodies, as described in the legend to Fig. 6. Anti-peptide titers of sera were determined by enzyme-linked immunosorbent assay, as described in Materials and Methods, by using a synthetic peptide carrying a trimer of the amino acid 93 to 103 fragment of VP1 as a coating antigen. The titer was calculated as the reciprocal of the dilution (\log_{10}) giving an optical density equal to three times that given by sera (1:25) of mice immunized with LAS HBsAg particles. Symbols: O, LAS particles; ●, BAP particles; △, PAP particles; ▲, DAP particles.

PAP particles (Fig. 7). A high PV-1-neutralizing anamnestic response was detected in mice previously immunized with PAP and DAP particles but not in those which had received LAS and BAP particles. However, a large increase in antibodies to the poliovirus peptide (trimer of PV-1 fragment) was detected in mice previously immunized with BAP particles but not in those immunized with HBsAg LAS particles. Analogous but lower anti-peptide responses were obtained in rabbits previously immunized with BAP particles and intravenously challenged with PAP particles on day 178 (rabbits immunized with LAS particles as control) (not shown).

Antigenic and immunogenic properties of particles carrying the PV-2 sequence. The antigenic site 1 sequence of PV-1 inserted at amino acid position 113 of HBsAg is recognized by the corresponding MAb C3. We therefore tested whether PAL particles, carrying the homologous PV-2 sequence (HBsPV2Ag) inserted at the same position of the HBsAg, could react with the anti-PV-2 MAbs Ilo and HO2. [³⁵S] methionine-labeled PAL particles were immunoprecipitated with HBsAg antiserum and the anti-PV-2 MAbs in the presence and absence of RIPA detergent. The precipitates were analyzed by SDS-PAGE (not shown). Under both physiological and denaturing conditions, only HBsAg antiserum precipitated PAL particles. The PV-2 MAbs did not react.

We then tested whether PAL particles could elicit an anti-poliovirus response. Three rabbits were immunized with purified HBsPV2Ag particles. The immunoprecipitating activity of one of the immune rabbit serum samples (LF 17) was tested against infectious or denatured [³⁵S]methionine-labeled poliovirus (Fig. 8). There was no difference between immune and nonimmune sera in binding to the infectious PV-2. However, the immune serum gave a stronger signal with SDS-denatured VP1 protein than the control. Therefore, poliovirus-specific anti-peptide antibodies had been synthesized. Moreover, the immune serum immunoprecipitated the infectious v510 chimeric poliovirus in which the

antigenic site 1 of PV-1 Mahoney had been replaced by the corresponding amino acid sequence of PV-2 Lansing (29). No difference was detected between the immune and non-immune LF 17 sera in immunoprecipitation experiments using purified [³⁵S]methionine-labeled infectious PV-1 (data not shown). This indicates that the PV-2 sequence, when exposed at the surface of the PV-1 virion, adopts a configuration which is somewhat different from that on the original PV-2.

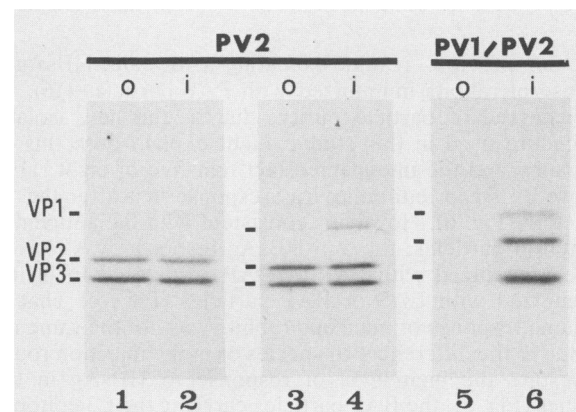


FIG. 8. Immunoprecipitation of poliovirus strains with an anti-HBsPV2Ag rabbit serum. Infectious (lanes 1 and 2) or denatured (lanes 3 and 4) [³⁵S]methionine-labeled poliovirus PV-2 Lansing strain and infectious chimeric PV-1/PV-2 poliovirus v510 (lanes 5 and 6) were immunoprecipitated with the serum of rabbit LF 17 immunized with two injections of HBsPV2Ag particles (PAL). The immunoprecipitated proteins were analyzed by SDS-PAGE followed by autoradiography. Autoradiograms of different gels are shown. o, Preimmune serum; i, immune serum collected 42 days following the first injection. The positions of poliovirus capsid proteins VP1, VP2, and VP3 are indicated. Preimmune serum shows a binding affinity for PV-2 which did not increase after rabbit immunization (lanes 1 to 4).

A		93	95	97	99	101	103	Neutralization						
PV1 (MAHONEY)		ASP	ASN	PRO	ALA	SER	THR	THR	ASN	LYS	ASP	LYS	LEU	-
PV 2 (LANSING)		ASP	ASN	ASP	ALA	PRO	THR	LYS	ARG	ALA	SER	LYS	LEU	-
PV1/PV2 (V510)		-----												
512D111		ASN-----												
511D111		----- GLY-----												
511H311		----- ASN-----												
513H111		----- GLU-----												
515H111		----- HIS-----												
516D111		----- ARG-----												
PV1/PV2 (V410)		VAL-----												
411H111		VAL----- ASN-----												
412H111		VAL----- SER-----												
411D1111		VAL----- ASP-----												

B		Rabbit LF 17				Rabbit LF41				Rabbit LF42				Rabbit LF28		
		0	14	28	42	0	14	28	42	0	14	28	42	0	14	28
Polio. Strain																
PV 1 (Mahoney)		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	3160	2000
PV 2 (Lansing)		-	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PV 1 / PV 2 (V510)		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
511D111		-	10	250	100	-	12	10	-	-	12	15	-	10	15	-
412 H 111		-	-	40	30	-	30	15	125	-	50	-	-	-	-	-

FIG. 9. Neutralization of poliovirus-antigenic variants by sera from rabbits immunized with HBsPV2Ag particles. Rabbits were immunized at days 0 and 14 with HBsPV2Ag PAL particles (LF 17, LF 41, and LF 42) or with HBsPV1Ag PAP particles (LF 28). (A) The poliovirus-neutralizing activity of the immune serum from rabbit LF 17, harvested 42 days after the first injection, was assayed against each of the indicated poliovirus strains. Numbers refer to amino acid residues in the poliovirus capsid protein VP1 of the PV-1 Mahoney strain in which the PV-1 sequence was substituted by that of the PV-2 Lansing strain. Amino acid substitutions in the PV-2 sequence are indicated for each antigenic variant derived from the chimeric v510 or v410 strains (29). Neutralization of 10 poliovirus 50% tissue culture infective doses at a serum dilution of 1:10 was considered positive (+). (B) The neutralization titers were measured by a standard plaque reduction assay and expressed as the reciprocal of the end-point dilution of serum samples giving 50% plaque reduction. Serum samples were considered negative (-) for titers <1:10, the lowest dilution tested. ND, Not determined; Days, days following the first injection and corresponding to the harvest of sera; 0, preimmune serum.

Neither the PV-2 virion nor the v510 chimera was neutralized in vitro by the immune LF 17 rabbit serum. This serum sample was tested for its ability to neutralize chimeric PV-1/PV-2 poliovirus mutants which had been isolated as strains escaping neutralization with anti-PV-2 MAbs Ilo or HO2 (T. Couderc, A. Martin, C. Wychowski, M. Girard, F. Horaud, and R. Crainic, submitted for publication; T. Couderc, unpublished results). These antigenic variants carry amino acid substitutions within (or close to) the PV-2 sequence equivalent to that expressed in PAL HBsPV2Ag particles. Two of the ten poliovirus variants tested were neutralized by the immune LF 17 rabbit serum in screening infectivity inhibition assays (Fig. 9A). The two sensitive antigenic variants and the parental v510 strain were tested against sera from all the rabbits (LF 17, LF 41, and LF 42) in plaque reduction neutralization assays (Fig. 9B). The two variants were neutralized with low efficiencies by all three sera. The parental v510 strain was unaffected. No neutralization was observed with the preimmune sera nor with the immune sera from a rabbit (LF 28) immunized with PAP HBsPV1Ag particles. These results indicate that HBsPV2Ag particles could express neutralization determinants present on the two sensitive poliovirus antigenic variants.

DISCUSSION

We have studied the activity of an antigenic peptide fragment, which is part of the neutralization antigenic site 1 of poliovirus, expressed on various hybrid HBsAg particles. Two different insertion sites on the HBsAg molecule and two homologous poliovirus sequences were used. The results showed how structural factors, such as the position on the HBsAg molecule, constraints from the carrier protein, and the nature of the inserted sequence, could favor or limit the expression of the antigenic fragment as a viral neutralization immunogen.

Critical role of the position of the heterologous antigenic fragment on the HBsAg molecule. When inserted at position 113 of the HBsAg, the PV-1 fragment was exposed at the surface of the hybrid PAP and DAP particles, as demonstrated both by reactivity with the MAb C3 and by trypsin cleavage experiments. The surface exposure of the PV-1 fragment correlates well with its capacity to induce poliovirus-specific antibodies. In contrast, when inserted at position 50 of the HBsAg, the PV-1 fragment was only slightly reactive with trypsin and MAb C3. The poor accessibility to these external agents of the PV-1 fragment in BAP particles

correlates with the low immunogenicity of this fragment in BAP particles. We cannot exclude that, when inserted at position 50 of the HBsAg, the PV-1 sequence is exposed in a different conformation at the surface of the BAP particles. This conformation, resulting in trypsin resistance and low reactivity with the MAb C3, might induce a different spectrum of antibodies and thus appear to be of low immunogenicity. This seems, nevertheless, unlikely because antibodies induced by BAP particles did not bind to denatured PV-1 proteins and barely recognized the corresponding PV-1 synthetic peptide. However, although the BAP particles did not induce anti-PV-1 antibodies, they primed animals for a subsequent challenge with PAP particles. A similar effect has also been described with equivalent poliovirus synthetic peptides (18).

Insertions at amino acid position 113 of HBsAg appear to favor the expression of heterologous antigenic sequences, whereas insertions at position 50 do not. This observation is also supported by the ability of the PV-2 fragment inserted at the same position of HBsAg to induce viral antibodies, even if these antibodies were not specifically active against the original PV-2. Moreover, a 13-amino-acid peptide fragment from the VP3 capsid protein of PV-1, at the same position on HBsAg particles, elicited rabbits antibodies reacting in immunoprecipitation with heated virions (C particles) and with denatured VP3 protein (unpublished data). Our results are in agreement with what is known about the antigenic structure of proteins showing that good accessibility of peptide fragments at the surface of protein complexes is critical for their reactivities with antibodies and, depending on host factors, for their immunogenicities (3, 22). Of the two major hydrophilic regions encompassing residues 45 through 72 and 110 through 145 of the HBsAg molecule, only the latter is significantly involved in forming type and subtype HBsAg determinants (38). The location of heterologous sequences around the major HBsAg determinants seems to favor the expression of the immunogenic properties of the inserted fragments. However, structural requirements that are more complex are needed to induce antibodies which would be biologically active against the target virus.

Relationship between antigenic fragments in particles carrying a double insertion. In spite of the high level of discrepancy between the activities of the PV-1 fragment when it was inserted at position 50 or 113 of HBsAg, a cooperative effect was observed when fragments were inserted at both sites of the same molecule. This was indicated by the reactivity of DAP particles with the anti-PV-1 MAb C3, which was higher than either of the BAP or PAP particles carrying a single insertion. Moreover, the reactivity of DAP particles seems higher than the expected sum of BAP and PAP reactivities. Structural modifications may have occurred in the carrier part of the hybrid molecule which improved the accessibility and/or conformation of one or both PV-1 fragments, thus favoring expression. The double insertion of the PV-1 sequence in DAP particles might also result in a different arrangement of the antigenic sites which, if they fit the span and the symmetry of the two antibody-binding sites of the MAb C3 immunoglobulin G molecule, would favor bivalent binding. The increased reactivity of the DAP particles with the MAb C3 correlates with the slight improvement of the anti-poliovirus immune response observed in mice immunized with these particles. These results illustrate that two antigenic fragments may have a functional relationship in hybrid particles when both are present on the same polypeptide.

Structural differences between PV-1- and PV-2-antigenic

fragments. In contrast to the PV-1 fragment, the homologous PV-2 segment, inserted at position 113 of HBsAg, did not express PV-2-specific neutralization determinants. The continuous nature of some neutralization-antigenic determinants of N-AgI was demonstrated in different ways for PV-1 and PV-3 (4, 8, 18, 20, 25, 49). The only efficient expression of the PV-2 fragment was demonstrated on chimeric PV-1/PV-2 poliovirus in which the homologous sequence of PV-1 (amino acids 94 to 102 of VP1) was replaced by that of PV-2 (29, 36). These chimeras were neutralized by PV-2 MAbs and induced PV-2-neutralizing antibodies.

Assuming that, when inserted at the same position of HBsAg, the two homologous PV-1 and PV-2 fragments are influenced by the same surrounding residues, the observed difference in their specific activities indicates that intrinsic structural factors differentiate these fragments at the antigenic and immunogenic level. Some antigenic fragments (e.g., the PV-2 sequence) could be more sensitive to surrounding sequences than others. Antigenic variants selected from PV-2 Lansing as escaping N-AgI-specific neutralizing MAbs have amino acid substitutions in the amino acid 93 to 101 VP1 sequence (27). Deletion at position 105, which is thought, by analogy with PV-1 (24), not to be part of the loop carrying the linear site, makes the PV-2-antigenic site resistant to MAbs (27). The PV-1 fragment seems to be more self-structured than its PV-2 counterpart. This might explain why the PV-1 sequence was always active when expressed in different vectors, including HBsAg (14, 16), diphtheria toxin protein (40), or membrane and periplasmic *Escherichia coli* proteins (7, 28).

An antigenic fragment of the foot-and-mouth disease virus (amino acids 141 to 160 of protein VP1) was also active as a synthetic peptide and as part of a hybrid recombinant *E. coli* protein or hybrid HBcAg particles (9). Three-dimensional structure analysis of the virus showed this antigenic fragment to be a disordered, flexible, prominent loop. Its characteristics may be what allow it to retain activity when expressed in various systems (1). The difference in activities between the PV-1 and PV-2 fragments on HBsAg particles can perhaps be explained by the relative prominence of such structural features.

Structural relationship between the immunogen and the target virus. The properties of the anti-HBsPV2Ag antisera indicated that the PV-2 sequence is expressed differently on HBsAg and on the PV-2 Lansing virus. However, experiments with these sera provided evidence that homologies may exist between HBsPV2Ag and the antibody-binding sites of the PV-1/PV-2 chimeric virus. The presentation of the PV-2 segment on the original PV-2 virion, therefore, differs from that on the chimera. This was also supported by the fact that PV-2 MAbs (e.g., MAbs I10 and HO2) had different neutralizing efficiencies when tested against the two viruses (29, 36). The viral neutralizing activities of antibodies were highly dependent on the virus structure, as indicated by the ability of HBsPV2Ag antibodies to neutralize only chimera mutants carrying amino acid substitutions in the PV-2 sequence. These results strengthen the idea that antibody binding is not sufficient to provide viral neutralization. Poliovirus epitopes which lose their neutralization properties without losing their antibody-binding capacities have already been described (4).

Antibodies elicited against synthetic peptides derived from N-AgI of PV-3 could neutralize the homologous strain and sometimes neutralized antigenic variants better than the parental strains (20). In contrast, HBsPV2Ag antibodies had a more narrow antigenic spectrum and deviated markedly

from the expected target. Stabilization of the PV-2 sequence on HBsAg may have caused this discrepancy. This observation correlates with results indicating that neutralizing antisera raised against a foot-and-mouth disease virus synthetic peptide have a wider antigenic spectrum than the corresponding anti-virion antisera (5).

In this and some of our previous work (14, 16), we have shown that a PV-1-antigenic fragment, when expressed at the surface of HBsAg particles, could be made highly immunogenic and could stimulate memory cells. Furthermore, if the quantity of injected antigen is taken into consideration, it would appear that HBsPV1Ag can induce poliovirus-neutralizing antibodies more efficiently than equivalent synthetic peptides (8, 18, 25). Foot-and-mouth disease virus or *P. falciparum* sequences are more immunogenic when expressed on HBV-derived particles than when they exist as synthetic peptides or hybrid monomeric proteins (9, 41). These findings confirmed the efficacies of recombinant hybrid particles in presenting foreign antigens. However, the results obtained with the PV-2 fragment suggest that structural constraints might limit the expression of some antigenic sequences as viral neutralization immunogens. In this case, the experiments which consisted in finding the right target virus for a given immunogen, nevertheless suggest that the right immunogen of a given target may be identified by introducing amino acid substitutions in the original peptide sequence. Mimotopes, short synthetic-peptide sequences that mimic discontinuous epitopes, are a good example for such an approach (21, 31).

Disulfide cyclization has been shown to increase the antigenic activities of synthetic peptides by locking their secondary structures (6, 17). The work described here illustrates the value of detailed analysis of antigenic fragments which are locked on heterologous particulate antigens.

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