JOURNAL OF VIROLOGY, May 1988, p. 1836–1839
0022-538X/88/051836-04$02.00/0

Vol. 62, No. 5

Presentation and Immunogenicity of the Hepatitis B Surface Antigen and a Poliovirus Neutralization Antigen on Mixed Empty Envelope Particles

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Insertion of a synthetic DNA fragment encoding a poliovirus neutralization epitope into the S gene encoding the major envelope protein of hepatitis B virus has yielded hybrid (HBsPolioAg) particles closely resembling authentic 22-nm antigen (HBsAg) particles by expression of the modified gene in mammalian cells. In mice, these hybrid particles induce neutralizing antibodies against poliovirus but only a very weak immune response to HBsAg (F. Delpeyroux, N. Chenciner, A. Lim, Y. Malpièce, B. Blondel, R. Crainic, S. Van der Werf, and R. E. Streeck, Science 233:472–474, 1986). By cotransfection with different plasmids carrying either modified or unmodified S genes, we have now obtained mixed particles presenting both HBsAg and HBsPolioAg. When such particles were inoculated into rabbits, antibodies to both poliovirus and to HBsAg were induced. Moreover, the titers of neutralizing antibodies to poliovirus induced by HBsPolioAg were much higher than those previously obtained in mice. The design of multivalent particles carrying various peptide sequences or presenting several heterologous epitopes may therefore be possible.

The hepatitis B surface antigen (HBsAg) appearing in the sera of patients infected by the hepatitis B virus (HBV) is carried by three morphologically distinct particles, 42-nm spherical particles representing infectious virions (Dane particles), filaments of about 22 nm in diameter, and 22-nm spherical particles corresponding to empty viral envelopes (18). The 22-nm particles consist of cellular lipids, the major envelope protein, and one or two minor envelope proteins. The proteins are present in both the glycosylated and nonglycosylated forms (17) and are linked by disulfide bridges into dimers and higher oligomers. Reduction of the inter- and intramolecular disulfide bridges and dissociation of the particles lead to a drastic decrease of the HBsAg antigenicity (12, 16). Expression of the S gene of the HBV genome encoding the 226-amino-acid sequence of the major envelope protein in mammalian cell lines yields defined lipoprotein particles similar to those from human serum which are secreted into the cellular medium (5, 13).

We have previously analyzed the formation and secretion of modified HBsAg particles by creating in-phase insertions of different lengths and sequences in various regions of the S gene. Expression of the modified genes was studied in mouse L cells (3). A hybrid HBsAg carrying a poliovirus neutralization epitope inserted next to the major antigenic region was shown to be organized into 22-nm particles which reacted with a poliovirus-specific monoclonal antibody. Mice immunized with these HBsPolioAg particles showed a poor response to HBsAg and a low but significant titer of poliovirus-neutralizing antibodies (4). The weak immune response to HBsAg suggested that an important conformational change of the hepatitis B surface antigen might have occurred as a result of the insertion of the poliovirus epitope. To further analyze the potential of the HBV envelope particles as an immunogenic carrier of heterologous antigens, we have carried out this study with the following objectives: to design mixed particles in which both HBsAg and HBsPolioAg could be presented in biologically active form, to analyze the immunogenicity of the mixed viral antigens, and to extend the system to other species knowing that mice are poor responders to poliovirus-neutralizing antigens.

To obtain expression of HBsAg and HBsPolioAg, we transfected two plasmids, pLAS and pPAP, into mouse L cells. pLAS and pPAP both carry the S gene of HBV, including the viral polyadenylation site, and the simian virus 40 early promoter. pPAP differs from pLAS by a 39-base-pair insertion of a synthetic DNA fragment in the unique *Bam*HI site within the S gene, encoding amino acid residues 93 through 103 of the VP1 capsid protein of poliovirus type 1 (Mahoney strain) and an additional Gly and Ser residue encoded by the *Bam*HI linker used for insertion. As previously shown by transfection of mouse L cells with pLAS or pPAP, stable clones secreting either HBsAg or hybrid HBs PolioAg particles could be obtained (4).

For the expression of HBsAg and HBsPolioAg from the same cells, pLAS and pPAP were mixed with plasmid pW (2) encoding neomycin resistance and were cotransfected into mouse L cells. For 10^6 cells, 5 μg each of pLAS and pPAP and 2 μg of pW were used. Of 12 G418-resistant clones isolated, 10 were HBsAg positive. To characterize the HBsAg products, proteins of seven clones were labeled in vivo with [^{35}S]methionine. Cellular supernatants were clarified at 12,000 × *g* for 5 min, and rabbit antiserum to human HBsAg particles (Behring) was added at a dilution of 1:100. Protein A-Sepharose (Pharmacia) was added as described previously (3), except that the Sepharose bed was washed three times with a solution consisting of 10 mM Tris hydrochloride (pH 7.2), 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 1% sodium deoxycholate and was washed twice with 125 mM Tris hydrochloride (pH 6.8). Proteins were eluted by boiling for 5 min in 40 μl of sample buffer and were submitted to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. One of these clones (MAP) produced HBsAg and HBsPolioAg in both the

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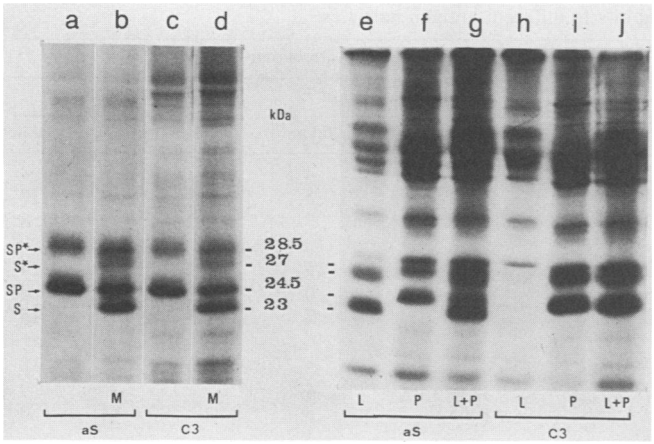

FIG. 1. Polyacrylamide gel electrophoresis of HBsAg and HBs PolioAg in the presence of sodium dodecyl sulfate. [^{35}S]methionine-labeled proteins from cellular supernatants were immunoprecipitated with antiserum to HBsAg (aS) or the antipoliovirus monoclonal antibody C3. The autoradiograms of two different (12.5%) gels are shown. HBsAg (S), HBsPolioAg (SP), and their glycosylated forms (★) are indicated. Their molecular sizes deduced from standards (Pharmacia) are indicated in kilodaltons (kDa) between lanes d and e. Lanes: a and c, two independent clones producing only HBsPolioAg; b and d, MAP clone producing both HBsAg and HBsPolioAg (M); e and h, LAS clone producing HBsAg (L); f and i, PAP clone producing HBsPolioAg (P); g and j, mixture of LAS and PAP supernatants (L+P).

glycosylated and nonglycosylated forms (Fig. 1, lane b). Moreover, all four polypeptides were immunoprecipitated from the supernatant of the MAP clone by the anti-poliovirus C3 monoclonal antibody reacting specifically with HBsPo lioAg (Fig. 1, lane d).

To check that the immunoprecipitation of HBsAg (lacking the polio insertion) by the polio-specific monoclonal antibody was not due to aggregation or to protein exchange between different particles, the supernatants of two cellular clones producing either HBsAg or HBsPolioAg alone were mixed and kept at 37°C for 36 h. Only HBsPolioAg was immunoprecipitated from the mixed supernatants by the monoclonal antibody C3, whereas both proteins reacted with the antiserum to HBsAg (Fig. 1, lanes e to j). This shows that immunoprecipitation of HBsAg by C3 from the supernatant of the MAP clone was due to the presence of HBsAg and HBsPolioAg in the same particle, probably linked by disulfide bridges (mixed particles). As expected, mixed HBsAg-HBsPolioAg particles had the same density (about 1.2 g/cm^3 in CsCl) as HBsAg particles and as HBsPolioAg particles. For the purification of antigens, HBsAg particles from the supernatants of cellular clones grown to confluence were precipitated by ammonium sulfate and were submitted to two successive centrifugations in CsCl using a Beckman VTi 50 rotor, followed by a 10 to 30% (wt/wt) linear sucrose gradient in a solution consisting of 10 mM Tris hydrochloride (pH 7.5), 150 mM NaCl, 1 mM EDTA (TNE) with a 0.5-ml cushion of 66% sucrose. Alternatively, HBsAg-containing fractions obtained after the first CsCl gradient were pooled and recentrifuged in a four-step CsCl gradient from 1.3 to 1.1 g/cm^3 with a Beckman SW41 rotor. Finally, HBsAg-containing fractions were pooled and dialyzed against TNE. An analysis of the proteins of partially purified mixed particles is shown in Fig. 2. In this preparation, HBsAg represented only 20% of the proteins of the mixed particles (Fig. 2e), although immunoprecipitation had previously suggested that roughly equal amounts of HBsAg and HBsPolioAg were present (Fig. 1). This discrepancy may be due to a certain instability of the particular clone used for the two experiments.

Antisera were raised in rabbits to HBsAg particles from human serum, HBsPolioAg particles, and mixed HBsAg-HBsPolioAg particles. The analysis of the sera harvested before and 2 weeks after the first and the second injections is shown in Table 1. Whereas HBsAg particles induced high anti-HBsAg titers of antibodies (approximately 15,000 to 20,000 radioimmunoassay units/μg of HBsAg inoculated, detected 2 weeks after the second injection in rabbits LF1 and LF2), the HBsAg response induced by HBsPolioAg was very low, as previously observed in mice (4). With the same amount of protein, only about 0.3 to 1% of the antibody titer elicited by HBsAg was observed with HBsPolioAg. However, in mixed HBsAg-HBsPolioAg particles, the immunogenicity of HBsAg was restored. Assuming that the mixed particles carried about 20% HBsAg and 80% HBsPolioAg, as suggested by the protein analysis shown in Fig. 2, the antibody titer per microgram of HBsAg obtained was of the same order as that obtained with unmodified HBsAg particles (10,000 to 15,000 radioimmunoassay units per μg of HBsAg inoculated in rabbits LF7 and LF8).

Neutralizing antibodies against Mahoney poliovirus were induced after the first injection in all but one (LF5) of the rabbits immunized with particles carrying HBsPolioAg. Moreover, three animals showed an increase of poliovirus-neutralizing antibody titers after the booster injection. One of the animals (LF6), immunized with a higher dose of HBsPolioAg, showed a particularly high titer of neutralizing antibodies (Fig. 3). The antiserum to poliovirus obtained was specific for the Mahoney strain. The Sabin type 1 strain, the Mahoney-derived attenuated strain, was not neutralized by the rabbit antisera (Table 1). This was rather surprising, since the C3 monoclonal antibody which was isolated from a mouse immunized with heated Mahoney virions and which

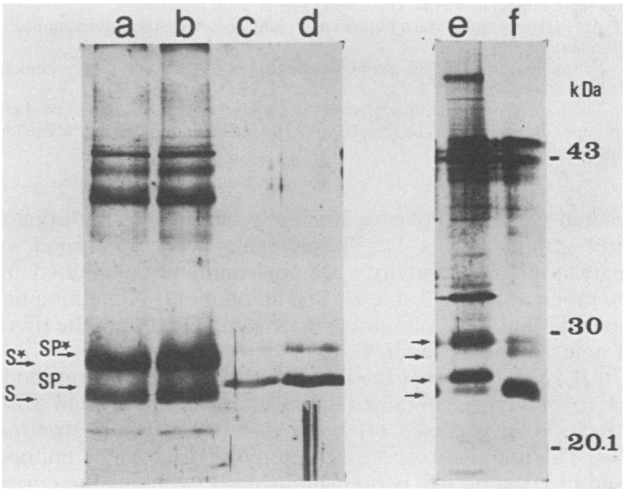

FIG. 2. Polyacrylamide gel electrophoresis of proteins from HBs Ag and HBsPolioAg particles revealed by silver staining. Two different 12.5% gels are shown. HBsAg (S) and HBsPolioAg (SP) and their glycosylated forms (★) are indicated. Lanes: a and b, 0.75 and 1 μg of human HBsAg particles from the Hevac B vaccine (Pasteur Vaccins); c and d, 0.25 and 0.5 μg of HBsPolioAg particles from a PAP clone; e, 1 μg of mixed particles from a MAP clone; f, HBsAg particles from a Vero cell clone (N. Chenciner, unpublished results). Molecular size standards (kilodaltons [kDa]) (Pharmacia) are indicated at the right of the gel.

TABLE 1. Immunogenic response in rabbits to different antigen particles

Rabbit	Antigen and amt (μg) injected[a]	Days after inoculation	Anti-HBsAg titer[b] (RIA U [10^{-3}])	Antipoliovirus activity			
				Immunoprecipitation %[c]		Neutralizing titer[d]	
				Mahoney	Sabin	Mahoney	Sabin
LF1	HBsAg (10)	0	0	4	4	0	4
		15	3	1	5	8	17
		30	140	0	7	6	8
LF2	HBsAg (20)	0	0	0	5	30	24
		15	26	3	0	0	0
		30	412	3	7	8	15
LF3	HBsPolioAg (10)	0	0	3	4	4	18
		15	0	46	4	133	0
		30	0.4	65	3	790	0
LF4	HBsPolioAg (20)	0	0	0	0	4	0
		15	0.4	18	4	64	11
		30	3.6	38	7	78	18
LF5	HBsPolioAg (20)	0	0	3	0	5	0
		15	0	2	4	0	0
		30	4.5	4	3	5	0
LF6	HBsPolioAg (40)	0	0	0	0	4	13
		15	0	62	1	1,513	13
		30	0.4	83	2	4,096	11
LF7	HBsAg-HBsPolioAg (15)	0	0	0	3	0	0
		15	0.7	87	5	635	0
		30	35	90	6	5,500	0
LF8	HBsAg-HBsPolioAg (15)	0	0	0	0	0	0
		15	0.2	23	3	82	0
	C3	30	48	16	5	74	0
	McAb Mahoney			90	96	512	128

[a] Female New Zealand White rabbits (EGAV, Centre Elevage Georges Achard, Saint Mars d'Egrenne, France) were immunized by intradermal injection of 22-nm particles of the specified antigen with a 50% emulsion of complete Freund adjuvant, which was repeated 2 weeks later with incomplete Freund adjuvant. Sera were collected before and 2 weeks after each injection.

[b] Anti-HBsAg titers were determined using the AUSAB radioimmunoassay (RIA) (Abbott Laboratories) by the semiquantitative method described by the manufacturer.

[c] [^{35}S]methionine-labeled purified poliovirions (20,000 cpm) were immunoprecipitated with 50 μl of each serum in 0.5 ml of phosphate-buffered saline as described previously (1).

[d] The titer of neutralizing antibodies in each serum was measured by a plaque reduction assay on Vero cells, with an input of 100 PFU of Mahoney or Sabin type 1 poliovirus strain. The reciprocal of the serum dilution giving 50% plaque reduction was computed from the regression curves of mean values obtained from duplicates.

reacted with HBsPolioAg efficiently neutralized both poliovirus strains (Table 1). These results were confirmed by analysis of the poliovirus proteins immunoprecipitated by the rabbit antisera (Table 1). The amount of [^{35}S]methionine-labeled Mahoney poliovirus was in agreement with the titers of neutralizing antibodies.

It is very likely that the low titers of neutralizing antibodies to poliovirus obtained in mice by immunization with HBsPolioAg particles (4) were due to antigen restriction (10). The immune response to the poliovirus type 1 epitope used in HBsPolioAg is poor in mice (9), whereas the corresponding sequence of poliovirus type 3 is immunodominant for mice (11). However, the low response to HbsAg found in mice immunized with HbsPolioAg cannot be attributed to antigen restriction. Mice normally respond well to HBsAg, and the low titer of antibodies to HBsAg observed in mice remained low in rabbits immunized by HBsPolioAg, as shown in this work. This is likely to be due to distortion of the surface antigen in the HBsPolioAg particles as a result of the insertion of the VP1 sequence. This is in agreement with previous observations that the antigen carried by the major HBsAg protein is highly sensitive to conformational changes (12).

Irrespective of antibody titer, antibodies to poliovirus induced by HBsPolioAg were neutralizing in both mice and rabbits. Neutralizing antibodies have also been obtained by immunization of rabbits with polypeptides overlapping the sequence used in HBsPolioAg (7–9). Apparently the polyclonal antibodies raised by HBsPolioAg in rabbits seemed to have a different specificity than the monoclonal antibody C3 directed against the very VP1 sequence used in HBsPolioAg. Whereas C3 neutralized both the Mahoney and the Sabin 1 strains, which differ in 2 of 11 amino acids of VP1 used, the rabbit antiserum was specific for the Mahoney strain. This suggests that different paratopes were preferentially induced by HBsPolioAg, as compared with the natural poliovirus epitope. This is in agreement with our previous observation that the two Lys residues present in the VP1 sequence are accessible to trypsin in HBsPolioAg but are resistant to proteolysis in infectious virions (4), indicating different

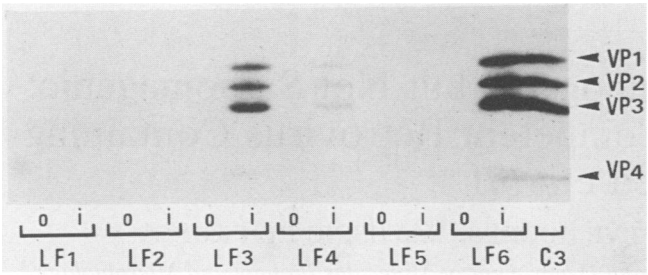

FIG. 3. Polyacrylamide gel electrophoresis of polypeptides of infectious poliovirus. [^{35}S]methionine-labeled virions were immunoprecipitated with antisera from rabbits LF1 to LF6 (Table 1) or with the antipoliovirus monoclonal antibody (C3). o, Pre-immune sera; i, sera taken 2 weeks after the second immunization. VP1 through VP4, the proteins of the poliovirus capsid. For immunoprecipitation, [^{35}S]methionine-labeled Mahoney virus (13,500 cpm) (1) was diluted in 500 μl of phosphate-buffered saline containing 2% bovine serum albumin. Rabbit sera or ascites fluid containing the C3 monoclonal antibody was added at a dilution of 1:50 or 1:100, respectively. Incubation was performed overnight at 4°C. Addition of protein A-Sepharose and the subsequent steps were carried out as described previously (3).

structural environments of these residues in the two macromolecular complexes.

Our work is the first description of 22-nm particles composed of heterologous proteins which differ in internal sequences. The obvious capacity of these proteins to mix in the membrane of the endoplasmic reticulum and to form mixed aggregates by invagination of this membrane (6, 14) lends credence to the notion that the internal heterologous region does not play a critical role in the formation and the secretion of these particles. Moreover, the capacity of the mixed particles to induce antibodies to both HBsAg and poliovirus shows that the two antigens do not structurally interfere with each other. By variation of the ratio of the two plasmids used for transfection, it may be possible to vary the relative concentrations of the two antigens on the 22-nm particles. Similarly, particles carrying a larger number of antigens may be produced. These various possibilities may allow the design of highly sophisticated vaccinating structures.

Paul et al. (15) have described HBsAg particles from human serum which carried normally mutually exclusive subtype determinants. This finding was explained by phenotypic mixing resulting from a two-hit infection of the same cell with viruses of different genotype. Our results confirm that the same HBsAg particle may carry HBsAg encoded by different S genes.

We thank P. Adamowicz for human HBsAg particles and N. Chenciner for encouragement and support. The expert technical assistance of M. Lambert and A. Candréa is gratefully acknowledged. We thank A. Galliot and N. Perrin for typing the manuscript.

This work was partly supported by grants 83.L.0936 and 84.V.816 from the Ministère de l'Industrie et de la Recherche and grants 83.3008 and 86.3003 from the Institut National de la Santé et de la Recherche Médicale.

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