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Reconstitution of hepatitis B surface antigen proteins into phospholipid vesicles

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Abstract

Hepatitis B surface antigen (HBsAg), devoid of 75% of its total lipids has been reconstituted with several phospholipids by the detergent dialysis method, using the non-ionic detergent β-D-octyl glucoside. Upon reconstitution with both neutral and acidic phospholipids, HBsAg particles had the same morphology and, as indicated by trypsin hydrolysis, the topology of the surface proteins was maintained. However, only negatively charged phospholipids were able to completely revert the conformational changes which had been induced by removal of the lipids. The helical content, as indicated by CD techniques, and the antigenic activity, as measured by binding to polyclonal antibodies, of HBsAg reconstituted with acidic phospholipids were practically identical to those of the native antigen. Cholesterol had no effect on the antigenic activity recovered by reconstitution with any of the phospholipids.

Key words: Hepatitis B surface antigen; Reconstitution; Acidic phospholipid; Vesicle

1. Introduction

Hepatitis B surface antigen (HBsAg) is the envelope of the hepatitis B virus (HBV). During HBV infection, hepatocytes synthesize and secrete large amounts of HBsAg, mainly in the form of spherical lipoprotein particles with a diameter of about 20 nm, which are readily observable by electron microscopy [1,2]. HBsAg is a complex macromolecular aggregate ($3.5 \cdot 10^6$ molecular mass) composed of protein (75% by weight), carbohydrates (in the form of glycoproteins) and host derived lipids (25%) [3]. SDS-polyacrylamide gel electrophoresis of purified HBsAg reveals the presence of two proteins, designated as S and gS (the glycosylated form of S), which account for more than 90% of the total protein of HBsAg. These two proteins have been demonstrated to be the translation product of a single gene, with the only difference between the two being the presence of the carbohydrates attached to gS at position Asn-146 [4,5].

Of the different components of HBsAg, the proteins are responsible for the induction of antibodies, and both the group and subtype specific antigenic determinants are localized within the proteins [6–9]. However, lipids play an important role in maintaining the structure and antigenic activity of HBsAg since when most of the lipids are removed the helical content of HBsAg proteins is reduced from 52% to 28% and the antigenic activity is reduced to less than 10% [10]. The lipids may exert their effects either by specific interactions with the proteins or by changing the structural state of the membrane.

The lipid compositions of the HBsAg obtained from human plasma and that produced by human hepatoma cell lines have been reported [3,11]. In both cases phospholipids were found to be the major lipid class

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Abbreviations: HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; CD, circular dichroism; SDS, sodium dodecylsulfate; OG, β-D-octyl glucoside; PC, egg phosphatidylcholine; PE, egg phosphatidylethanolamine; PS, bovine brain phosphatidylserine; PI, bovine liver phosphatidylinositol; CL, bovine heart cardiolipin.

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present, accounting for 67% and 91.5%, respectively, phosphatidylcholine being the most abundant component. Small amounts of negatively charged phospholipids were also found. In general, the envelopes of enveloped viruses have phospholipid compositions essentially identical to that of the host cell membranes [12,13]. However, in the case of HBV, it has been postulated that the HBsAg proteins may be responsible for the preferential incorporation of PC into the 20 nm particles [11].

Reconstitution of viral proteins into lipids has been widely used to examine protein-lipid interactions, to study the mechanism of fusion with either the cell or endosomal membranes, and even for vaccination purposes [14–16]. In this paper we describe the reconstitution of HBsAg proteins into phospholipid vesicles. The process is shown to be specific, with the acidic phospholipids being the only ones that can completely reverse the conformational changes induced by removal of lipids. This report describes the structural and antigenic properties of such reconstituted particles.

2. Materials and methods

Materials. Phospholipids were purchased from Avanti Polar Lipids. Cholesterol and cholesteryl linoleate were from Sigma. β-D-Octylglucoside (OG) was purchased from Calbiochem. Electrophoresis reagents were obtained from BioRad. Trypsin was obtained from Sigma. Cesium chloride was from Aldrich and solutions of cesium chloride were filtered through GSWP04750 Millipore filters prior to use.

Purification and reconstitution of HBsAg. HBsAg was purified from the plasma of high titre chronic carriers of HBsAg, adw subtype, following previously described procedures [4]. The protein concentration was determined either from the absorbance spectrum, using the value of 3.726 as the absorbance at 280 nm of a 0.1% (w/v) solution of HBsAg [17] or by amino acid analysis in which aliquots of protein (10–20 μg) were hydrolized in sealed evacuated tubes with constant boiling HCl for 24 h at 110°C. Amino acid analysis was performed with a Beckman 6300 amino acid analyzer.

Lipid phosphorus was determined by the micromethod of Barlett [18] as previously described [3]. Lipid homogeneity was assayed by thin layer chromatography [19]. HBsAg was delipidated as previously described [10], except that 2.2% (w/v) OG was used. Under these conditions 75% of the total lipids was removed. The reconstitution procedure consisted of incubating delipidated HBsAg (60–100 μg/ml) in 10 mM Tris (pH 7.0), containing 50 mM NaCl, with phospholipid vesicles in the presence of 1% (w/v) OG for 16–20 h at 4°C. These vesicles were obtained by a detergent dialysis method [20]. Detergent/phospholipid/protein mixed vesicles were dialyzed for 48 h against four 2-liter changes of 10 mM Tris/HCl (pH 7.0), containing 50 mM NaCl. The phospholipid concentration was adjusted so that the lipid/protein ratio varied between 2 and 100 ng phospholipid phosphorus/μg protein. In some experiments [*glucose*-U-^{14}C]octyl glucoside (New England Nuclear) was added to determine the extent of its removal during dialysis. In some instances, after dialysis, the mixture was centrifuged through CsCl gradients (1.15–1.25 g/ml) in a Beckman SW65 rotor at 45 000 rpm for 3 h. When the effect of cholesterol was tested, it was added to the dried lipid film prior to vesicle formation. The lipids derived from HBsAg were also used for reconstitution. These were obtained from the band which is observed at the top of the gradient, in addition to the delipidated protein band, when HBsAg is treated with 2.2% OG and centrifuged through CsCl gradients. This band contains the lipids derived from the native antigen [10]. These lipids were recovered by extraction according to the procedure of Bligh and Dyer [21] and subsequently used to prepare liposomes for reconstitution purposes.

Circular dichroism studies. CD spectra were obtained on a Jobin Yvon Mark III dichrograph by using a 0.1 cm path-length quartz cell and 60–120 μg/ml of protein. A minimum of three scans were collected for every sample. The values of the mean residue weight ellipticities were calculated on the basis of 110 as the average molecular weight per residue and they are reported in terms of Θ_{MRW} (degrees cm^2 dmol^{-1}). The secondary structure estimations were performed by computer fit according to the ellipticity reference values of Bolotina et al. [22], which are based on proteins of known three-dimensional structure. The fitting was accomplished by the analytical method based on a matrix formulation previously described [23]. The spectra shown are representative of those obtained for three different preparations.

Trypsin digestion. HBsAg was digested with trypsin in 10 mM Tris (pH 7.0), containing 50 mM NaCl, at a protein/trypsin weight ratio of 50:1 as previously described [4].

Polyacrylamide gel electrophoresis. Analytical 15% polyacrylamide slab gel electrophoresis was performed with a BioRad Mini Protean II apparatus. A 4% acrylamide stacking gel was used. The gel and the buffer formulations were those of O'Farrel [24]. Gels were stained according to the silver nitrate staining method as described [25].

Electron microscopy. Samples of HBsAg either native or reconstituted (20–30 μg/ml) were applied to a 400 mesh Formvar-carbon-coated copper grid for approximately 10 min. Excess fluid was drawn away with filter paper. The sample was then negatively stained with 2% (w/v) phosphotungstic acid at pH 7.2 and

examined using a Zeiss EM 902 transmission electron microscope.

Antigenic activity assays. The antigenic activity of HBsAg was determined using polyclonal anti-HBsAg antibodies obtained after immunization of New Zealand rabbits with purified plasma derived HBsAg. Microtiter plates (Costar) were coated with increasing amounts of HBsAg, either native, delipidated or reconstituted (between 0–10 ng of protein/well) for 15–18 h at 4°C. The plates were extensively washed with 0.1% (v/v) Tween 20 and blocked with 1% (w/v) BSA in PBS at 37°C for 2 h. Rabbit anti-HBs (1:7000 in PBS, 0.5% (w/v) BSA) was added after extensive washing and the plate incubated for 1 h and 45 min at 37°C; peroxidase labelled goat anti-rabbit (Tago Laboratories) (1:1000) was allowed to react for 1 h and 30 min and after washing, *o*-phenylenediamine (0.5 mg/ml in 0.1 M citrate buffer (pH 5.0), containing 4% (v/v) methanol and 0.03% (v/v) H_2O_2) was added. Colour was allowed to develop for 5–7 min and then the reaction was stopped by addition of 3 N H_2SO_4. The absorbance at 492 nm was measured in a Multiskan Titertek. The absorbance values were referred to those obtained for native HBsAg. Thus, the antigenic activity of both delipidated and reconstituted HBsAg was calculated as amount of native antigen and finally expressed as percentage of the total amount of antigen assayed. In some cases, the antigenic activity was determined using the AUSRIA II-125 kit from Abbott Laboratories, which is a 'sandwich' solid-phase radioimmunoassay, as previously described [26]. Since no differences were observed between the two procedures, most of the assays were performed according to the former one. The values reported are representative of those obtained for three different preparations.

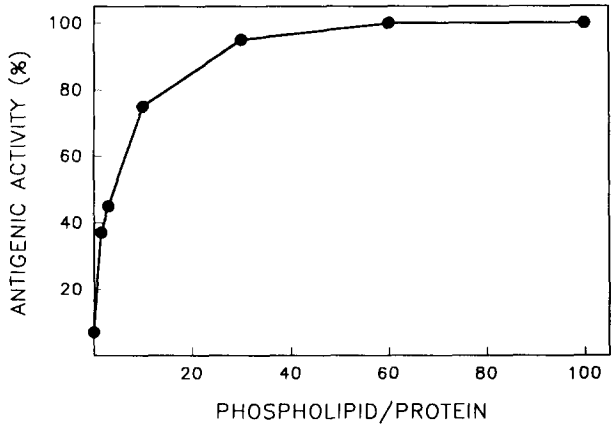

Fig. 1. Antigenic activity of HBsAg as a function of increasing concentration of PS. Reconstitution was performed at different phospholipid/protein ratios. The phospholipid/protein ratio is expressed as ng phosphorus/μg protein. The antigenic activity was measured after dialysis of the protein-lipid-detergent complexes against rabbit polyclonal antibodies as described in Materials and methods.

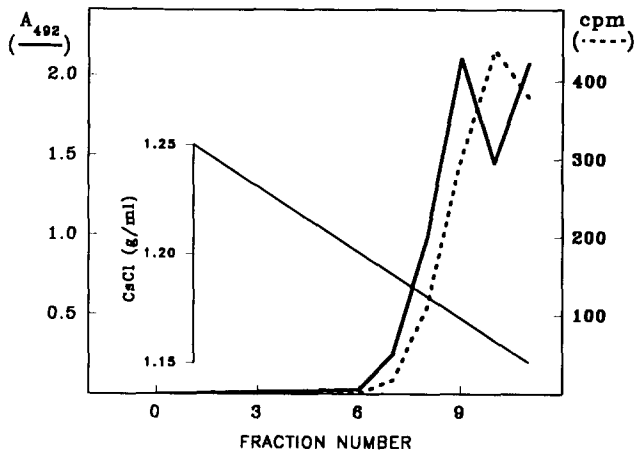

Fig. 2. Centrifugation of reconstituted HBsAg on CsCl linear gradient. Delipidated HBsAg (60 μg/ml) was incubated with PC (60 ng phosphorus/μg protein) in the presence of 1% (w/v) OG and dialyzed against four 2-liter changes of 10 mM Tris (pH 7.0), containing 50 mM NaCl. Centrifugation was performed for 3 h at 45000 rpm in a Beckman SW65 rotor. After centrifugation, fractions of 0.4 ml were collected from the bottom of the tube and assayed for antigenic activity as described in Materials and methods (A_{492}). Dotted line represents radioactivity of di[^{14}C]palmitoyl phosphatidylcholine (New England Nuclear) included in the initial mixture as a lipid marker.

3. Results

3.1. Reconstitution method

HBsAg devoid of 70–75% of the lipids was used as the starting material for reconstitution studies. The structural and antigenic properties of this HBsAg were virtually identical to those previously described [10].

Reconstitution was carried out by incubating delipidated HBsAg with different exogenous lipids in the presence of 1% OG followed by exhaustive dialysis to remove the detergent. The effect on the antigenic activity of varying the amount of phospholipid in the reconstitution mixtures is shown in Fig. 1. These data correspond to PS but similar results were obtained with other phospholipids. The antigenic activity of the reconstituted sample increased with increasing PS concentrations. At 10 ng phosphorus/μg protein there was a recovery of 75–80% of the antigenic activity, and the maximum recovery occurred at 60 ng phosphorus/μg of protein. Higher phospholipid/protein ratios did not improve antigenicity. Hence, all reconstitution experiments were carried out at a ratio of 60 ng phosphorus/μg protein.

Regardless of the lipid used, when the lipid-protein complexes were centrifuged through CsCl linear gradients (1.15–1.25 g/ml) all of the antigenic activity ap-

peared at the top of the gradient (Fig. 2), as two bands with slightly different densities (1.15 and 1.17 g/ml). The phospholipids are also present in the fractions containing HBsAg-antigenic activity (Fig. 2). Moreover, no antigenic activity was detected at a density of 1.25 g/ml, which is the density at which delipidated antigen banded. These results indicate that under these conditions all surface antigen interacted with the lipids. The buoyant density value is consistent with the presence of a lipid-protein complex in the reconstituted samples. Since the two peaks which were observed were not well resolved, the lipid-protein complexes obtained after dialysis were used without any further purification. On the other hand, the residual detergent concentration in the reconstituted samples was lower than 0.01%, as determined by the use of [^{14}C]OG.

3.2. Structural properties of reconstituted HBsAg

CD spectra of representative samples are shown in Fig. 3. Reconstitution with PS yielded a sample with ellipticity values ($\Theta_{222} = -14050 \pm 350$) very similar to those of native HBsAg ($\Theta_{222} = -14400 \pm 200$). The sample reconstituted with PC exhibited a CD spectrum intermediate between those of native and delipidated HBsAg ($\Theta_{222} = -11700 \pm 400$ for PC-reconstituted and -9370 ± 500 for delipidated). These data indicate a similar helical content of HBsAg native and reconstituted with PS while the sample reconstituted with PC had an helical content intermediate between those of native and delipidated antigen. Hence, only PS was able to completely reverse the conformational changes induced by removal of the lipids.

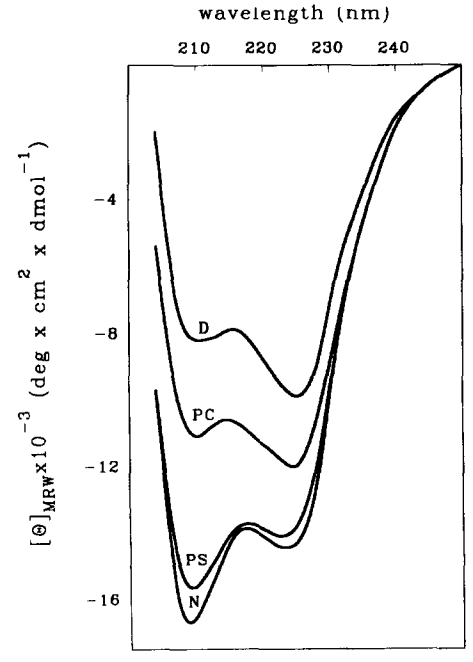

Fig. 3. CD spectrum of reconstituted HBsAg. N, native antigen; D, delipidated HBsAg after treatment with 2.2% (w/v) OG; PC and PS represent the spectra of the antigen reconstituted with the corresponding phospholipid at a lipid/protein ratio of 60 ng phosphorus/μg protein. The protein concentration was 60–120 μg/ml and it was determined by amino acid analysis as described in Materials and methods after reconstitution.

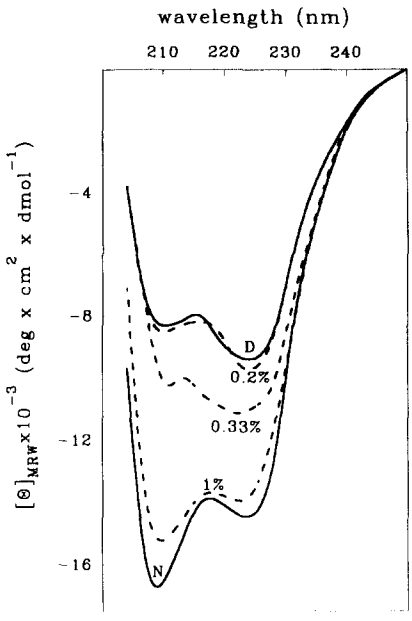

Fig. 4. Effect of OG on CD spectrum of delipidated HBsAg. The amount of OG is indicated in each spectrum. N, native antigen; D, delipidated HBsAg. The protein concentration was 60–120 μg/ml.

As shown in Fig. 4, OG concentrations up to 0.2% did not affect the CD spectrum of the delipidated antigen and, thus, the residual OG concentration after dialysis, lower than 0.01%, could not be responsible for the observed recovery of structural properties. Concentrations of 1% OG or greater were required to recover a CD spectrum similar to that of the native HBsAg (Fig. 4). Hence, we can conclude that the lipids are responsible for the recovery of the properties observed upon reconstitution, and not the detergent.

Trypsin hydrolysis has also been used to examine the structural changes induced upon reconstitution of the HBsAg. Lys-122 is the only peptide bond accessible to the protease in the intact particle although there are other potential cleavage sites. Hydrolysis at this bond gives rise to two peptides which are observed by SDS-PAGE together with the two main HBsAg proteins, S and gS [5]. The electrophoretic pattern observed following digestion of the antigen reconstituted with either neutral or acidic phospholipids was identical to that of native HBsAg (data not shown). These results indicated that the exposed regions of the polypeptide chains exhibit the same accesibility to the protease in the native and the reconstituted particles, regardless of the phospholipid.

Electron microscopy revealed that the morphology of HBsAg did not differ following reconstitution with either neutral or negatively charged phospholipids (Fig. 5). The size of HBsAg reconstituted with zwitterionic phospholipids was found to be close to that of the detergent solubilized HBsAg (18.6 ± 2.3 and 17.7 ± 2.2 nm for PC and PE, and 17 nm for delipidated) while that reconstituted with acidic phospholipids had a diameter almost identical to native HBsAg (20 ± 1.2 and 19.7 ± 2.5 nm for PS and cardiolipin and 20 nm for native).

3.3. Antigenic properties of reconstituted HBsAg

The antigenic activity of HBsAg reconstituted with different phospholipids, as measured with polyclonal antibodies, is depicted in Fig. 6. As can be seen, the incorporation of neutral and acidic phospholipids increased the antigenic activity of HBsAg compared to that of the delipidated antigen. However, with neutral phospholipids, such as PC and PE, only 42–45% of the original activity was recovered while with PS, PI and cardiolipin the recovery was 80–96%. Hence, the dif-

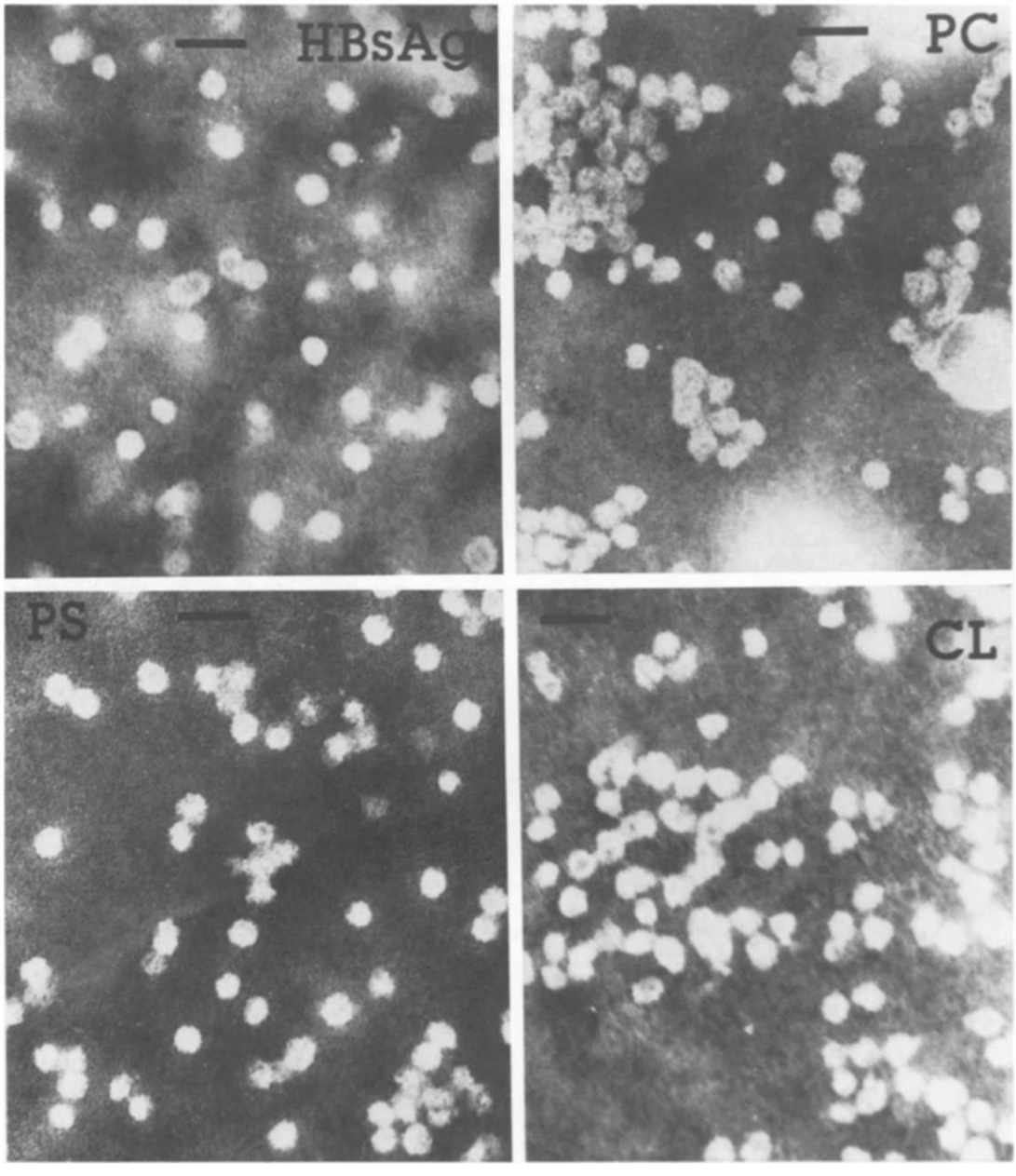

Fig. 5. Electron micrograph of HBsAg. HBsAg was reconstituted with phosphatidylserine (PS), cardiolipin (CL), and phosphatidylcholine (PC) at a phospholipid/protein ratio of 60 ng phosphorus/μg protein. Sample labelled HBsAg corresponds to purified plasma derived antigen. Samples (20–30 μg/ml) were negatively stained with 2% potassium phosphotungstate. Bar represents 50 nm.

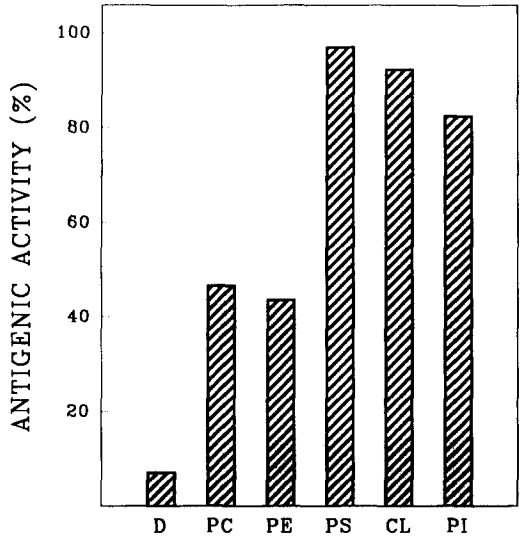

Fig. 6. Antigenic activity of HBsAg reconstituted with different phospholipids. D, delipidated antigen; in all other cases the phospholipid used for reconstitution is indicated. The phospholipid/protein ratio was 60 ng phosphorus/μg protein. The antigenic activity was measured against rabbit polyclonal antibodies as described in Materials and methods.

ferences observed in the CD spectra correlated well with the difference found for the antigenic activity. Since the fatty acid compositions of all of the phospholipids used were similar (average length between 17.9 and 18.9 and average degree of unsaturation between 1.5 and 1.9) it could be argued that these observed differences are related specifically to the nature of the polar headgroup.

The effect of PC on the reconstitution by PS was tested by using different mixtures of the two while always maintaining the ratio of 60 ng phosphorus/μg protein. As the amount of PS increased the antigenic activity also increased (Fig. 7). Moreover, the antigenic activity observed with mixed vesicles is slightly lower than that observed with pure vesicles, at the same absolute amount of PS. Thus, at 15 ng phosphorus/μg protein, the recovery was 80% with pure PS (Fig. 1) while with mixed vesicles it was 67% (Fig. 7). Reconstitution was therefore dependent on the absolute amount of PS and the presence of neutral phospholipids did not greatly affect the recovery of antigenic activity.

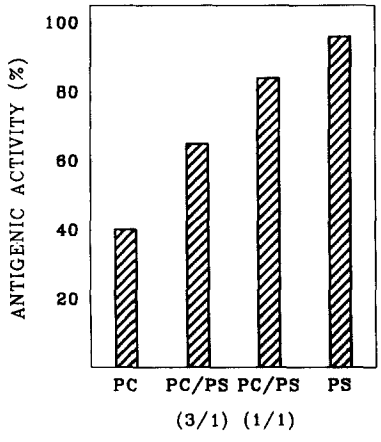

Fig. 7. Effect of PC on antigenic activity of HBsAg reconstituted with PS. The phospholipid/protein ratio was always maintained at 60 ng phosphorus/μg protein. Then, the amount of PS present in each case, expressed as ng phosphorus/μg protein, was: 60 (PS), 30 (PC/PS, 1:1) and 15 (PC/PS, 3:1). PC corresponds to HBsAg reconstituted with pure PC at the same phospholipid/protein ratio.

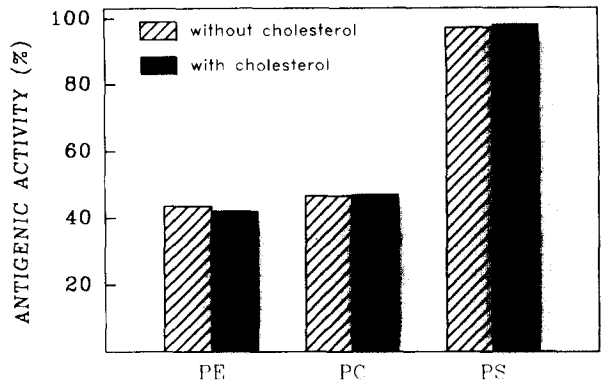

Fig. 8. Effect of cholesterol on antigenic activity of reconstituted HBsAg. HBsAg was reconstituted with the indicated phospholipid at a phospholipid/protein ratio of 60 ng phosphorus/μg protein in the presence or absence of cholesterol. Where indicated, phospholipid vesicles were prepared containing 50 μM cholesterol (25 μM free and 25 μM cholesteryl linoleate).

Since cholesterol accounts for 30% of the lipid content of HBsAg (15% free and 15% esterified) [3] its effect on the reconstitution with different phospholipids has been studied. As can be seen in Fig. 8, cholesterol did not affect significatively the extent of recovery of the antigenic activity with either neutral and negatively charged phospholipids.

4. Discussion

We have previously shown that the removal of most of the lipids from HBsAg produced conformational changes that rendered the antigen practically devoid of antigenic activity [10]. However, it could be argued that the process of removal itself rather than the absence of the lipids that was responsible for the observed changes. Reconstitution with several phospholipids has been carried out and the fact that some of them reversed the conformational changes which had been induced by delipidation indicates that phospholipids are important in maintaining the antigenic structure of HBsAg.

Detergent dialysis has been the reconstitution method selected. The non-ionic detergent β-D-octyl glucoside was chosen because of its high critical micellar concentration, which facilitates its removal by dialy-

sis. This detergent has been widely used for reconstitution of membrane proteins [27,28] as well as viral spike glycoproteins [20,29].

Maximum recovery of antigenic activity was obtained when the lipid/protein ratio in the initial detergent-phospholipid-protein complexes was 60 ng phosphorus/μg protein. When the reconstituted material was centrifuged through CsCl gradients, all HBsAg antigenic activity was detected at the top of the gradient, regardless of which phospholipid was used. The density of the reconstituted HBsAg (1.15–1.17 g/ml) indicated a higher lipid/protein ratio than the native plasma derived antigen (1.21 g/ml).

Both neutral and acidic phospholipids induced formation of stable lipid-protein complexes which, in addition to their density, shared other common properties. Their morphology, as revealed by electron microscopy, was very similar. Although samples treated with acidic phospholipids resulted in HBsAg particles which were slightly bigger than those reconstituted with neutral ones, all of the particles had a size close to that of native HBsAg. Also, following reconstitution there was no increase in the number of trypsin accesible bonds in the HBsAg particles. Lys-122 remained the only accesible one, as in the native antigen [5]. This is consistent with the conclusion that the proteins possess the same orientation of exposed regions in reconstituted particles with either PS or PC. Moreover, proteins are not just entrapped in the lipid vesicles but rather interact with the lipids in a topologically correct manner similar to that in intact particles. Thus, the reconstitution process, defined only as the formation of lipid-protein complexes, would not be specific since all of the phospholipids examined were able to induce it. However, when related to the recovery of native properties, reconstitution is specific for acidic phospholipids. Only these were able to completely restore the structural and antigenic properties of the delipidated HBsAg. CD studies indicated that the secondary structure of HBsAg reconstituted in the presence of PS was virtually identical to that of native HBsAg, at least in terms of its helical content. Samples treated with neutral phospholipids recovered an helical content that was intermediate between those of native and delipidated HBsAg. Similar effects have been ascribed to the interaction of acidic phospholipids with several proteins. For instance, antitumour protein α-sarcin increases its helical content upon interaction with acidic phospholipids [30]. Also, a signal peptide of cytochrome *c* has a non-ordered structure when associated with PC, while in the presence of negatively charged phospholipids it has 49% α-helix [31].

Reconstitution with other acidic phospholipids (PI and cardiolipin) resulted in HBsAg particles with antigenic activities, as measured with polyclonal antibodies, very similar to that of native HBsAg. Thus the recovery of antigenic activity appears to be specific for acidic phospholipids and not just specific for PS. The only particles which had been reconstituted with acidic phospholipids for which the CD spectrum was measured were those reconstituted with PS. However, based on the observed relationship between the helical content of the protein and the antigenic activity [26], the specificity for acidic phospholipids can probably be defined not only in terms of antigenic activity but also in terms of secondary structure. Such specificity for negative phospholipids has been described for several membrane proteins [32,33] and for some of them the interaction is specific for only one type, phosphatidylserine [34]. This is not the case of HBsAg since all acidic phospholipids tested rendered HBsAg fully active. Ionic interactions have a definite role in determining this specificity since an increase in the ionic strength during reconstitution caused a decrease of antigenic activity (Gómez-Gutiérrez, J. and Gavilanes, F., unpublished data).

The presence of neutral phospholipids did not significantly alter the specific interactions observed since the binding of polyclonal antibodies to HBsAg that had been reconstituted with PS was only slightly lower when different amounts of PC were included during preparation of the lipid vesicles. Therefore, it is not necessary for the lipid molecules to be completely free or present as independent domains as has been described for other membrane proteins such as the carbamoyl phosphate synthetase I from rat liver, where only cardiolipin inhibited the enzyme whereas mixed liposomes composed of neutral phospholipids and cardiolipin did not [33]. However, it could also be possible that HBsAg proteins induce clustering of the acidic phospholipids from mixed vesicles composed of PS and PC as it has been observed in the case of protein kinase *c* [35] or the hydrophobic myelin protein, lipophilin [36].

Cholesterol is a major lipid component of human plasma derived HBsAg, accounting for 30% of the lipid content, with 15% free and 15% esterified [3]. However, cholesterol had no further effect on the degree of reconstitution shown by any phospholipid tested. Hence, the modification of the physical state of the bilayer, i.e., the perturbation of the packing of the fatty acyl chains and decrease in the freedom of the polar headgroup, which is induced by cholesterol [37] had no measurable effect on the antigenic activity. When the reconstitution was carried out with the lipids derived from the HBsAg (those extracted from the low density band which is obtained during solubilization of HBsAg and which must account for, at least, 75% of native lipids [10]), only 40% of the original antigenic activity was recovered (data not shown). This can be explained since with PC, which represents 90% of HBsAg phospholipids, only 45% antigenic activity is recovered. The

fact that the major HBsAg lipids (PC and cholesterol) did not induce full recovery of antigenic activity could indicate that the conformational changes produced upon removal of the lipids are not completely reversible (when using the same kind of lipids) and only additional forces, those imparted by acidic phospholipids, could induce the proper folding of the polypeptide chain during reconstitution.

From the results shown it is obvious that HBsAg proteins can interact with different phospholipid classes but have a marked preference for interaction with negatively charged phospholipids, compounds which are minor components of HBsAg both derived from human plasma [3] and produced by human hepatoma cell lines [11]. This seems to indicate that the proteins are not responsible for the preferential incorporation of PC into 20 nm particles as proposed by Satoh et al. [11], and that the lipid composition of HBsAg only reflects that of the host cell membrane. Lipid-protein interactions render the proper structure of the polypeptide chain, but specific interactions with acidic phospholipids can alter local substructures.

Acknowledgements

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