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Antigenicity of hepatitis B surface antigen proteins reconstituted with phospholipids

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Abstract

Hepatitis B surface antigen (HBsAg) has been reconstituted with different phospholipid classes. All epitopes defined by a panel of monoclonal antibodies which recognize both group- and subtype-specific antigenic determinants showed specificity for acidic phospholipids. Electrostatic interactions between HBsAg proteins and acidic phospholipids are partly responsible for the complete recovery of the antigenic properties. In addition to the nature of the polar head group, the fatty acid composition of the phospholipid also influenced the recovery of the antigenic activity. Negatively charged phospholipids must bear at least one unsaturated fatty acid in order to be effective in recovering full antigenic activity of HBsAg. The results reported herein support the conclusion that the antigenic activity is dependent on the physical state of the phospholipid moiety. The appropriate membrane fluidity is required for optimum conformation but, once this conformation is established, additional interactions imparted by the various phospholipids give a difference in the patterns of antigenicity. The analysis of binding of the monoclonal antibodies allowed the classification of the epitopes into two groups according to their dependence on the lipid moiety. Of all the antigenic determinants only those close to the lipid-protein interface would change upon direct interaction with the phospholipids. The rest would depend on the correct protein conformation determined by the appropriate phospholipid composition.

Keywords: Hepatitis B surface antigen; Reconstitution; Acidic phospholipid; Antigenicity

1. Introduction

Hepatitis B surface antigen (HBsAg), the envelope of the Hepatitis B virus (HBV), is a complex macromolecular structure composed of proteins (75% by weight), carbohydrates (in the form of glycoproteins) and host derived lipids (25%) [1]. During infection, hepatocytes synthesize and secrete HBsAg in excess, mainly in the form of lipoprotein particles of 20 nm diameter [2,3]. SDS-polyacrylamide gel electrophoresis of HBsAg reveals the existence of two proteins, designated S and gS, which account for more than 90% of the protein content. These two proteins have identical amino acid sequence and differ only by the presence of carbohydrates attached to gS at position Asn-146 [4,5].

HBsAg is also an antigenically complex structure. All HBsAg have group-specific antigenic determinants designated 'a', which have been demonstrated to be composed of at least three different antigenic sites [6]. In addition, there are two sets of normally mutually exclusive subtype-specific determinants designated 'd/y' and 'w/r'. Thus, there are four major antigenic subtypes of HBsAg, adw, ayw, adr and ayr [7]. Additional complexities have led to the recognition of 10 different serotypes of HBsAg [8].

Thermal stability and chemical modification studies support the idea that S protein-related antigenic determinants are critically dependent on the conformation of HBsAg proteins [9–12]. Moreover, there are epitopes which show a consistent increase of antibody binding with in-

Abbreviations: HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; OG, β-D-octylglucoside; DPH, 1,6-diphenyl-1,3,5-hexatriene; PC, egg phosphatidylcholine; PE, egg phosphatidylethanolamine; PS, bovine brain phosphatidylserine; PI, bovine liver phosphatidylinositol; CL, bovine heart cardiolipin; DMPC, POPC and DOPC, 1,2-dimyristoyl-, 1-palmitoyl-2-oleoyl- and 1,2-dioleoylphosphatidylcholine; DPPS, DMPS, POPS and DOPS, 1,2-dipalmitoyl-1,2-dimyristoyl-, 1-palmitoyl-2-oleoyl- and 1,2-dioleoylphosphatidylserine; DPPG, DMPG and DOPG, 1,2-dipalmitoyl-, 1,2-dimyristoyl- and 1,2-dioleoylphosphatidylglycerol.

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creasing temperature. Such an increase in antibody binding occurs simultaneously with an increase in the fluidity of surface lipid regions, as monitored by fluorescence depolarization measurements of 1-(trimethylammoniophenyl)-6-phenyl-1,3,5-hexatriene [12]. This correlation, along with the observation that lipids play an important role in maintaining the structure and antigenic activity of HBsAg [13], is consistent with the conclusion that certain epitopes of HBsAg which are close to the lipid-protein interface, are dependent on the fluidity of the surface lipid regions. Thus, any change in the physical state of the lipids could confer a different degree of exposure to such antigenic determinants.

HBsAg proteins can be reconstituted into phospholipid vesicles. Negatively charged phospholipids are able to completely revert the conformational changes induced by removal of the lipids [14]. Reconstitution has been shown to enhance the immune response against viral proteins as demonstrated for influenza [15,16], herpes simplex virus [17], rabies virus [18] or human immunodeficiency virus [19]. When HBsAg is incorporated into liposomes composed chiefly of egg phosphatidylcholine and cholesterol and this material is used to immunize guinea pigs, the mean titre of induced antibodies is higher than that obtained in animals injected with free antigen [20]. Also, HBsAg polypeptide micelles obtained upon removal of lipids with Triton X-100 have been reported to be more immunogenic than the intact particle [21–25]. In this paper we describe the effect of the fatty acid composition of the phospholipid used to reconstitute HBsAg proteins on the antigenicity of the surface antigen, using both poly- and monoclonal antibodies.

2. Materials and methods

2.1. Materials

Phospholipids were purchased from Avanti Polar Lipids, except DPPS which was obtained from Sigma. β-D-Octylglucoside (OG) was purchased from Calbiochem.

2.2. 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]. 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 [26] or by amino acid analysis in which aliquots of protein (10–20 μg) were hydrolysed in sealed evacuated tubes with constant boiling HCl for 24 h at 110°C. Amino acid analysis was performed on a Beckman 6300 amino acid analyzer.

HBsAg was delipidated as previously described [13], except that 2.2% (w/v) OG was used. Under these conditions 75% of the total lipids were removed. Reconstitution was carried out by the detergent dialysis method as previously described [14]. The phospholipid/protein ratio was always maintained at 60 ng of phospholipid phosphorus/μg protein for maximum recovery of antigenic activity. When the effect of ionic strength was studied, dialysis was performed against 10 mM Tris, pH 7.0, containing 200 mM NaCl instead of 50 mM. Lipid phosphorus was determined by the micromethod of Barlett [27] as previously described [1]. Lipid homogeneity was assayed by thin-layer chromatography [28].

2.3. Fluorescence polarization measurements

DPH (1 mM) was dissolved in tetrahydrofuran and added to phospholipid vesicles or reconstituted HBsAg (60 μg protein/ml) at a ratio of 1 molecule of fluorophore for every 200 phospholipid molecules. In this range, fluorescence polarization (P) was not dependent upon concentration and/or light-scattering. Steady-state fluorescence polarization was measured on a Perkin-Elmer MPF 44-E spectrofluorimeter equipped with polarizers in both excitation and emission beams. P was calculated according to

$$P = (I_{VV} - G \cdot I_{VH})/(I_{VV} + G \cdot I_{VH})$$

where I_{VV} and I_{VH} are respectively the vertical and horizontal fluorescence intensities when the sample is excited with the excitation polarizer oriented vertically. The emission grating factor was calculated as $G = I_{HV}/I_{HH}$. Fluorescence anisotropy (r) was calculated from P values according to

$$r = 2P/(3 - P)$$

Temperature was controlled by a Lauda circulating water bath. The probe was excited at 365 nm and the emission was measured at 425 nm.

2.4. Antigenic activity assays

The antigenic activity of HBsAg was determined by using polyclonal (rabbit) anti-HBsAg antibodies as previously described [14] and also by using monoclonal antibodies as it follows. Microtiter plates (Costar) were coated with 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. Mouse anti-HBs (diluted in 0.5% (w/v) BSA in PBS, at the optimum dilution determined in a similar assay with a fixed amount of 100 ng/ml of HBsAg) was added after extensive washing and the plate incubated for 1 h and 45 min at 37°C. Biotinylated goat anti-mouse (Pierce) (1:4000 in PBS, 0.5% (w/v) BSA) was allowed to react for 1 h and 30 min at 37°C and the plates were exhaustively washed with 0.1% Tween 20. Avidin:biotinylated horse radish peroxidase complex

(Vectastain Elite ABC kit from Vector Laboratories) (1:200) was added and allowed to react for 1 h at 37°C. 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, monoclonal antibody-binding capacity was determined using an inhibition assay as previously described [13], which does not involve washing with 0.1% Tween 20. Since the same trend was observed, the assays were performed according to the former procedure. The absence of nonspecific binding of the different monoclonal antibodies to the phospholipids was assessed by coating plates with native or delipidated HBsAg in the presence of increasing amounts of the phospholipids and performing the assay as described above. The presence of phospholipids had no appreciable effect on the antigenicity of HBsAg. Monoclonals were prepared and characterized as previously described [6]. The six monoclonals are all directed against the HBsAg protein and have the following specificities: H35, H5 and H166 are directed against the group specific 'a' determinant but not against the same site since they do not compete with each other; H95 has the 'd' specificity and H12 and H53 can not be assigned to determinants classifiable by their reactions against the accepted NIH panel of subtypes [6].

3. Results

3.1. Reconstitution with natural phospholipids

The effect of the reconstitution procedure on the different epitopes of HBsAg was assessed with a panel of monoclonal antibodies which recognize both group and subtype specific antigenic determinants. When the reconstitution was performed with natural phospholipids the results shown in Fig. 1 were obtained. The antigenicity of all delipidated HBsAg epitopes (between 10–21%) increased upon reconstitution with both neutral and acidic phospholipids. However, the binding of all monoclonal antibodies was higher for HBsAg reconstituted with negatively charged phospholipids, PS, CL and PI (between 70–150%), than for the antigen reconstituted with neutral phospholipids, PC and PE (between 35–75%). Hence, all epitopes defined by the panel of monoclonal antibodies showed specificity for acidic phospholipids. The antibody binding results of HBsAg reconstituted with acidic phospholipids allowed to identify two groups of antigenic determinants: that defined by H35 and H95 with recoveries close to native HBsAg values (70–95%) and that defined by monoclonals H5, H12, H53 and H166 for which reconstitution with acidic phospholipids yielded values of antigenic activity higher than those of plasma derived antigen:

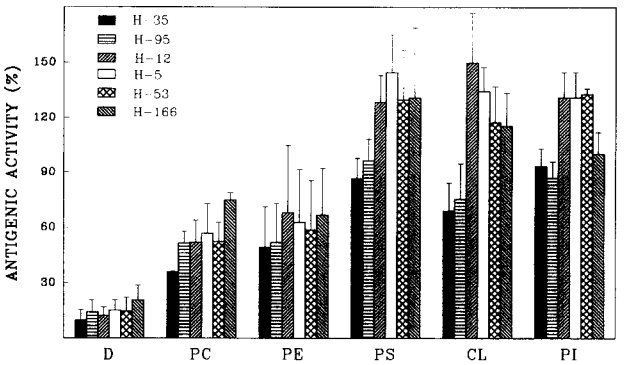

Fig. 1. Antigenic activity of HBsAg reconstituted with different natural phospholipids. The antigenic activity was measured with the indicated monoclonal antibody as indicated in Materials and methods. D, delipidated HBsAg; in all other cases, the phospholipid used for reconstitution is indicated. The reconstitution was performed at a phospholipid/protein ratio of 60 ng phosphorus/μg protein. The results are expressed as means ± S.D. of four different experiments.

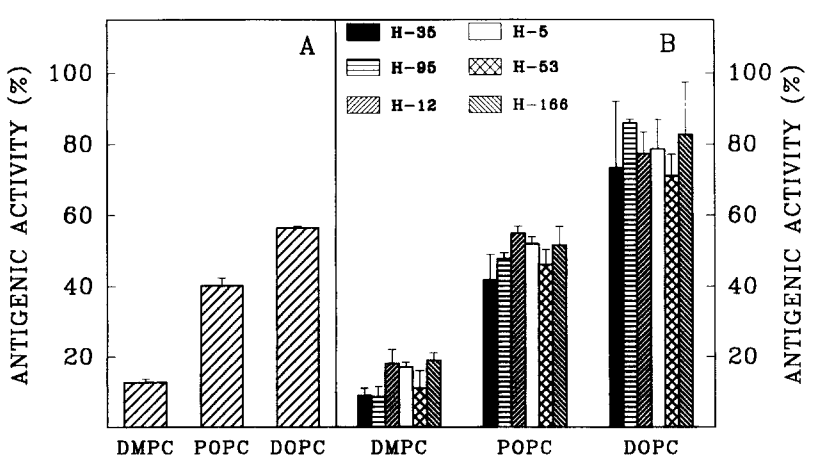

Fig. 2. Effect of acyl chain composition on antigenic activity of HBsAg reconstituted with phosphatidylcholine. Antigenic activity of HBsAg reconstituted with the indicated phospholipid was measured with polyclonal (A) and monoclonal antibodies (B). The reconstitution was performed at a phospholipid/protein ratio of 60 ng phosphorus/μg protein. The results are expressed as means ± S.D. of four different experiments.

129–150% for H12, 131–145% for H5, 117–133% for H53 and 100–131% for H166. Based on these results it is clear that the four epitopes recognized by these four monoclonals are the most dependent on the lipid moiety and, therefore, a higher binding to these determinants can be observed with the appropriate lipid composition.

On the other hand, the binding pattern of these four monoclonal antibodies was not maintained among acidic phospholipids-reconstituted HBsAg. Thus, reconstitution with PS induced the highest antigenic activity of H5 (145%), with CL that of H12 (150%) and PI-reconstituted gave similar results for H12 (131%), H5 (131%) and H53 (133%). These differences should reflect a different phospholipid-protein interaction in the reconstituted HBsAg.

3.2. Reconstitution with synthetic phospholipids

The role of the acyl moiety of the phospholipid on the recovery of antigenic activity upon reconstitution has been studied with phosphatidylcholine and two acidic phospholipids, phosphatidylglycerol and phosphatidylserine. When synthetic phosphatidylcholines were used the results of Fig. 2 were obtained. Reconstitution of HBsAg with phosphatidylcholine with two saturated fatty acids, DMPC, resulted in a slight increase of polyclonal antibody binding with respect to delipidated HBsAg (from 7% for delipidated HBsAg to 13% for DMPC-reconstituted antigen) (Fig. 2A). The presence of one or two unsaturated bonds in the phospholipid used for reconstitution resulted in a higher increase of polyclonal binding (40% and 57% for POPC and DOPC, respectively) (Fig. 2A). When the antigenicity was determined using monoclonal antibodies the same trend was observed (Fig. 2B) although reconstitution with DOPC induced higher recoveries of all antigenic determinants (between 71–86%) than those obtained with polyclonal antibodies (57%). Moreover, reconstitution with synthetic phosphatidylcholine did not induce any significant difference in the binding of the different monoclonals (between 42–55% for POPC and 71–86% for DOPC).

The results obtained with synthetic phosphatidylserines confirmed the importance of the fatty acid composition of the phospholipid used for reconstitution. The antigenic activity, measured with polyclonal antibodies (Fig. 3A), increased as the chain length of saturated phospholipids decreased (34% for DPPS and 53% for DMPS). Higher antigenicity values, similar to those of native HBsAg, were only observed when the phospholipid contained unsaturated fatty acids (96% and 84% for POPS and DOPS, respectively). Thus, it is clear that the nature of the fatty acid (its length and unsaturation degree) is important for the recovery of the antigenic properties of HBsAg. Similar conclusions can be drawn from the binding studies of monoclonal antibodies (Fig. 3B).

The specificity for acidic phospholipids observed upon reconstitution with natural phospholipids is clearly seen when comparing the results obtained with DMPC- and DMPS-reconstituted HBsAg. The former induced binding of all monoclonal antibodies similar to delipidated HBsAg (9–19%) while the latter induced much higher values (between 52–84%).

With POPS- and DOPS-reconstituted HBsAg two groups of determinants could be defined: the one defined by H35 and H95, with recoveries similar to those observed with polyclonal antibodies (80–100%) and that recognized by H5, H12, H53 and H166, with values higher than the native antigen (between 110 and 145%). The epitope defined by monoclonal H5 is the most active in all the phosphatidylserine-reconstituted HBsAg, with the exception of H166 when reconstituted with DOPS (145%). The epitope defined by monoclonal H166 is the only one which showed a consistent increase of binding with the degree of unsaturation of the fatty acid and, hence, the antigenic determinant defined by H166 is the most affected by this parameter.

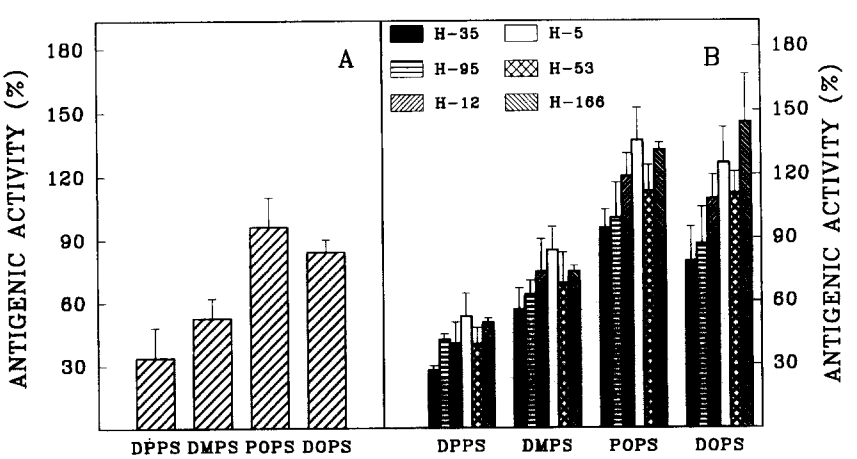

Fig. 3. Effect of acyl chain composition on antigenic activity of HBsAg reconstituted with phosphatidylserine. Antigenic activity of HBsAg reconstituted with the indicated phospholipid was measured with polyclonal (A) and monoclonal antibodies (B). The reconstitution was performed at a phospholipid/protein ratio of 60 ng phosphorus/μg protein. The results are expressed as means ± S.D. of four different experiments.

The results obtained when synthetic phosphatidylglycerols were used for reconstitution are depicted in Fig. 4. As occurred with other acidic phospholipids, reconstitution with some of the phosphatidylglycerols resulted in an antigenic activity similar to that of native HBsAg (86 and 98% for DMPG and DOPG, respectively) (Fig. 4A). When compared with the results obtained with phosphatidylserine, there was a marked difference in one of the phospholipids. While DMPS-reconstituted antigen had an antigenic activity of 52% of native HBsAg, the one reconstituted with DMPG yielded a higher value (86%). Thus, in the case of phosphatidylglycerol-reconstituted HBsAg, unlike those containing phosphatidylserine, the presence of unsaturation is not essential in order to obtain high recovery of antigenic activity.

Monoclonal antibodies binding studies of HBsAg reconstituted with DOPG and DMPG indicated a high recovery, higher than 100%, of all antigenic determinants (Fig. 4B). The highest binding corresponded to H12 and H166 when reconstitution was performed with DOPG. In this case the existence of the two groups of antigenic determinants that could be defined with other acidic phospholipids was not readily apparent.

The relationship between antigenic activity, measured with polyclonal antibodies, and the phase transition temperature of acidic phospholipids is depicted in Fig. 5. At 37°C, the temperature at which antigenic activity assays were performed, maximum antigenicity was observed with those phospholipids containing unsaturated fatty acids as well as for DMPG, all of which were in a liquid-crystalline state.

On the other hand, the effect of the increase of ionic strength during reconstitution with acidic phospholipids on the binding of polyclonal antibodies was tested (Fig. 6). It is clear from these results that electrostatic interactions are important in determining the degree of recovery of antigenic activity. If the ionic strength was increased, there

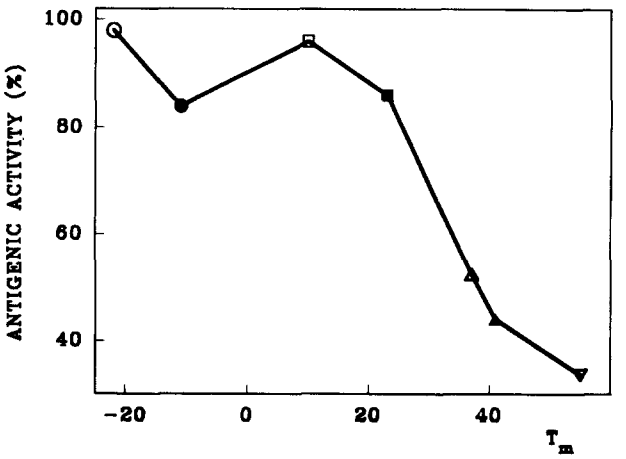

Fig. 5. Relationship between antigenic activity and phase transition temperature (T_m) of the phospholipid used for reconstitution. The antigenic activity was measured with polyclonal antibodies. The reconstitution was performed at a phospholipid/protein ratio of 60 ng phosphorus/μg protein. The symbols correspond to: (○), DOPG; (●), DOPS; (□), POPS; (■), DMPG; (△), DMPS; (▲), DPPG; (▽), DPPS. The results are representative of those obtained for four different experiments.

was a significant decrease in the antigenic activity that was recovered. Thus, DMPS-reconstituted HBsAg yielded a similar antigenicity to that of delipidated HBsAg; DOPG- and POPS-reconstituted antigen gave results similar to those obtained upon reconstitution with phosphatidylcholine of the same fatty acid composition. Moreover, the decrease in recoverable activity was greater as the degree of unsaturation decreased; in the case of DOPG-reconstituted the activity decreased from 98% to 61% while for POPS-reconstituted the decrease was from 96% to 42%. Hence, as indicated above, in addition to the electrostatic interactions in the polar head group region, the nature of the fatty acid of the phospholipid is important in determining the antigenic activity.

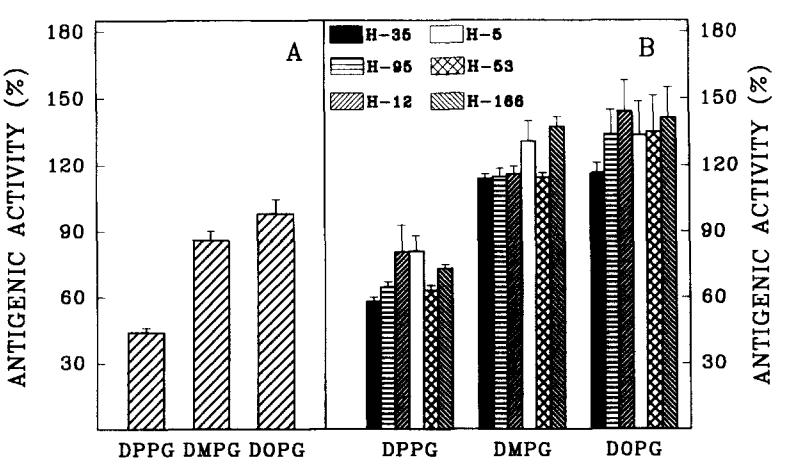

Fig. 4. Effect of acyl chain composition on antigenic activity of HBsAg reconstituted with phosphatidylglycerol. Antigenic activity of HBsAg reconstituted with the indicated phospholipid was measured with polyclonal (A) and monoclonal antibodies (B). The reconstitution was performed at a phospholipid/protein ratio of 60 ng phosphorus/μg protein. The results are expressed as means ± S.D. of four different experiments.

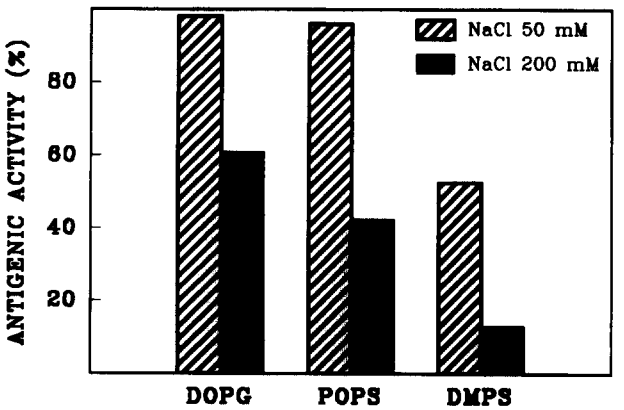

Fig. 6. Effect of ionic strength on antigenic activity of reconstituted HBsAg. After incubation of delipidated antigen (60 μg protein/ml) with the indicated phospholipid, at a phospholipid/protein ratio of 60 ng phosphorus/μg protein, in the presence of 1% OG, the mixtures were dialyzed against 10 mM Tris, pH 7.0, containing either 50 mM or 200 mM NaCl. Antigenic activity was determined with polyclonal antibodies. The results are representative of those obtained for four different experiments.

3.3. Effect of HBsAg proteins on the thermotropic behaviour of phospholipids

To verify the existence of phospholipid-protein hydrophobic interactions, the effect of HBsAg proteins on the thermotropic behaviour of synthetic phospholipids has been studied by fluorescence depolarization of the probe DPH. The results for three phospholipids which induced low, medium and high recovery of antigenic activity, DMPC, DMPS and DMPG, respectively, are shown in Fig. 7. Upon reconstitution with DMPC, the transition temperature of the phospholipid (20.5°C) increased to 26°C (Fig. 7A). Moreover, HBsAg proteins produced a decrease in the order of the lipid molecules below the phase transition temperature and an ordering on the liquid-crystalline state, thus decreasing the enthalpy of the phase transition. Similar conclusions can be drawn for DMPG, although the increase in the phase transition temperature was only 1°C with respect to pure DMPG vesicles (Fig. 7C). However, the effect was much more pronounced when reconstitution was performed with DMPS, since HBsAg proteins practically abolished the phase transition of pure vesicles (Fig. 7B).

4. Discussion

HBsAg proteins can be reconstituted into lipid-protein complexes with both neutral and acidic phospholipids resulting in particles with the same morphology and topology of surface proteins than plasma derived antigen. However, only negatively charged phospholipids are able to completely revert the conformational changes induced by removal of the lipids [14]. Moreover, the antigenic activity of reconstituted HBsAg, measured with polyclonal antibodies, is virtually indistinguishable from that of native antigen when PS, PI or CL were employed. In order to study the effect of reconstitution on the different antigenic determinants and to more clearly define the role of the phospholipid moiety, reconstitution with various phospholipids has been carried out, and the antigenic activity has been measured with a panel of monoclonal antibodies.

Electrostatic interactions between HBsAg proteins and acidic phospholipids are partly responsible for the complete recovery of the structural and antigenic properties. The presence of 200 mM NaCl during reconstitution with DMPS, POPS and DOPG brought the antigenic activity levels to those obtained with neutral phospholipids of the

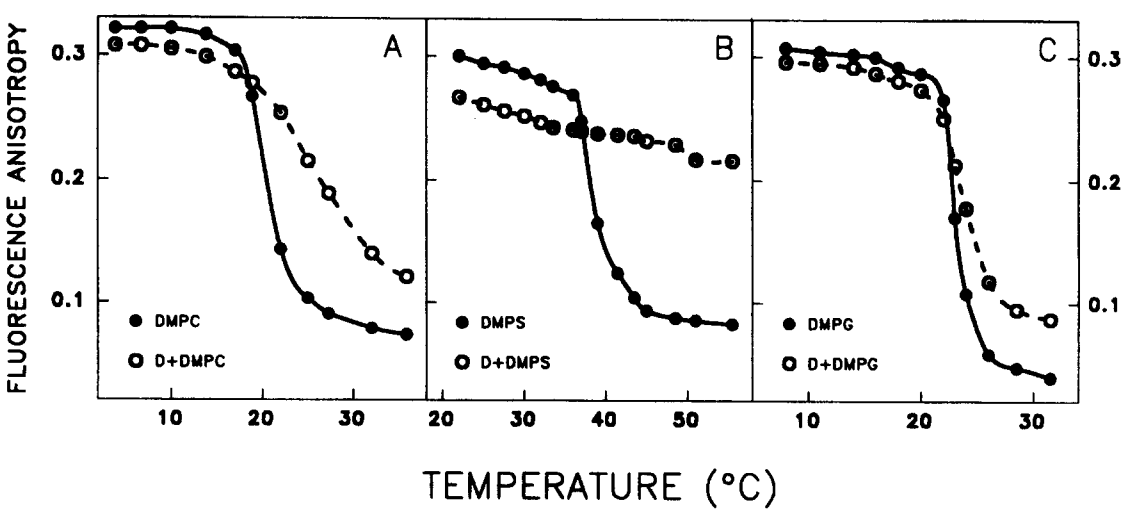

Fig. 7. Temperature dependence of fluorescence anisotropy of DPH labeled phospholipid vesicles and reconstituted HBsAg. Phospholipid vesicles (closed symbols) or HBsAg reconstituted with the corresponding phospholipid (open symbols) were labeled with DPH and fluorescence anisotropy was measured at the indicated temperature. The phospholipid concentration was 80 μM and the probe to lipid ratio was 1 to 200. The reconstitution was performed at a phospholipid/protein ratio of 60 ng phosphorus/μg protein. The results are representative of those obtained for three different experiments.

same fatty acid composition. Basic amino acid side chains in the exposed regions of HBsAg proteins would be most likely responsible for the interaction with acidic phospholipids. When this interaction was diminished by increasing the ionic strength during reconstitution, the recovery of antigenic activity would then depend on the fatty acid composition, regardless of the polar head group. Consistent with this interpretation is the fact that reconstitution with the natural acidic phospholipids PS, PI and CL, with similar average chain lengths and degree of unsaturation, induced similar antigenic activity (82–97%) [14]. However, the polar head group must have some differential effect since the antigenic activity of HBsAg reconstituted with phosphatidylserine is lower than that obtained with phosphatidylglycerol, of the same fatty acid composition. These observed differences could result from distinct electrostatic interactions between the lipids and the S protein due to differences in charge distribution, even though the phospholipids have the same overall net charge. Also, the greater interactions between HBsAg proteins and phosphatidylglycerol near the lipid-water interface, due to the lower capacity of this phospholipid to form intermolecular hydrogen bonds [29], could explain the differences.

In addition to the electrostatic interactions, fluorescence depolarization studies of the probe DPH suggest that HBsAg proteins interact hydrophobically with the phospholipids. This interaction would be greater for DMPS since the thermotropic behaviour of this phospholipid was the one most affected by the interaction with HBsAg proteins. However, the fact that this phospholipid is not the one which induced the highest recovery of antigenicity would suggest that part of these hydrophobic interactions are not involved in determining the most active conformation. The interactions of the phospholipids which modify the antigenic activity should take place at the surface of the phospholipid, near the exposed region of the protein where the antigenic determinants must be located and, therefore, not monitored by DPH which is assumed to probe internal regions, aligned with the phospholipid acyl chain [30]. Therefore, some specific interaction must confer the correct conformation of the antigenic determinants.

In addition to the nature of the polar head group, the fatty acid composition of the phospholipid also influenced the recovery of the antigenic activity. Except for DMPG, all of the saturated phospholipids which were tested induced lower antigenic activity than those containing unsaturated fatty acids. The phospholipid must possess at least one unsaturation to efficiently reverse the conformational changes that result from delipidation. Similar observations have been described in other systems. For example, fully active cytochrome P450 is only obtained by reconstitution with unsaturated phosphatidylcholine [31]; also T antigen from SV40 virus has a higher affinity for unsaturated than for saturated phosphatidylglycerol [32].

HBsAg reconstituted with saturated phosphatidylserine and phosphatidylglycerol, although not fully active, showed increased antigenic activity with decreasing chain length of the fatty acid. It could be argued that, as described for leucine transport system of *Lactococcus lactis* [33], lipid-protein interactions are a function of the thickness of the lipid bilayer which can alter thickness of the protein's hydrophobic portions. However, phospholipids of plasma derived HBsAg have an average chain length (17.6) [1], closer to that of palmitic than myristic acid and the latter induced higher polyclonal binding. It is accepted that the physicochemical properties imparted by its various lipids have a definite influence on the functional aspects of any given membrane. The results reported herein support the view that antigenic properties of HBsAg are dependent upon the physical state of the lipid moiety. Full antigenic activity is only recovered upon reconstitution with those phospholipids which are in the liquid-crystalline state at 37°C, the temperature at which antigenic activity is measured. Hence, the degree of exposure of the antigenic determinants would be a function of the fluidity of the lipid moiety. The appropriate membrane fluidity is required for optimum conformation but, once this conformation is established, additional interactions imparted by the different phospholipids give a difference in the patterns of monoclonal antibodies binding. On this same regard, the binding of monoclonal antibody H53 to native HBsAg shows a consistent increase with increasing temperatures [12].

Monoclonal antibodies binding studies allowed the classification of the different epitopes into two groups, based on their dependence on the phospholipid moiety: that defined by H35 and H95 which are the most difficult to reconstitute and that corresponding to H5, H12, H53 and H166 which reached levels with some acidic phospholipids much higher than those of native HBsAg. Of all the antigenic determinants only those close to the lipid-protein interface would change upon direct interaction with the phospholipids. The rest would depend on the correct protein conformation determined by the appropriate phospholipid composition. It has been demonstrated that Lys-122 of the S protein is essential for the 'd' determinant recognized by H95 and that monoclonals H35 and H95 compete for binding to HBsAg [6]; the epitopes recognized by these must then be located close to Lys-122. On the other hand, this region of the S protein is exposed in the intact particle [5] and does not change upon reconstitution [14]. These facts would indicate that the antigenic determinants that bind H35 and H95 are far from the lipid-protein interface and therefore would be the most difficult to reconstitute.

The second group of determinants interacted differently with the phospholipids. Upon reconstitution with acidic phospholipids the four monoclonals reached higher binding levels than those obtained with native antigen. As evidenced by circular dichroism, after reconstitution with negative phospholipids the overall structure is similar to that of native HBsAg [14] while the antigenic activity of these four determinants is higher than 100%. Therefore,

some direct interaction between these epitopes and the phospholipids must account for the increased antigenicity; their degree of exposure must be modulated by the lipid moiety either through interaction between basic residues of these epitopes and acidic phospholipids or through localized changes in these determinants which have to be close to the lipid moiety. In either case, electrostatic interactions must play an important role. Among those basic residues susceptible to interaction with negatively charged phospholipids, Lys-141 and 160 as well as Arg-169 are good candidates; they belong to one of the hydrophilic regions of the S protein where most of the antigenic determinants are predicted [34] and they are not cleaved by trypsin, probably because of their interaction with phospholipids.

Finally, it should be noted that an altered distribution of antigenic determinants could be responsible for the enhanced immune response observed upon reconstitution of subunit viral proteins of influenza [15,16], herpes simplex virus [17], rabies virus [18] and human immunodeficiency virus [19] or HBsAg polypeptide micelles obtained upon removal of lipids with Triton X-100 [21–25]. The reconstitution of HBsAg with acidic phospholipids clearly alters the conformation of the HBsAg epitopes, at least those recognized by the panel of monoclonal antibodies, with the binding to some of them higher than in native antigen. These reconstituted particles could have a higher immunogenicity as it has been demonstrated for HBsAg which was incorporated into liposomes composed chiefly of PC and cholesterol [20]. Under the conditions which these authors used (sonication of HBsAg in the presence of neutral phospholipids) some interchange of lipids could have taken place. Hence, since our results show that negatively charged phospholipids are more effective in reconstituting surface antigen, it could be possible to obtain a phospholipid-protein complex even more immunogenic by deliberately interchanging the natural lipids with acidic phospholipids.

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