Major Polar Lipids of Hepatitis B Antigen Preparations: Evidence for the Presence of a Glycosphingolipid

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The major phospholipids of hepatitis B antigen, subtype *adw*, were characterized as phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine. In addition, two carbohydrate-containing lipids were observed, one of which was characterized as a non-sialic acid-containing, water-soluble glycosphingolipid. Upon hydrolysis the glycosphingolipid yielded both hydroxy and non-hydroxy fatty acid as well as a long-chain base which was putatively identified as dihydrosphingosine.

The association between hepatitis B surface antigen (HB_sAg) and serum hepatitis is well established and has led to investigations of the nature of the antigen (1, 3, 4, 6, 8, 12, 15). It has been demonstrated (12, 15) that the serological activity of HB_sAg is not destroyed after digestion with proteolytic enzymes or after treatment with protein denaturants, but that the serological activity is destroyed after lipid extraction (12). More recently, Burrell et al. (3) have reported that carbohydrate is necessary for the serological activity of HB_sAg. On the basis of the above reports and because glycolipids are well established antigenic determinants (for review see ref. 16), the possibility that an antigenic determinant of HB_sAg is a glycolipid or is lipid-associated prompted us to examine the polar lipid composition of HB_sAg. In this paper we present evidence for at least one and probably two glycosphingolipids in purified HB_sAg.

MATERIALS AND METHODS

HB_sAg purification and criteria for purity. The method for purification of HB_sAg has previously been described (6). Briefly, HB_sAg positive serum, subtype *adw* (HB_sAg/*adw*), was pelleted at high speed, the resulting pellet acidified to pH 2.4 at 20°C for 1 h, suspended in a small volume of buffer, and banded twice isopycally on CsCl gradients. The material was further purified by rate zonal centrifugation in a linear sucrose gradient (20 to 40%, wt/wt). The ultrastructural and antigenic criteria for purity have been previously detailed (6). Briefly, each purified preparation used in this study was emulsified in Freund complete adjuvant and injected on days 0 and 14 into three guinea pigs. The antisera were tested for the presence of antibody to both HB_sAg and to normal human serum (HB_sAg negative) by immunodiffusion in gels and by two-dimensional complement fixation

titrations. None of the purified HB_sAg preparations elicited detectable antibody to normal human serum components. Electron microscopic examination of negatively stained preparations revealed only the presence of spherical particles measuring 17 to 25 nm in diameter. The plasma were subtyped by agar gel diffusion as described by Le Bouvier (13). Specific antisera for the *d* and *y* determinants were prepared in guinea pigs (11). Specific rabbit anti-*r* and anti-*w* sera were supplied by J. Gerin (Oak Ridge National Laboratory, Rockville, Md.).

Polyacrylamide gel electrophoresis (PAGE). Solubilization of the sample and preparation of the polyacrylamide gels as well as visualization of the carbohydrate-containing components with the periodate-Schiff reagent have been recently described (4).

Extraction and chromatography of the lipid. The lipids were extracted by using chloroform-methanol (20). In some instances the CHCl₃:CH₃OH extract was adjusted to a ratio of 2:1 by volume and then partitioned with water according to the method of Folch et al. (7). After extraction the lipids were taken to dryness under N₂ and redissolved in solvent A—CHCl₃:CH₃OH:H₂O:pyridine (20:28:6:1, by volume). For phospholipid analysis, the lipid extract was applied in a small spot to a sheet (5 by 7 in.) of EDTA-dipped, silicic acid-impregnated paper (SG81) and chromatography was carried out in two dimensions: (i) solvent B—CHCl₃:CH₃OH:NH₄OH:H₂O (66:27:3:0.9, by volume); (ii) solvent C—CHCl₃:CH₃OH:glacial acetic (16:2:3, by volume) (M.R. Steiner and S. Steiner, unpublished data). Authentic standards were chromatographed in a similar manner. The two-dimensional chromatograms were routinely visualized with I₂ vapor, although ninhydrin reagent and rhodamine 6G (20) were also used. After the above chromatography, each compound was individually excised and eluted, and the eluate was applied to a silicic acid thin-layer plate (Q-5, Quantum Industries, Fairfield, N.J.). Chromatography of the above was accomplished in one dimension by using solvent D—CHCl₃:CH₃OH:H₂O (60:35:8, by volume). Authentic standards were chro-

matographed in parallel lanes, and the phospholipids were visualized by use of the Dittmer-Lester reagent for phospholipid (5). Lipid phosphorus was estimated by the method of Bartlett (2).

For glycolipid analysis, either the total lipid extract or the "Folch aqueous phase" was applied in a streak to a silicic acid thin layer plate (Q-5, Quantum Industries, Fairfield, N.J.) and chromatographed in solvent D. Visualization of the glycolipid was generally accomplished by spraying a portion of the plate with the orcinol reagent (9). Additional chemical detection methods for carbohydrate were also employed, including resorcinol reagent (22), phenol-sulfuric reagent (21) and anthrone reagent (17).

Pronase treatment. A portion of the "Folch aqueous phase" which contained the carbohydrate-containing component was taken to dryness and resuspended in a solution of Pronase (0.1 mg/ml in 0.01 M Tris-hydrochloride, pH 8.0) and incubated at 37 C for 10 h. Controls with buffer alone and with heat-inactivated Pronase were also performed.

Alkaline degradation. Mild alkaline methanolysis was performed by using 0.2 N KOH in CH₃OH (14). Hydrolysis of the ceramide to fatty acid and long-chain base using Ba(OH)₂-dioxane was carried out as previously described (20).

Chromatography of long-chain base fraction. Chromatography of long-chain base fractions was carried out in one dimension on silica gel-impregnated paper (Whatman SG81) with solvent E—CHCl₃:CH₃OH:2 N NH₄OH (40:10:1, by volume) (18) and was visualized with the ninhydrin reagent.

Chromatography of the fatty acids obtained from glycosphingolipid. Fatty acids were obtained from the glycosphingolipid after hydrolysis in 2.9 N HCl in methanol at 75 C for 24 h followed by extraction into Et₂O or after Ba(OH)₂-dioxane hydrolysis (2), acidification to pH 1.0 with HCl, and extraction with Et₂O. The methyl esters of the fatty acids were chromatographed on SG81 paper in solvent F—benzene:CHCl₃:CH₃COOH (90:10:1, by volume) (18).

Chemicals. Phospholipid standards, i.e., phos-

phatidylcholine, sphingomyelin, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, diphosphatidylglycerol, and lysophosphatidylcholine, were obtained from Applied Science Co., Inc. (State College, Pa.) and Sigma Chemical Co. (St. Louis, Mo.). Ganglioside standards, ceramide standards with sphingosine base, sphingosine, and dihydrosphingosine were obtained from Supelco, Inc. (Bellefonte, Pa.). Ceramide standard containing a dihydrosphingosine base was obtained from Mile Laboratories (Kankakee, Ill.). Pronase was obtained from California Biochemical Co. (San Diego, Calif.).

RESULTS

Characterization of the phospholipids. The results of one- and two-dimensional chromatography (Table 1) show that the identity of the major phospholipids of HB_sAg are: phosphatidylcholine (PC), sphingomyelin (SPH), and lysophosphatidylcholine (LPC). The approximate percentage composition (i.e., based on lipid-phosphorus) was found to be PC (65%), SPH (30%), and LPC (5%). Even when a relatively large amount of phospholipid was chromatographed as above, ninhydrin-positive material could not be observed, thus making it unlikely that significant amounts of phosphatidylethanolamine or phosphatidylserine were present.

Characterization of the carbohydrate-containing lipid. Previous examination of glycoproteins of HB_sAg by periodate-Schiff staining of polyacrylamide gels revealed that in addition to the presence of several glycoproteins there was a periodate-Schiff-positive region near the gel front (4). This front material did not stain with the protein stain Coomassie blue and therefore the possibility that it was a glycolipid was considered. Extraction of the HB_sAg

TABLE 1. Identification of the major phospholipids^a of HB_sAg

Presumed parent phospholipid	Two-dimensional chromatography (1st dimension, solvent B ^b ; 2nd dimension, solvent C ^c)				One-dimensional chromatography (solvent D ^d)	
	Standard R _f values		Observed R _f values		Standard R _f values	Observed R _f values
	1st dimension	2nd dimension	1st dimension	2nd dimension		
Phosphatidylcholine	0.58	0.09	0.58	0.09	0.81	0.81
Sphingomyelin	0.33	0.10	0.33	0.10	0.64	0.64
Lysophosphatidylcholine	0.17	0.02	0.17	0.02	0.43	0.43

^a Additional phospholipid standards and their two-dimensional chromatography R_f values (i.e., 1st and 2nd, respectively) include: phosphatidylethanolamine, 0.60, 0.31; phosphatidylserine, 0.25, 0.12; diphosphatidylglycerol, 0.67, 0.62; and phosphatidylinositol, 0.15, 0.03. See text for the details of the chromatography.

^b Solvent B, CHCl₃:CH₃OH:NH₄OH:H₂O (66:27:3:0.9, by volume).

^c Solvent C, CHCl₃:CH₃OH:glacial CH₃COOH (16:2:3, by volume).

^d Solvent D, CHCl₃:CH₃OH:H₂O (60:35:8, by volume).

using chloroform-methanol completely removed the periodate-Schiff-positive front material. On the other hand, the extraction did not affect the gel profile of the glycoproteins. When the lipid extract was applied to thin layer plates, chromatographed in solvent D, and the plates sprayed with the orcinol reagent, two carbohydrate-positive compounds were revealed—glycosphingolipid-1 (GSL-1) and glycosphingolipid-2 (GSL-2) (Fig. 1). Parallel chromatograms revealed these components to also be positive with other carbohydrate reagents (i.e., anthrone and phenol-sulfuric acid). On the other hand, GSL-1 and GSL-2 were found to be

negative when tested for sialic acid (22), for α -amino nitrogen, and for lipid-phosphorus (5). In addition, the chromatographic mobility of both compounds was unaffected by Pronase digestion, thereby supporting the previous observations (i.e., Coomassie blue stain) that GSL-1 and GSL-2 were not proteins.

Since most higher animal cell glycolipids are sphingolipids, we set forth to examine the possibility that GSL-1 was also a sphingolipid. Two primary criteria were used to establish the sphingolipid nature of GSL-1: (i) resistance to mild alkali treatment and (ii) release of fatty acid and long-chain base as the lipid hydrolysis

FIG. 1. Chromatograph of orcinol-positive compounds. Chromatography was carried out on silica gel thin layer plates in solvent D and sprayed with orcinol reagent. Glycosphingolipid-1 (GSL-1) and glycosphingolipid-2 (GSL-2) are the compounds obtained from the HB_sAg lipid extract. GD-1, i.e., disialoganglioside, and lactosylceramide are authentic standards used for R_f comparison.

products. With respect to the first criterion, it was observed that treatment of GSL-1 under mild alkali conditions did not result in the release of any detectable lipid nor did it affect the chromatographic properties of GSL-1. In contrast, treatment of authentic phosphatides under parallel conditions resulted in release of the fatty acid moiety and a dramatic alteration in their chromatographic properties. With respect to the second criterion, the release of fatty acid and long-chain base as the lipid degradation products was used to identify the lipid portion of GSL-1 as ceramide. Ceramide is the characteristic lipid moiety of sphingolipids. Both acid and base hydrolysis conditions, suitable to hydrolyze ceramides, were employed to catalyze the release of the fatty acid from GSL-1. On the other hand, acid-catalyzed hydrolysis resulted in conversion of a significant portion of the long-chain base to a high R_f artifact (20) and was therefore not employed in studies of the long-chain base. The fatty acid pattern obtained from acid or base degradation of GSL-1 can be seen in Fig. 2. Based on the chromatographic patterns, GSL-1 appears to have both hydroxy and non-hydroxy fatty acid and a third class of fatty acid as yet unidentified. All the rhodamine and I_2 vapor-positive material in the fatty acid fraction was observed in these three components. More sensitive analytical methods (e.g., gas liquid chromatography) will have to be employed in order to identify the individual fatty acids. The results of chromatography of the long-chain base fraction of GSL-1 (Fig. 3) after base hydrolysis indicated it to be dihydrosphingosine. No other ninhydrin or I_2 vapor-positive material was observed on the chromatograph of this fraction. Thus, the results are consistent with the interpretation that the major carbohydrate-containing organic solvent extractable compound (i.e., GSL-1) of HB_sAg is a glycosphingolipid.

DISCUSSION

Kim and Bissell (12) reported the presence of phosphatidylcholine, sphingomyelin, and phosphatidylethanolamine in HB_sAg but did not report the presence of organic extractable carbohydrate-containing material. On the other hand, in studies of the glycoproteins of HB_sAg we observed carbohydrate-containing material which was believed to be glycolipid (4). On that basis we undertook an examination of the polar lipids (i.e., phospholipid and glycolipid) of HB_sAg. In contrast to the phospholipid results reported by Kim and Bissell (12), we report the major phospholipids to be phosphatidylcholine,

FIG. 2. Silicic acid paper chromatography of fatty acid portion of glycosphingolipid GSL-1. GSL-1 was purified by aqueous Folch partition of the lipid extract, followed by thin-layer chromatography on Q-5 plates in solvent D. After excision, elution and concentration of the material under N_2 , it was further purified by rechromatography in solvent D. A small portion of the purified material was examined for purity by chromatography in solvent D followed by charring with 50% H_2SO_4 . Samples were hydrolyzed either in methanolic-hydrochloride or in $Ba(OH)_2$ and partitioned. The samples were chromatographed on silicic acid-impregnated paper (SG81) in solvent E and visualized with I_2 vapor. None of the compounds were positive with ninhydrin. Lane 1, authentic methyl-2 hydroxystearate; lane 2, methyl stearate; lane 3, fatty acid fraction after methanolic-hydrochloride methanolysis; lane 4, fatty acid fraction following $Ba(OH)_2$ hydrolysis and methylation of free fatty acids.

sphingomyelin, and lysophosphatidylcholine (65, 30, and 5%, respectively). We did not observe the presence of phosphatidylethanolamine nor did we observe any ninhydrin-positive material in the total lipid extract. The differences in the phospholipid composition between these two studies could stem from differences in the HB_sAg preparations used or from differences in the method of analysis. For example, the identification of the phospholipids in our study was based on chromatography in three separate solvent systems with visualization accomplished by use of specific (i.e., phospholipid spray, ninhydrin reagent) and less specific rea-

FIG. 3. Chromatography of the long-chain base fraction of glycosphingolipid GSL-1. For purification of GSL-1, see the legend for Fig. 2. Chromatography of long-chain base products from compound GSL-1 was carried out on silicic acid-impregnated paper (SG81) in solvent F and visualized with ninhydrin. Open circles denote purple color. Lane 1, authentic ceramide containing sphingosine base hydrolyzed in $\text{Ba}(\text{OH})_2$ -dioxane as described in the text. Lane 2, authentic ceramide with dihydrosphingosine base hydrolyzed in $\text{Ba}(\text{OH})_2$ -dioxane as described in the text. Lane 3, GSL-1 hydrolyzed in $\text{Ba}(\text{OH})_2$ -dioxane.

gents (i.e., I_2 vapor, rhodamine 6G). In contrast, Kim and Bissell (12) based the phospholipid identification of HB_sAg on chromatography in a single solvent system and used only less specific reagents for visualization of the compounds (i.e., I_2 vapor and charring with H_2SO_4).

In addition to the phosphorus-containing lipids, two chloroform-methanol extractable carbohydrate-containing compounds were also observed. One of these compounds (GSL-1) has been putatively identified as a glycosphingolipid based on: (i) extractability with organic solvents, (ii) presence of carbohydrate, (iii) mild alkaline stability, (iv) Pronase resistance, and (v) the presence of long-chain base and fatty acid as the lipid hydrolysis products. An insufficient amount of the other carbohydrate-containing compound (i.e., GSL-2) was available for as complete an analysis, although its

extractability with organic solvents, its mild alkaline stability, the presence of carbohydrate, and the resistance to Pronase suggest that it is also a glycolipid. While the nature of the oligosaccharide moiety of GSL-1 is unknown, based on its chromatographic mobility as compared to glycolipid standards, it would be predicted to be relatively large. Similar to gangliosides, GSL-1 was found to be water-soluble, but, unlike gangliosides, it was found to lack sialic acid. Such properties (i.e., water solubility and the absence of sialic acid) are reminiscent of the fucosylglycolipids or blood group glycolipids (10). Although most of the studies were performed by using $\text{HB}_s\text{Ag}/\text{adw}$, preliminary examination of $\text{HB}_s\text{Ag}/\text{ayw}$ revealed the presence of carbohydrate-containing lipid components of similar R_f values to those observed with $\text{HB}_s\text{Ag}/\text{adw}$ as well as the same phospholipid pattern.

Because of the reported involvement of carbohydrate and lipid in the antigenicity of HB_sAg (3, 12), the finding of glycosphingolipid is particularly noteworthy. We are presently accumulating sufficient quantities of glycosphingolipids GSL-1 and GSL-2 to determine whether or not they possess HB_sAg antigenic determinants.

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