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Physicochemical Properties of Recombinant Hepatitis B Surface Antigen Expressed in Mammalian Cell (C127)

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The physicochemical properties of recombinant hepatitis B surface antigen (r-HBsAg), which was expressed in C127 mammalian cell were studied. Using roller bottle culture in DMEM supplemented with fetal bovine serum, 10-15 mg/L of r-HBsAg was produced with about 31% of purification yield. The purity of r-HBsAg by HPLC was 99.8% and electron microscopic examination showed homogeneous spherical particle with 22 nm in diameter, a morphological characteristic of HBsAg. The density of r-HBsAg by CsCl density gradient method was 1.19 g/ml and the isoelectric point by Mono P™ HR 5/20 column was 4.6. The analysis of subunit protein pattern using SDS-PAGE followed by scanning densitometry gave 81.3% of S protein and 18.7% of pre-S protein. Fluorophore-assisted-carbohydrate-electrophoresis analysis showed the relative amount of carbohydrate to protein was 1.7% and its major component was N-acetyl glucosamine, which was about 39% of total carbohydrate. The relative amount of lipid to protein determined by vanillin phosphoric acid method was 32.5% and its major component was phospholipid, which was about 70% of total lipid. The physicochemical properties of C127 mammalian cell-derived r-HBsAg are similar to those of p-HBsAg, suggesting that the r-HBsAg can be used in developing a new preventive vaccine against hepatitis B.

Key words: Recombinant hepatitis B surface antigen, Physicochemical properties, Mouse fibroblast cell (C127), Fluorophore-assisted carbohydrate electrophoresis

INTRODUCTION

Hepatitis B virus (HBV) has been known to cause hepatitis B (Blumberg *et al.*, 1965; Blumberg *et al.*, 1968), and epidemiological study has showed hepatitis B is, in turn, a major causative agent of liver cirrhosis and hepatocellular carcinoma (Beasley *et al.*, 1984). The number of chronic patients of hepatitis B in the world is about 350 million, of which 90% is in developing countries. Hepatitis B virus can be found in the serum of HBV chronic carrier as 42 nm Dane particle (virion), 22 nm spherical particle and 100 nm filamentous particle (Dane *et al.*, 1970). Dane particle consists of an envelope carrying HBsAg, a capsid, carrying HBcAg and circular DNA molecule. Among these, 22 nm spherical particle is used for the preparation of preventive vaccine. The HBsAg in viral envelope has one well characterized common determinant, '*a*', and two sets of mutually exclusive subtype determinants, d/y and w/r. Thus four major subtypes of HBsAg are adw, ayw, adr and ayr. These subtypes have an unequal distribution worldwide (Courouce-Pauty *et al.*, 1983). HBsAg particle is a complex macromolecular structure comprised of lipids and proteins, some of which are glycosylated (Tiollais *et al.*, 1985). Glycoproteins are anchored in a lipid bilayer. Lipid components play a role in the helical structure of HBsAg proteins and in its antigenicity (Gavilanes *et al.*, 1990). The particles contain three surface proteins, called the major, the middle and the large, produced by a single long open reading frame. The major protein encoded by S gene in two forms, non-glycosylated (P 24) and glycosylated (GP27). The middle protein encoded by pre-S2 region and S gene is present in two forms, monoglycosylated (GP33) and diglycosylated (GP36). The large protein encoded by the pre-S1 region, pre-S2 region and S gene is present in non-glycosylated (P39) and glycosylated (GP42) (Stibbe and Gerlich, 1983; Heerman *et al.*, 1984). It has been postulated that HBV has binding sites for polymerized human serum albumin (pHSA) and that it attaches to the surface of the hepatocytes via polymeric albumin (Gerlich *et al.*, 1993). Part of pre-S and all of S encode the middle protein component carrying the pHSA receptor (Machida *et al.*, 1984). Pre-S specific antibodies to the polyalbumin receptor may interfere directly with the binding of HBV to hepatocytes and

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therefore be capable of preventing the initial step of infection. Therefore, pre-S protein of HBsAg particle is required for polyalbumin receptor and provides a method for the preparation of a potentially efficacious vaccine (Neurath *et al.*, 1986). Currently available commercial hepatitis B vaccines are prepared from HBV carrier plasma-derived HBsAg, yeast origin (*Saccharomyces*- or *Hansenula*-derived) HBsAg, or mammalian cell origin (CHO cell-derived) HBsAg (Michel *et al.*, 1985). Although plasma-derived particles serve as an effective vaccine, their use is limited, first, by the supply of HBV carrier plasma, later, also by the risk of using human plasma component. Today there are efficient recombinant vaccines, based on HBsAg particles derived from yeast cells and CHO cells. In HBsAg expressed in yeast cells, the immunodominant region contains little of middle protein, which is encoded by pre-S (Valenzuela *et al.*, 1982). HBsAg expressed in CHO cells has limitation in its clinical use by low productivity and high cost. In this study, we examined the physicochemical properties of HBsAg expressed in mouse fibroblast cell (C127), regarding morphological characteristic, density, isoelectric point (IEP), subunit protein pattern, carbohydrate content and lipid content.

MATERIALS AND METHODS

Transfected cell line preparation

Mouse fibroblast cell line C127, developed by Dr. Howley of NIH (U.S.A.) was used for transfection of plasmid pHPS1 encoding pre-S and S of human hepatitis B virus (HBV). The selection of HV1-4 cell that produced the high level of HBsAg was accomplished and generously provided by Dr. Samanta of Eugenetech International (Samanta *et al.*, 1989).

Roller bottle culture

HV1-4 cells were grown in DMEM (Dulbecco's Modified Eagles Medium) supplemented with 10% fetal bovine serum, 40 μM $CdCl_2$ and 1% penicillin-streptomycin. 5×10^7 cells of HV1-4 were passaged in a roller bottle (490 cm^2 in surface area), followed by rotation of 0.5 rpm at 37°C with a change of culture media on every 3-4 days for 2 months. The level of HBsAg in each harvest was assessed by enzyme-linked immunosorbent assay (ELISA) and the levels of glucose and lactate were measured for examining a correlation between the cellular metabolic rate and HBsAg production.

Purification of HBsAg

The cell-free supernatant was brought by filtering through cartridge filter (0.2 μm) and ultrafiltration (Millipore Pellicon System, mol wt 300 kd cutoff), then processed for further purification using KBr gradient ultracentrifugation, Sephacryl S-500 gel chromatography and Mono Q-Sepharose chromatography.

SDS-PAGE analysis

The purified HBsAg were analyzed by SDS-PAGE under the denaturation and reduction condition. Subsequently subunit proteins were quantitated by using scanning densitometry of SDS-PAGE bands.

N-terminal amino acid sequence analysis

The purified r-HBsAg (5-30 nmole) was further analyzed by Western blotting using guinea pig polyclonal antibody against HBsAg for the location of each subunit of r-HBsAg. The area of S antigen was dissected out for the determination of N-terminal amino acid sequence using amino acid sequence analyzer (Applied Biosystems).

Monosaccharide analysis

Two hundred μg of purified r-HBsAg was deglycosylated by incubation with peptide N-glycosidase F (PNGase F). The deglycosylated r-HBsAg was hydrolyzed by the addition of various agent for different monosaccharide, such as, 0.1 N TFA for neuramic acids (N-acetylneuraminic acid and N-glycosylneuramic acid), 2 N TFA for neutral sugar (glucose, mannose, fucose and galactose), and 4 N HCl for amino sugar (N-acetylglucosamine and N-acetylgalactosamine). The hydrolyzed product was labeled with ANTS (8-amino naphthalene 1,3,6 tri-sulfonic acid) and separated by FACE (fluorophore-assisted-carbohydrate-electrophoresis) method. Subsequently, monosaccharides were quantitated by using scanning densitometry (Hu *et al.*, 1994; Starr *et al.*, 1996).

Lipid analysis

Total lipid: Lipid standard Merck-O-test standard (1~500 μg) and samples (1 mg) were hydrolyzed in test tubes by adding 2 ml of conc. sulfuric acid, and heating in the boiling water for 10 minutes. The hydrolyzed lipid standards and samples (10 μL) were mixed with 2 ml of colorizing agent (vanillin, 8 mmole/L; phosphoric acid, 11.9 mole/L) and reacted for 40~50 minutes. The total lipid was calculated by measuring absorbance at 530 nm.

Cholesterol: The lipid components of HBsAg were extracted by the method of Bligh-Dyer (Bligh and Dyer, 1959) and the level of cholesterol was measured by enzyme assay (Cholesterol analyzing enzyme reaction kit, Merck, U.S.A.) (Ansari and Smith, 1979).

Triglyceride: In the presence of p-chlorophenol, 4-amino antipyrine and peroxidase, H_2O_2 generates color-

ed compound, quinonimine, which can be measured using spectrophotometric method. Samples and standards (10 μL of each) were added to 1 ml of reaction buffer containing enzyme and substrate, then reacted for 10 minutes at 20~25 °C. The amount of triglyceride is determined by measuring the absorbance of the developed color at a wavelength of 505 nm.

Phospholipid: Sample solution extracted by Bligh Dyer method was evaporated with N_2 gas. The dried sample were mixed with 0.4 ml of ashing reagent (60% perchloric acid:95% sulfuric acid=1:1). The reaction product was mixed with 2.0 ml of molybdate reagent (1 g ammonium molybdate/100 ml) and 0.1 ml of reducing agent (sodium bisulfite 15 g, 1-amino-2-naphthol-4-sulfonic acid 0.25 g and sodium sulfite 1 g/100 ml). The absorbance at 820 nm was measured for quantitation of phospholipid.

RESULTS AND DISCUSSION

Cell culture

The cultivation of the HV1-4 cell line was performed for 2~3 months continuously. This duration of the culture was about 3~4 times longer than that could be done in CHO cells. The yield of HBsAg from HV1-4 cells were 10~20 mg/L on every three days since the 20th day of the culture (Fig. 1), which was comparable to 5~30 mg/L in CHO cell culture(Michel *et al.,* 1984). During the 60 days of culture in roller bottle, glucose consumption was increased to 90~95% of input glucose by 20~25 days post-cultivation. Lactate concentration was increased to 1.3~1.5 g/L by day 15 but then decreased to 0.2~0.3 g/L in production phase (after 15~20 days), in which HBsAg production was rapidly increased to 17 mg/L by day 50. In this study, after the 2 months of culture, cell membrane was degraded after 2 months of cultivation with the increase of lactate production, which induces cell death and reduction of HBsAg production.

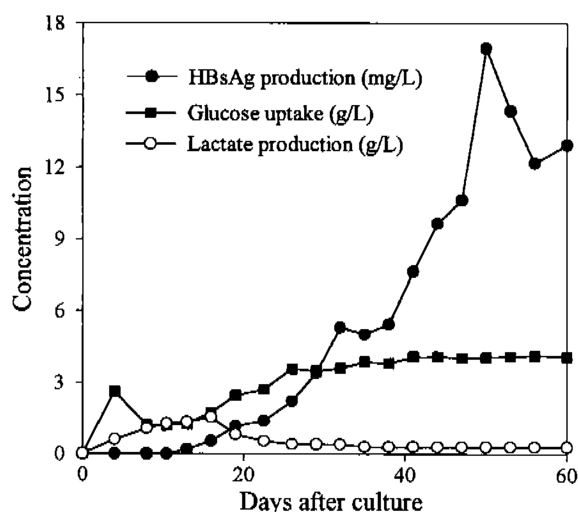

Fig. 1. Periodic glucose uptake, and lactate and HBsAg production during HV1-4 cell culture in roller bottle.

Purification

HBsAg was purified by ultrafiltration using a 300 kd cutoff membrane filter, in which about 95% of foreign proteins were efficiently removed. The harvested concentrate was treated with 4% PEG, followed by the treatment of the supernatant with 10% PEG, then the pellet was further purified by KBr-gradient ultracentrifugation to remove lipids and lipoproteins. Sephacryl S-500 gel filtration was performed to remove high molecular weight proteins (albumin etc). HBsAg was extracted by applying Mono-Q chromatography with 0.15 N NaCl. The estimated total purification yield was about 31% (Table I). The analysis of the sample using HPLC (column: TSK gel G6000 PW) gave 99.8 % purity. In terms of physicochemical characterization of HBsAg, UV spectrophotometry, CsCl density gradient centrifugation and isoelectric point (IEP) were performed. The UV spectrum of r-HBsAg shows absorbance peak at 280 nm and the shoulder at 290 nm. The IEP was pH 4.6, which is almost identical to that of p-HBsAg and the density of r-HBsAg was 1.186, which is also similar to 1.215 of p-HBsAg, indicating that r-HBsAg has physicochemical characteristics which are almost identical to those of p-HBsAg.

Electron microscopy

The purified r-HBsAg and p-HBsAg were examined by transmission electron microscopy with magnification of 100,000. The electron microscopy showed both r-

Table I. Purification of r-HBsAg expressed in mammalian cell (C127)

	Volume (ml)	Protein (mg/ml)[a]	Total protein (mg)	HBsAg (μg/ml)[b]	Total HBsAg (mg)	Recovery (%)
Culture medium	46,500	5.85	272,025	7.6	353.4	100
Diafiltration	2,000	4.85	9,700	166.0	332.0	93.9
PEG 8000 fractionation	550	4.18	2,300	486.0	267.3	75.6
KBr gradient	570	0.50	285.0	310.4	177.0	50.1
Sephacryl S-500	1,530	0.16	244.8	104.6	160.0	45.3
Mono-Q Sepharose	160	0.70	112.0	692.0	110.7	31.3

[a]Protein assay was carried out by Lowry method.
[b]HBsAg content was measured by ELISA using monoclonal antibody DDI-1.

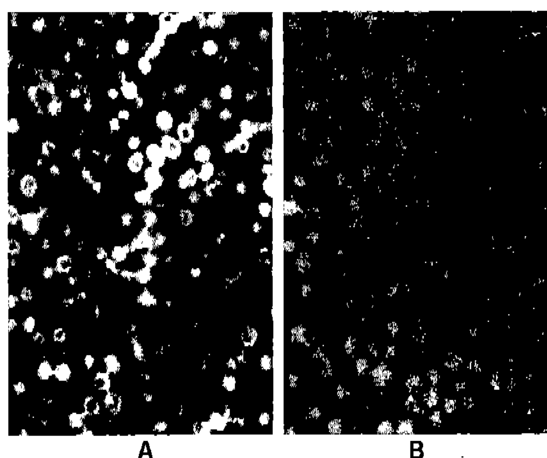

Fig. 2. Electronmicroscopic investigation of r-HBsAg (A) and p-HBsAg (B). Negative staining method. The concentration of the samples was adjusted to 1 mg/ml of r- and p-HBsAg, respectively, in pH 7.4 PBS. Magnification, (100,000. Homogeneous spherical particles (22 nm in diameter) were present.

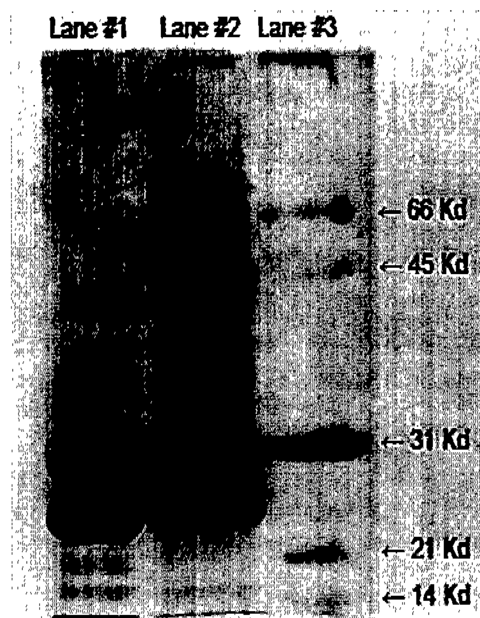

Fig. 3. Comparison of subunit protein pattern between r-HBsAg and p-HBsAg. Lane 1, r-HBsAg; lane 2, p-HBsAg; lane 3, molecular weight marker.

HBsAg and p-HBsAg have similar spherical particle shape of 22 nm in diameter, which is one of the morphological characteristics of HBsAg (Fig. 2).

SDS-PAGE

SDS-PAGE patterns were shown in Fig. 3 and subunit protein patterns analyzed by scanning densitometry were presented in Table II. The previous studies showed that the composition of pre-S of HBsAg from different sources was quite various, 0% (*Hansenula*), 23.7% (CHO) (Diminsky *et al.*, 1997). Pre-S composition of C127-derived HBsAg was 18.7%, indicating that it is similar to that of CHO-derived HBsAg. Thoma *et al.* (1990) reported that the duration of protection by pre-S antigen persists longer than that by S antigen. Akahane *et al.* (1993) and Sherlock *et al.* (1983) also reported pre-S vaccine is effective in non-responder that had vaccination with S antigen only. Also, vaccination with pre-S vaccine is known effective against vertical infection since pre-S antibody is uprising before S antibody (Neurath *et al.*, 1986; Krone *et al.*, 1990). The analysis of the C127-derived r-HBsAg in this study demonstrated that it contains high level of pre-S, suggesting that it might be useful for the high risk personale such as new-born babies in potential maternal vertical infection and medical employees as well as general public.

N-terminal amino acid sequence

The N-terminal amino acid sequence analysis on purified r-HBsAg from HV1-4 cell was performed by Edman degradation method.

NH_2-Met Glu Ser Thr Thr Ser Gly Phe Leu Gly
Pro Leu Leu Val Pro Gln Ala Gly-COOH

Our results showed that r-HBsAg of HV1-4 had a similar sequence with the previously reported sequence of adr type of p-HBsAg. One amino acid substitution was at the third position, replacing Asn to Ser.

Monosaccharide analysis

The relative amount of carbohydrate to protein (w/w) was 1.7% and N-acetylglucosamine known as

Table II. Quantitative analysis of HBsAg protein in SDS-PAG

Source	Protein (weight %)[a]								
	Small (S)			Middle (M)			Large (L)		
	24 kd	27 kd	Total	33 kd	36 kd	Total	39 kd	42 kd	Total
r-HBsAg	42.7	38.6	81.3	12.5	0.8	13.3	4.1	1.3	5.4
p-HBsAg	37.4	33.2	70.6	8.0	2.2	10.2	6.6	12.5	19.1

[a]The amount of protein was measured by scanning densitometry following SDS-PAGE. Small protein (S), 226 amino acids long and encoded by the S gene; Middle protein (M), 281 amino acids long and encoded by the pre-S2 region; Large protein, 389 or 400 amino acids long and encoded by the pre-S1 region, pre-S2 region and S gene.

Table III. Monosaccharide analysis of r-HBsAg

Sugar[a]	Mol. weight	Carbohydrate (p mole)[b]	Carbohydrate (μg)[c]	Molar ratio (%)
GalNAc	203.2	558	0.113	3.2
Mannose	162.2	2833	0.460	16.8
Fucose	146.2	1273	0.186	7.5
Glucose	162.2	393	0.064	2.3
Galactose	162.2	3013	0.489	17.8
GlcNac	203.2	6372	1.295	37.7
NANA	291.3	2474	0.721	14.7
NGNA	307.3	0	0.000	0.0
Total			3.328 (1.7%)	100

[a]GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; NANA, N- acetylneuramic acid; NGNA, N-glycosylneuramic acid.
[b]Carbohydrate content was measured by fluorophore-assisted-carbohydrate electrophoresis.
[c]The values were expressed in carbohydrate content (μg) per 200 μg protein.

Table IV. Monosaccharide analysis of p-HBsAg[a]

Sugar[a]	Mol. weight	Carbohydrate (p mole)	Carbohydrate ((g)	Molar ratio (%)
GalNAc	203.2	722	0.147	4.0
Mannose	162.2	3319	0.538	18.3
Fucose	146.2	0	0.000	0.0
Glucose	162.2	0	0.000	0.0
Galactose	162.2	3326	0.539	18.4
GlcNac	203.2	9128	1.855	50.3
NANA	291.3	1634	0.476	9.0
NGNA	307.3	0	0.000	0.0
Total			3.555 (1.8%)	100

[a]See the footnote in Table III.

bacterial cell wall component was recognized as a major carbohydrate with 39% of total carbohydrate (Table III). The glycosylation level of r-HBsAg (1.7%) was comparable to that of p-HBsAg (1.8%)(Table IV), while lower than that of CHO-derived r-HBsAg (2.1%) (Diminsky *et al.*, 1997). The composition of glycosylation shows N-acetylglucosamine is a major component as 39% in r-HBsAg and 52% in p-HBsAg, and particularly small amount of fucose was found in r-HBsAg but not in p-HBsAg.

Lipid analysis

The particles of r-HBsAg and p-HBsAg were analyzed for their cholesterol, triglyceride, phospholipid and total lipid level. As shown in Table V, total lipid were 325 and 260 μg/mg protein, cholesterol was 62.4 and 42.9 μg/mg protein, respectively, in r-HBsAg and p-HBsAg. Phospholipid was 226.3 and 176.8 μg/mg protein and triglyceride was 44.3 and 37 μg/mg protein in r-HBsAg and p-HBsAg, respectively. Gavilanes *et al.* (1990) reported that most of the lipid components could be removed by beta-D-octyl glucoside, which

Table V. Lipid composition of r-HBsAg[a]

Batch No	Total lipid	Cholesterol	Phospholipid	Triglyceride
1	318	61.8	228	38.5
2	305	51.0	213	30.0
3	327	69.2	241	54.0
4	349	67.6	223	54.5
Average	325	62.4	226.3	44.3
p-HBsAg	260	42.9	176.8	37.0

[a]Methods for lipid analysis were as follows: Total lipid, vanillin-phosphoric acid method; Cholesterol, enzymatic kit (Merck); Phospholipid, phosphate analysis (ammonium molybdate method); Triglyceride, enzymatic kit (Merck). The values were expressed in the unit of μg/mg protein.

is non-ionic, non-denaturing surfactant and CsCl ultracentrifugation. He found that the elimination of lipid from HBsAg induce the change of structural conformation of HBsAg which has a reduced helical content and the decrease of antigenicity of HBsAg against polyclonal antibody. Lipid analysis shows total lipid content of r-HBsAg was 325 μg/mg protein, which is a little bit higher than that of p-HBsAg (260 μg/mg protein). Also, phospholipid, the highest lipid component of r-HBsAg was estimated as 70%, which is almost identical to 68% of p-HBsAg. These were slightly higher than that of the previous result (Gavilanes *et al.*, 1982) that was 60% and the difference could be due to the variance in their antigen purification method.

REFERENCES CITED

Akahane, Y., Clinical trial of a pre-S2 containing hepatitis B vaccine: Early response of anti pre-S2 in vaccines and efficacy in non-responders to conventional vaccines. *International Symposium on Viral Hepatitis and Liver Disease.* May 10-14, Tokyo, Japan (1993).

Ansari, G. A. S. and Smith, L., High performance liquid chromatography of cholesterol autooxidation products. *J. Chromatogr.*, 175, 307-315 (1979).

Beasley, R. P. and Hwang, L. Y., In *viral hepatitis and liver disease.* Grune & Stratton, New York. pp 209-224 (1984).

Bligh, E. G. and Dyer, W. J., A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.*, 37, 911-917 (1959).

Blumberg, B. S., Alter, H. J. and Visnich, S., A new antigen in leukemia sera. *JAMA,* 191, 541-546 (1965).

Blumberg, B. S., Sutnick, A. L. and London, W. T., Hepatitis and leukemia: their relation to Australia antigen. *Bull. N. Y. Acad. Med.*, 44, 1566-1586 (1968).

Courouce-Pauty, A. M., Plancon, A. and Soulier, J. P.,

Distribution of HBsAg subtypes in the world. *Vox Sang,* 44, 197-211 (1983).

Dane, D. S., Cameron, C. H. and Briggs, M., Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. *Lancet,* 1, 695-698 (1970).

Diminsky, D., Schirmbeck, R., Reimann, J. and Barenholz, Y., Comparison between hepatitis B surface antigen (HBsAg) particles derived from mammalian cells (CHO) and yeast cells (*Hansenula polymorpha*): composition, structure and immunogenicity. *Vaccine,* 15, 637-647 (1997).

Gavilanes, F., Gomez-Gutierrez, J., Aracil, M., Gonzalez-Ros, J. M., Ferragut, J. A., Guerrero, E. and Peterson, D. L., Hepatitis B surface antigen. Role of lipids in maintaining the structural and antigenic properties of protein components. *Biochem. J.,* 265, 857-864 (1990).

Gavilanes, F., Gonzalez-Ros, J. M. and Peterson, D. L., Structure of hepatitis B surface antigen. Characterization of the lipid components and their association with the viral proteins. *J. Biol. Chem.,* 257, 7770-7777 (1982).

Gerlich, W. H., Lu, X. and Heermann, K. H., Studies on the attachment and penetration of hepatitis B virus. *J. Hepatol.,* 17 (Suppl 3), 10-14 (1993).

Heermann, K. H, Goldmann, U., Schwartz, W., Seyffarth, T., Baumgarten, H. and Gerlich, W. H., Large surface proteins of hepatitis B virus containing the pre-S sequence. *J. Virol.,* 52, 396-402 (1984).

Hourvitz, A., Mosseri, R., Solomon, A., Yehezkelli, Y., Atsmon, J., Danon, Y.L., Koren, R. and Shouval, D., Reactogenicity and immunogenicity of a new recombinant hepatitis B vaccine containing Pre S antigens: a preliminary report. *J. Viral. Hepat.,* 3, 37-42 (1996).

Hu, G. F. and Vallee, B. L., A gel retardation assay for the interaction of proteins and carbohydrates by fluorophore-assisted carbohydrate electrophoresis. *Anal. Biochem.,* 218, 185-191 (1994).

Krone, B., Gerlich, W. H., Heermann, K. H., Lenz, A., Lu, X., Seiter, M. and Tomslen, R., Significance of pre-antigens for hepatitis B vaccines. *Progress in hepatitis B immunization.* 194, 21-25 (1990).

Michel, M. L., Sobczak, E., Malpiece, Y., Tiollais, P. and Streeck, R. E., Expression of amplified hepatitis B virus surface antigen genes in chinese hamster ovary cells. *Biotechnology,* 3, 561-566 (1985).

Michel, M. L., Pontisso, P., Sobczak, E., Malpiece, Y., Streek, R. E. and Tiollais, P., Synthesis in animal all of hepatitis B surface antigen particles carrying a receptor for polymerized human serum albumin. *Proc. Natl. Acad. Sci. USA.,* 81, 7708-7712 (1984).

Neurath, A. R., Antibodies to a synthetic peptide from the pre-S 120-145 region of the hepatitis B virus envelope are virus neutralizing. *Vaccine,* 4, 35-37 (1986).

Samanta, H. and Youn, B. W., Expression of hepatitis B virus surface antigen containing the pre-S region in mammalian cell culture system. *Vaccine,* 7, 69-76 (1989).

Sherlock, S., Fifth international symposium on viral hepatitis summary. *J. Hepatol.,* 17 (Suppl 3), S2-S5 (1993).

Starr, C. M., Masada, R. I., Hague, C., Skop, E. and Klock, J. C., Fluorophore-assisted carbohydrate electrophoresis in the separation, analysis, and sequencing of carbohydrates. *J. Chromatogr. A.,* 720, 295-321 (1996).

Stibbe, W. and Gerlich, W. H., Structural relationships between minor and major proteins of hepatitis B surface antigen. *J. Virol.,* 46, 626-628 (1983).

Thoma, H. A., Hemmerling, A. E. and Hotzinger, H., Evaluation of immune response in a third generation hepatitis B vaccine containing pre-S proteins in comparative trials. *Progress in hepatitis B immunization.* 194, 35 (1990).

Tiollais, P., Paucel, C. and Dejean, A.., The hepatitis B virus. *Nature,* 317, 489-495 (1985).

Valenzuela, P., Medina, A., Rutter, W. J., Ammerer, G. and Hall, B. D., Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature,* 298, 347-350 (1982).