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Human Liver Plasma Membranes Contain Receptors for the Hepatitis B Virus Pre-S1 Region and, via Polymerized Human Serum Albumin, for the Pre-S2 Region

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Hepatitis B virus particles contain three related viral envelope proteins, the small, middle, and large S (surface) proteins. All three proteins contain the small S amino acid sequence at their carboxyl terminus. It is not clear which of these S proteins functions as the viral attachment protein, binding to a target cell receptor and initiating infection. In this report, recombinant hepatitis B surface antigen (rHBsAg) particles, which contain only virus envelope proteins, were radioactively labeled, and their attachment to human liver membranes was examined. Only the rHBsAg particles containing the large S protein were capable of directly attaching to liver plasma membranes. The attachment was saturable and could be prevented by competition with unlabeled particles or by a monoclonal antibody specific for the large S protein. In the presence of polymerized human serum albumin, both large and middle S protein-containing rHBsAg particles were capable of attaching to the liver plasma membranes. Small S protein-containing rHBsAg particles were not able to attach even in the presence of polymerized human serum albumin. These results indicate that the large S protein may be the viral attachment protein for hepatocytes, binding directly to liver plasma membranes by its unique amino-terminal (pre-S1) sequence. These results also indicate that polymerized human serum albumin or a similar molecule could act as an intermediate receptor, attaching to liver plasma membranes and to the amino acid sequence (pre-S2) shared by the middle and large S proteins but not contained in the small S protein.

The envelope of the DNA-containing hepatitis B virus (HBV) particle is composed of three related surface (S) proteins. These S proteins are the only viral components found in the noninfectious hepatitis B surface antigen (HBsAg) particles which are produced in great abundance during HBV infection of an individual. The HBsAg particles occur in spherical and tubular forms which have a smaller diameter (22 nm) than the DNA-containing HBV particles (42 nm).

The major or small S protein is 226 amino acids in length (39). The two other S proteins include the small S sequence at their carboxyl termini. In addition, the middle S protein contains an amino-terminal extension to the small S protein of 55 amino acids (pre-S2) (26). The large S protein contains a further amino-terminal extension to the middle S protein of 108 to 119 (depending on the strain) amino acids (pre-S1) (14, 57). All three S proteins exist in two forms differing in the extent of glycosylation.

Since pre-S amino acid sequences are contained in all HBV isolates, as well as in related duck (46), woodchuck (48), and ground squirrel (48) hepatitis viruses, their translation products probably have some functional importance. The middle and large S proteins have been found in higher concentrations in the DNA-containing HBV particles than in the spherical HBsAg particles (14, 53), suggesting a role for these proteins in infectious HBV particle assembly or function.

Which of the three S proteins performs the attachment function for HBV and initiates infection is not clear. It has been suggested (16) that the middle S protein, which binds to polymerized human serum albumin (pHSA) (26, 27, 38), may use pHSA to attach to hepatocytes, which express albumin receptors (55, 58). Alternatively, it has been suggested that the large S protein performs the attachment function since a synthetic peptide containing a pre-S1 amino acid sequence and an antiserum raised against this peptide inhibited the attachment of cultured hepatoma (HepG2) cells to immobilized HBsAg particles (31). Recently, it was also shown that the small S protein can serve as an attachment protein, since recombinant HBsAg (rHBsAg) particles containing only the small S protein bind to the Vero, African green monkey kidney-derived cell line (21, 37).

No direct evidence has yet been presented to demonstrate the receptor activity of normal human hepatocytes for HBV envelope proteins. Using rHBsAg particles containing small, middle, or large S proteins, designated S-rHBsAg, M-rHBsAg, and L-rHBsAg, respectively, we have tested human liver-derived plasma membranes for receptor activity. Not only do these recombinant particles have the advantage of a defined composition, but they are free of HSA, a consistent contaminant of serum-derived particles. We present evidence for two different receptors on human liver plasma membranes. One receptor directly binds the pre-S1 amino acid sequence which is unique to the large S protein, while the other receptor binds the pre-S2 amino acid sequence, via pHSA.

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MATERIALS AND METHODS

Isolation of hepatocyte membranes. Plasma membranes were prepared from human liver tissue essentially by the method of Hubbard et al. (15). Liver tissue from cadaver kidney donors was collected into minimal essential medium (GIBCO Laboratories) as soon as possible after death and was refrigerated. All membrane isolation procedures were performed at 4°C. Within 6 h of death, 10 g of liver tissue was dissociated by teasing and Dounce homogenization in STM buffer (0.25 mM sucrose, 5 mM Tris hydrochloride [pH 8], 0.5 M $MgCl_2$). The suspension was centrifuged at 280 × *g* for 5 min in a TJ6 centrifuge (Beckman Instruments, Inc.) to remove large aggregates and unbroken cells. The supernatant was centrifuged again at 1,500 × *g* for 10 min. This supernatant was processed as described below for internal membranes. The pellet was resuspended in STM buffer, and 2 M sucrose in STM buffer was added to yield 25 ml of solution with a density of 1.18 g/cm^3. This solution was overlaid with 8 ml of STM buffer and was centrifuged for 1 h at 78,000 × *g* in an SW27 rotor in an L8 ultracentrifuge (Beckman). The plasma membrane enriched fraction floated up to the interface which was collected. Mouse liver plasma membranes were isolated in the same manner.

Internal membranes were isolated from human liver tissue, starting with the supernatant from the centrifugation step (1,500 × *g*) described above. This supernatant was centrifuged at 33,000 × *g* for 5 min in an SW41 rotor. The pellet was discarded, and the supernatant was again centrifuged in the same rotor at 78,000 × *g* for 100 min. The pellet was suspended in 57% sucrose in TM buffer (5 mM Tris hydrochloride [pH 8], 0.5 mM $MgCl_2$), and solutions of 37 and 4% sucrose in STM buffer were overlaid to form a step gradient. The gradient was centrifuged for 18 h at 78,000 × *g* in an SW27 rotor. The internal-membrane enriched fraction was recovered from the 4%-37% interface. All membrane preparations were stored at −80°C.

Liver membranes were visualized by electron microscopy. They were placed directly on a 0.25% Formvar-coated, glow-discharged, carbon-coated, 400-mesh copper grid. The grid was negative stained with 0.5% uranyl acetate, air dried, and viewed with a 100CX electron microscope (JEOL). Liver membranes were also characterized by their enzymatic activities. 5′-Nucleotidase activity was measured by a kit (Sigma Chemical Co.), using the instructions of the manufacturer. Glucose-6-phosphatase was assayed by the method of Baginski et al. (3). Protein concentration was determined with a protein assay (Bio-Rad Laboratories), using bovine serum albumin (BSA) as a standard.

rHBsAg particles. S-rHBsAg particles containing only the small S protein were the yeast-derived Engerix-B vaccine from SmithKline Biologicals. M-rHBsAg particles containing the middle S protein were a gift from M. L. Michel of the Pasteur Institut, Paris, France. These particles were secreted by CHO cells stably transfected with the HBV middle S gene (28). L-rHBsAg particles containing the large S protein were a gift from Merck Sharp & Dohme Research Laboratories. These particles were expressed in yeast cells stably transfected with the large S gene and were harvested by disruption of the yeast cells (20). All three of these rHBsAg preparations were purified by the producers. ^{125}I was covalently linked to the particles by the Iodobead (Pierce Chemical Co.) method, and free ^{125}I was removed by passage over a 6DPG (Bio-Rad) column.

Solid-phase membrane-binding assay. Liver membranes were pelleted in an Eppendorf centrifuge for 10 min and were suspended in phosphate-buffered saline (PBS) to a concentration of 300 μg/ml, unless otherwise noted. Wells of polyvinyl assay plates were incubated with 50 μl of membrane solutions overnight at 20°C. The plates were covered to prevent evaporation. The plates were rinsed with PBS, and each well was incubated with 60 μl of 20% BSA for 3 h at 20°C. After being washed, 50 μl of ^{125}I-L-rHBsAg particles (approximately 300 ng/ml) was added and the plates were incubated for 3 h at 20°C. The supernatant was removed and the wells were washed with PBS containing 0.05% Tween 20. The radioactivity associated with each well was determined with a gamma counter. The radioactivity bound to identically treated wells lacking membranes was subtracted as background.

For pHSA experiments, 300 μg of liver membranes per ml was incubated with PBS or with 300 μg of pHSA per ml in PBS for 45 min at 20°C. pHSA was prepared as described previously (41). After being washed three times by being pelleted and resuspended in PBS, the membranes were allowed to attach to wells of an assay plate as described above. Plates were blocked and binding assays were performed as described above.

Liquid-phase membrane-binding assay. Twenty μl of ^{125}I-M-rHBsAg particles (2 μg/ml, approximately 20,000 cpm) in PBS was mixed with 20 μl of liver membranes (3 mg/ml) in PBS and was incubated at 20°C for 45 min. The membranes were washed three times with 1% BSA in PBS by being pelleted in an Eppendorf centrifuge at 12,000 rpm for 5 min. Radioactivity which pelleted from identically treated tubes lacking membranes was subtracted before the percent bound was determined. These background counts per minute generally amounted to less than 5% of the total input particle counts per minute.

For pHSA experiments, 20 μl of membranes was preincubated for 45 min at 20°C with 4 μl of pHSA solution (3 mg/ml) in PBS. After extensive washing as described above to remove unattached pHSA, the membranes were mixed with ^{125}I-M-rHBsAg particles and the binding assay was performed as described above.

RESULTS

Plasma membranes were isolated from the liver of cadaver kidney donors by the procedure of Hubbard et al. (15). Internal liver membranes were isolated from the remaining membranes by differential centrifugation as described in Materials and Methods. Electron micrographs of each membrane preparation are shown in Fig. 1. The plasma membrane enriched fraction contains large connected vesicles similar to those described by Hubbard et al. (15). Of the 5′-nucleotidase activity (a plasma membrane enzyme marker), 15.4% was found in the plasma membrane fraction (Table 1), similar to the recovery described by Hubbard et al. (15). In addition, the plasma membrane fraction was rich in 5′-nucleotidase relative to glucose-6-phosphatase (an endoplasmic reticulum enzyme marker), while the internal membranes were richer in glucose-6-phosphatase than in 5′-nucleotidase. As found by Hubbard et al. (15), the plasma membranes remained somewhat contaminated with endoplasmic reticulum.

Direct binding of L-rHBsAg particles to human liver plasma membranes. Membrane preparations were examined for HBV receptor activity by their ability to bind L-rHBsAg particles which contain the HBV large S protein. The L-rHBsAg particles were derived from yeast which had been transfected with the large S gene (20). Unlike the highly

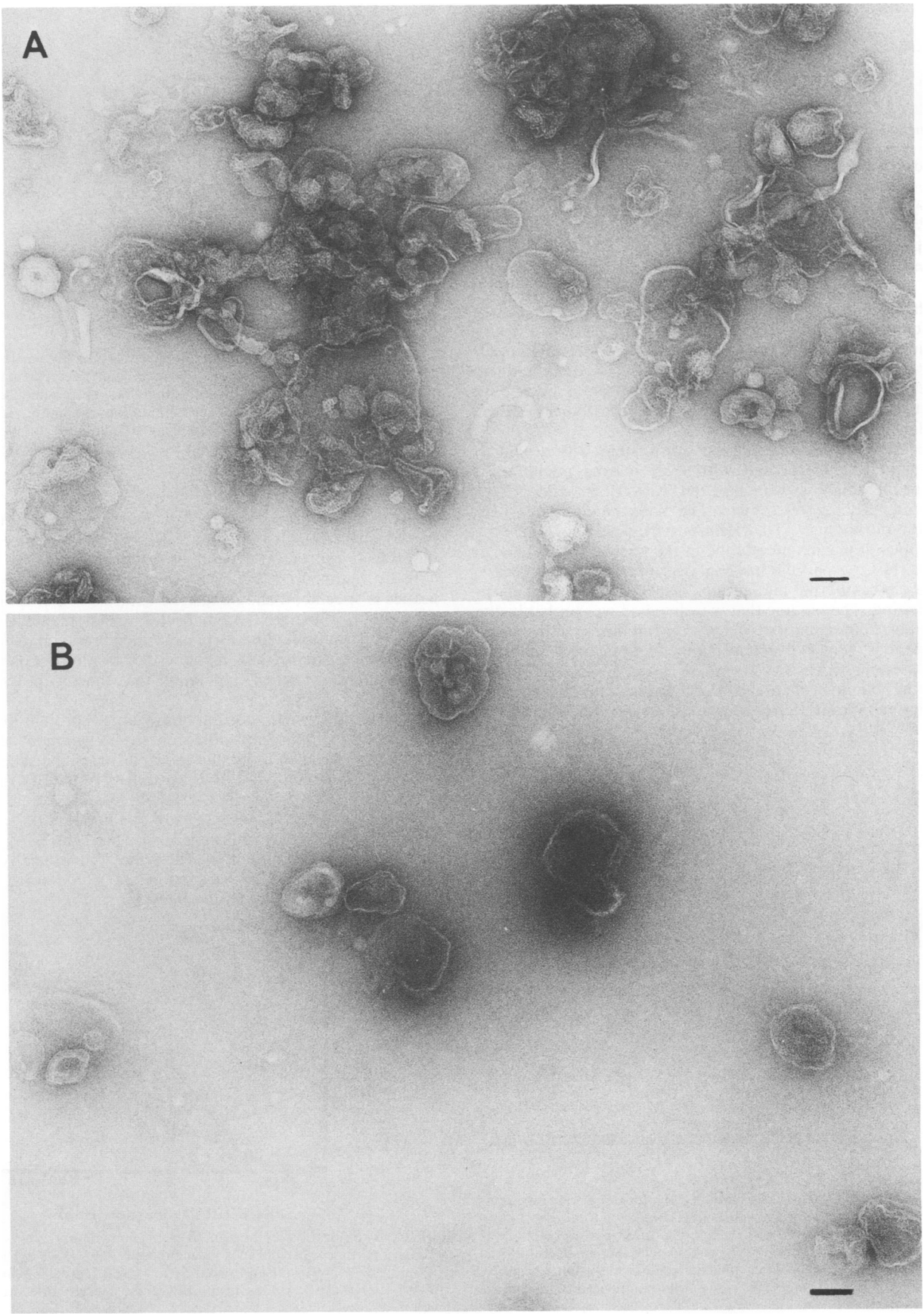

FIG. 1. Electron micrographs of enriched plasma membranes (A) and internal membranes (B) derived from human liver. Magnification, ×66,000. Bar, 100 nm.

TABLE 1. Biochemical characterization of human liver membrane fractions

Enzyme	Yield (sp act)[a] for membrane		% Activity in plasma membrane
	Plasma	Internal	
5'-Nucleotidase	21.9 (48.8)	112 (112)	16.4
Glucose-6-phosphatase	9.3 (20.7)	175 (175)	5.0
5'-Nucleotidase/glucose-6-phosphatase	2.35	0.64	

[a] Yield was determined as milliunits per gram of wet liver. Specific activity was determined as milliunits per milligram of protein.

regular serum-derived HBsAg particles, these L-rHBsAg particles vary widely in diameter and shape (20). To quantify binding, ^{125}I was covalently linked to the L-rHBsAg particles. Initial attempts to design a liquid-phase binding assay were unsuccessful since the centrifugal force needed to pellet the membrane vesicles also pelleted a portion of the L-rHBsAg particles.

An alternative solid-phase assay system was designed. Assay wells were coated with liver plasma membranes, nonspecific binding was blocked with BSA, and ^{125}I-L-rHBsAg particles were added. The plasma membranes were capable of binding ^{125}I-L-rHBsAg particles (Fig. 2). As the quantities of plasma membrane in the wells were increased, ^{125}I-L-rHBsAg particle binding also increased. In contrast, human liver internal membranes displayed much less particle binding. The binding that was detected was probably due to minor contamination of these membranes with plasma membranes. Mouse liver plasma membranes were unable to bind these particles.

If the binding of L-rHBsAg particles to human liver plasma membranes is specific, it should be inhibited by the addition of excess unlabeled particles. Unlabeled L-rHBsAg particles were able to prevent ^{125}I-L-rHBsAg particle binding in a concentration-dependent manner (Fig. 3).

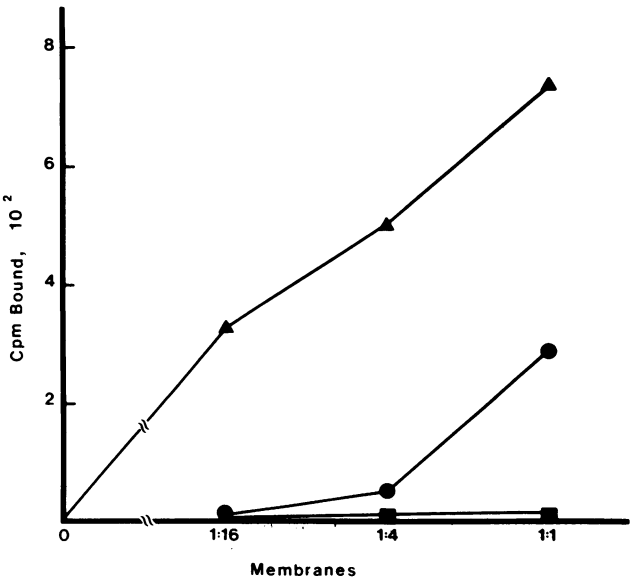

FIG. 2. L-rHBsAg particles binding to increasing concentrations of liver membranes. Assay wells were coated with 50 μl of membranes at a concentration of 0.75 mg of protein per ml (1:1) or the indicated dilution thereof. Wells coated with human liver plasma membranes (▲), human liver internal membranes (●), or mouse liver plasma membranes (■) were then incubated with ^{125}I-L-rHBsAg particles.

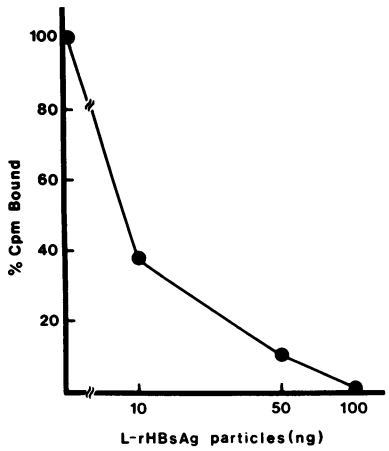

FIG. 3. Competition between unlabeled L-rHBsAg particles and ^{125}I-L-rHBsAg particles for binding to human liver plasma membranes. Assay wells coated with membranes were incubated with 50-μl volumes containing the same amount (approximately 15 ng) of ^{125}I-L-rHBsAg particles and increasing amounts of unlabeled L-rHBsAg particles. Results are expressed relative to binding in the absence of unlabeled particles.

If the binding of ^{125}I-L-rHBsAg particles to plasma membranes is specific, it should also be saturable. Increasing the amount of ^{125}I-L-rHBsAg particles added resulted in increased particle binding to plasma membrane-coated wells (Fig. 4, open symbols). At higher concentrations of particles, a plateau was reached, indicating that the receptors were saturated.

To determine which portion of the large S protein, pre-S1, pre-S2, or S, is responsible for the attachment of the L-rHBsAg particles to human liver plasma membranes, the binding activities of ^{125}I-S-rHBsAg and ^{125}I-M-rHBsAg were compared with those of ^{125}I-L-rHBsAg particles. Neither the S-rHBsAg nor the M-rHBsAg particles bound efficiently to human liver plasma membranes, while the L-rHBsAg particles did (Table 2, column 1). The major binding activity of the L-rHBsAg particles, therefore, must be contained in the unique pre-S1 sequence of the large S protein.

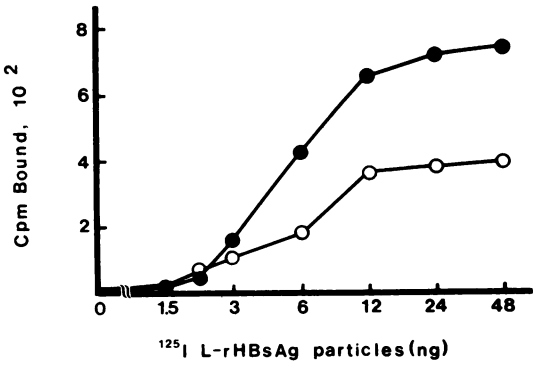

FIG. 4. Saturation of human liver plasma membranes with L-rHBsAg particles. Membranes preincubated with pHSA in PBS (●) or only PBS (○) were used to coat assay wells. Increasing amounts of ^{125}I-L-rHBsAg particles in 50-μl volumes were added to each well.

TABLE 2. Comparison of ^{125}I-rHBsAg particle binding to human liver plasma membranes in the solid-phase assay

Particle	Particles bound (cpm) to membranes preincubated with:	
	PBS	pHSA
^{125}I-S-rHBsAg	105	98
^{125}I-M-rHBsAg	360	5,080
^{125}I-L-rHBsAg	2,559	3,895

The binding of ^{125}I-L-rHBsAg particles to human liver plasma membranes was also examined with pre-S1-specific monoclonal antibodies, 18/7 (14) and T0606 (54), both of which are capable of binding to these L-rHBsAg particles. ^{125}I-L-rHBsAg particles were preincubated with each of these antibodies before being tested for plasma membrane-binding activity. Monoclonal antibody 18/7 prevented ^{125}I-L-rHBsAg particles from binding to the liver membranes in a concentration-dependent manner, whereas T0606 had no effect (Fig. 5). These results confirm the role of a portion of the pre-S1 peptide in the direct attachment of L-rHBsAg particles to human liver plasma membranes. In similar experiments, monoclonal antibodies specific for the pre-S2 peptide (F124 and F376) (6, 7, 29, 40) or small S protein (Hybritech, Inc.) did not block ^{125}I-L-rHBsAg particle binding to the human liver-derived plasma membranes (data not presented).

pHSA-mediated binding of rHBsAg particles to human liver plasma membranes. To test whether pHSA is capable of acting as an "intermediate receptor" between liver membranes and virus particles, human liver plasma membranes were preincubated with pHSA, washed, attached to polyvinyl assay wells, and examined for ^{125}I-L-rHBsAg particle binding. More particles were capable of binding to pHSA-incubated plasma membranes (Fig. 4, closed symbols) than

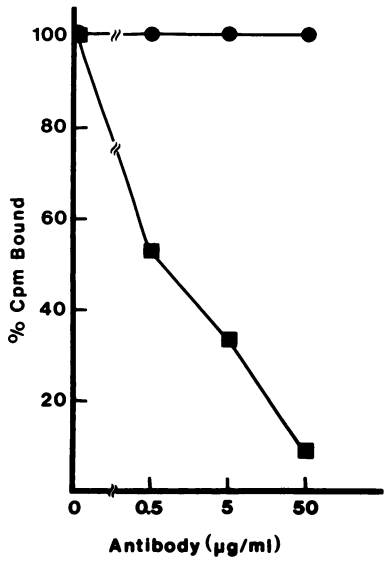

FIG. 5. Effects of pre-S1-specific monoclonal antibodies on the binding of L-rHBsAg particles to human liver plasma membranes. ^{125}I-L-rHBsAg particles were mixed with equal volumes of dilutions of antibody 18/7 (■) or T0606 (●) and were incubated for 45 min at 20°C before the mixtures were added to human liver plasma membrane-coated wells in a solid-phase binding assay. The L-rHBsAg particle binding in the absence of antibody is 100%.

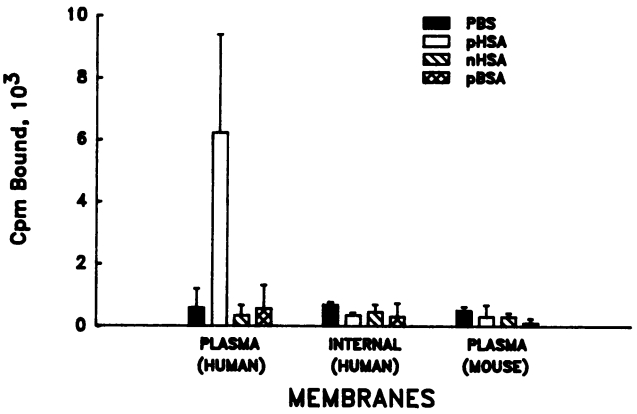

FIG. 6. M-rHBsAg particle binding to liver membrane fractions in solution. Equivalent amounts of liver membranes were preincubated with PBS, pHSA, normal HSA (nHSA), or polymerized BSA (pBSA) and were washed to remove free albumin before being mixed with ^{125}I-M-rHBsAg particles. Results are expressed as the mean counts per minute bound ± standard deviation.

to the same membranes lacking pHSA (open symbols). The binding to pHSA-incubated plasma membranes was also saturable.

To examine whether pHSA was interacting with the pre-S2 peptide, similar binding experiments were performed with ^{125}I-S-rHBsAg and ^{125}I-M-rHBsAg particles. The ^{125}I-S-rHBsAg particles did not bind efficiently to the human liver plasma membranes, regardless of whether the membranes had been preincubated with pHSA (Table 2, column 2). Neither did the ^{125}I-M-rHBsAg particles bind efficiently to these membranes in a direct manner. However, the ^{125}I-M-rHBsAg particles did bind to membranes preincubated with pHSA. Therefore, pre-S2, the region found in the middle S protein but not in the small S protein, is responsible for the attachment of M-rHBsAg particles to pHSA-coated human liver plasma membranes. Since the large S protein also contains pre-S2, it is not surprising that L-rHBsAg particles bound to human liver plasma membranes more efficiently when these membranes were preincubated with pHSA.

To examine the membrane and albumin specificities of this M-rHBsAg particle interaction, human liver plasma or internal membranes or mouse liver plasma membranes were preincubated with pHSA, HSA, or polymerized BSA. For these experiments, a simpler liquid-phase binding assay was used, since the small homogeneous size of M-rHBsAg particles allowed unbound particles to remain in suspension at centrifugal forces required to pellet the membrane vesicles. ^{125}I-M-rHBsAg particles bound efficiently only to human plasma membranes and only after the membranes had been incubated with pHSA (Fig. 6). Internal liver membranes and mouse liver plasma membranes were unreactive with ^{125}I-M-rHBsAg particles, regardless of the presence of native or polymerized human or bovine albumins. These results indicate that albumin-mediated attachment of M-rHBsAg particles to membranes is specific for pHSA and for human liver plasma membranes.

The binding of ^{125}I-M-rHBsAg particles to pHSA-incubated plasma membranes was further characterized with three pre-S2-specific monoclonal antibodies. Antibodies F124 and F376 recognize epitopes near the amino terminus of the middle S protein (6, 7, 29, 40). Antibody F52 recognizes an epitope near the carboxyl terminus of the pre-S2

TABLE 3. Effect of anti-pre-S2-specific antibodies on ^{125}I-M-rHBsAg particle binding to human liver plasma membranes[a]

Monoclonal antibody	cpm bound (%)
0	7,951 (45)
F124	2,733 (7)
F52	3,576 (17)
F376	5,559 (34)

[a] ^{125}I-M-rHBsAg particles were mixed with equal volumes of PBS (antibody 0) or three pre-S2 specific monoclonal antibodies in PBS and were incubated at 20°C for 30 min. The final concentration of each antibody was 80 μg/ml. These mixtures were added to Eppendorf tubes containing human liver plasma membranes, and the liquid-phase binding assay was performed. In a separate experiment, monoclonal antibody F124, at a concentration of 10 μg/ml, efficiently inhibited ^{125}I-M-rHBsAg particle binding.

region (40). Particles were incubated with each antibody before plasma membranes were added. All three antibodies inhibited ^{125}I-M-rHBsAg particles from binding to human liver plasma membranes (Table 3). Antibody F124 was the most effective inhibitor. These results confirm that the M-rHBsAg particles bind to the pHSA-incubated membranes via the pre-S2 amino acid sequence. Antibody F376 was the least efficient inhibitor of ^{125}I-M-rHBsAg particle binding, probably due to its poor recognition of native M-rHBsAg particles (6), even though it reacts well with a linear pre-S2 peptide (29).

DISCUSSION

The data presented in this report demonstrate that only L-rHBsAg particles, which contain the HBV large S protein, are capable of efficiently binding directly to human liver plasma membranes. M-rHBsAg or S-rHBsAg particles, which lack the pre-S1 amino acid sequence of the large S protein, are not able to efficiently bind directly to these membranes. In addition, a monoclonal antibody against pre-S1 specifically prevents this binding. Since the DNA-containing HBV particles incorporate the large S protein (14, 53), they may also bind directly to human liver membranes via the pre-S1 peptide. We are presently testing this possibility. We have found that L-rHBsAg particles will bind directly to the human hepatoma cell line HepG2 (M. Kaplan, M. Bankowski, and M. Peeples, manuscript in preparation).

Our finding that L-rHBsAg particles can bind directly to human liver membranes is consistent with the findings of Neurath et al. (31) that a peptide representing amino acids 21 to 47 from the amino terminus of the pre-S1 sequence, as well as antiserum to that peptide, was able to prevent HepG2 cells from binding to serum-derived HBsAg particles. However, an advantage of the recombinant over serum-derived HBsAg particles is that the recombinant particles have never been in contact with HSA. Not only is HSA a common contaminant of serum-derived HBsAg particles, but also it could act to attach the particles to hepatocyte membranes, thereby confusing the results.

Previous reports have demonstrated that pHSA binds to HBsAg particles (13, 41) within the pre-S2 amino acid sequence (26, 27, 38). Other reports have demonstrated that native and glutaraldehyde-polymerized albumins bind to hepatocytes (24, 55, 58, 60). However, we have shown that pHSA is necessary and sufficient for the attachment of pre-S2-containing M-rHBsAg particles to human liver plasma membranes. We have also shown that pHSA will enhance the L-rHBsAg particle binding to these membranes. Whether or not pHSA exists in vivo and might play a role in HBV infection is not clear. If so, it would have to be generated by a natural process. Aging, heating, or even another cross-linking agent, carbodiimide, is incapable of generating pHSA which binds efficiently to HBsAg (60). However, it has been recently reported that the pre-S2 domain reacts with naturally occurring human serum components (K.-H. Heermann, F. Waldeck, and W. H. Gerlich, Int. Symp. Viral Hepatitis Liver Dis. J. Med. Virol. **21**:56A, 1987).

The hepatocyte is a polarized epithelial cell in which the sinusoidal front, the lateral surface, and the bile canalicular front are functionally and morphologically distinct. In a preliminary report (42) of some of our findings, we stated that L-rHBsAg and M-rHBsAg particles bound to sinusoidal front membranes rather than to lateral surface and bile canalicular membranes which had been preincubated with pHSA. In fact, while these domains can be distinguished by electron microscopy, they cannot be separated physically by the method (15) used. This method does, however, allow isolation of a liver plasma membrane enriched fraction which contains both large S (pre-S1) protein receptor sites and pHSA receptor sites, as described in the present report. Perhaps electron microscopic studies of L-rHBsAg particles bound to these liver plasma membranes or of L-rHBsAg and M-rHBsAg particles bound to pHSA-coated liver plasma membranes will allow identification of the hepatocyte domain(s) which contains these receptors.

If, indeed, the pre-S1 and possibly the pre-S2 peptide sequences are involved in virus attachment in vivo, they would be good targets for protective immunization. In fact, antibodies reacting with the intact virion (1, 2), pre-S2 (5, 32), and pre-S1 (19), as well as antibodies blocking the pHSA-binding activity of HBV (45), have been described in the early phase of recovery from acute HBV infection. In addition, a synthetic peptide vaccine containing the pre-S2 amino acid sequence is protective in chimpanzees (18). Similarly, rabbit antiserum to a synthetic pre-S2 peptide has been shown to neutralize HBV from infectious serum such that injecting the mixture into chimpanzees did not cause disease (30).

An antibody response to the small S protein is also protective (34, 50). Peeples et al. (37) have found that S-rHBsAg particles containing only the small S protein (35, 36) are capable of specific binding to Vero cells in culture and that antibodies to the small S protein block this binding. Since neither hepatocyte membranes (Table 2) nor cultured hepatoma cells (37) are capable of efficiently binding S-rHBsAg particles, it seems unlikely that the Vero cell small S protein receptor exists on the hepatocyte surface, at least not in large numbers. This receptor may, however, exist on extrahepatic tissues, which are also infected by hepadnaviruses (4, 8–12, 22, 23, 25, 43, 44, 47, 49, 51, 52, 59). If HBV must infect one of these organs or cell types prior to infecting hepatocytes, anti-small S antibodies might prevent that initial infection, thereby protecting the individual.

It is also possible that antibodies to the pre-S1, pre-S2, or small S regions neutralize virus through a mechanism other than direct inhibition of binding. People and chimpanzees immunized with small S proteins are protected from infection (34, 50), presumably by circulating antibodies. Antibodies to middle S proteins have been shown to neutralize HBV (15, 30), and we have demonstrated here that an antibody to pre-S1 can block at least L-rHBsAg particle attachment. Perhaps there are several mechanisms for neutralizing HBV. This possibility is not surprising since other viruses can be neutralized by antibodies to different epitopes on their

attachment protein (17, 56) or to virion surface proteins other than the attachment protein (33).

In summary, using rHBsAg particles containing either middle or large S proteins, we have found two distinct receptors for HBV on human liver plasma membranes: one for the pre-S2-encoded amino acid sequences, which is mediated by pHSA, and another which directly binds the pre-S1 amino acid sequence. Using this system, we may be able to determine the nature of both of these receptors.

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