LPS-binding protein and CD14-dependent attachment of hepatitis B surface antigen to monocytes is determined by the phospholipid moiety of the particles

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It was observed recently that recombinant yeast-derived hepatitis B surface antigen (rHBsAg) particles, which contain the S protein only, bind almost exclusively to monocytes. It is shown here that binding requires the presence of the LPS receptor CD14. Furthermore, evidence is presented that a domain on CD14 that is identical to or largely overlaps with the LPS-binding pocket is instrumental for the attachment of rHBsAg. Additionally, it is shown that the heat-labile LPS-binding protein (LBP) catalyses the binding of rHBsAg to the cells. Remarkably, natural plasma-derived HBsAg (pHBsAg) does not have this property. pHBsAg devoid of its lipids and reconstituted with phosphatidylserine or phosphatidylglycerol acquires the characteristic of yeast-derived HBsAg. Clearly, the interaction of rHBsAg with the cell membrane is determined by the presence of charged phospholipids that are absent in pHBsAg. Although a lipid–receptor interaction is suggested, antibody-inhibition experiments suggest a possible involvement of the C-terminal region of the S protein in the interaction with monocytes. The possible implications of these observations for hepatitis B virus (HBV) infection and HBV vaccine efficiency are discussed.

Introduction

Hepatitis B virus (HBV) is without any doubt one of the most successful human viruses. Two billion of the six billion people alive today show evidence of past or current infection with HBV. Worldwide, HBV causes more than 1 million deaths per year and about 350–400 million people are persistently infected with this agent, with an estimated annual increase of 1 million. Chronic infection may lead to cirrhotic liver failure and infected people have a 100-fold increased risk of developing hepatocellular carcinoma (Chisari, 2000). Nevertheless, infection with HBV has become a vaccine-preventable disease.

One of the most remarkable features of HBV is its production of three different virus particles: infectious virions (Dane particles) and two types of non-infectious sub-virus particles, termed hepatitis B surface antigen (HBsAg). HBsAg consists mainly of spherical particles and a small amount of filamentous particles. These spheres and filaments can accumulate to several hundred µg/ml in blood of HBV-infected patients. The spherical particles contain virally encoded membrane proteins and approximately 30% (by weight) of host cell-derived lipids. The S protein accounts for more than 90% of the protein contained in HBsAg. The L and M protein forms the remainder. S, M and L share 226 C-terminal amino acids. The M protein contains an N-terminal extension of 55 amino acids, termed the pre-S2 sequence. The L protein is similar to M but is elongated at its N-terminal end with another 120–128 amino acids (pre-S1 sequence). Both glycosylated and non-glycosylated forms of these viral membrane proteins are present in the particles (Ganem, 1996; Seeger & Mason, 2000). Several cellular membrane and serum proteins have been shown to interact with the viral membrane proteins or HBsAg.
The pre-S2 region is considered to be involved in the attachment to polymerized human serum albumin, an unusual glycan structure, fibronectin and the transferrin receptor (Ilmai et al., 1979; Franco et al., 1992; Gerlich et al., 1993; Budkowska et al., 1995). In recent years, it has been suggested that the pre-S1 domain contains an attachment site for glyceraldehyde-3-phosphate-dehydrogenase, an IgA receptor, IL-6, asialoglycoprotein, an 80 kDa protein and an SCCA1 homologue (Petit et al., 1992; Pontisso et al., 1992; Neurath et al., 1992; Treichel et al., 1994; Ryu et al., 2000; De Falco et al., 2001). Attachment of the S protein to annexin V and apolipoprotein H has been reported (Hertogs et al., 1993; Mehdi et al., 1994).

The lipid composition of HBsAg has been determined as well. The main lipid components are phospholipids (≈ 67%), cholesterol ester (≈ 15%), cholesterol (≈ 14%) and triglycerides (≈ 3%). Phosphatidylcholine (PC) accounts for approximately 90% of the phospholipids, while phosphatidylethanolamine accounts for 2–4%. Trace amounts of phosphatidylserine (PS), sphingomyelin, lysophosphatidylcholine and lysophosphatidylethanolamine are present (Kim & Bisell, 1971; Gavilanes et al., 1982). Phospholipid components of HBsAg have been suggested to be involved in the binding of particles to two proteins, apolipoprotein H and annexin V (Neurath & Strick, 1994; De Meyer et al., 1999; Stefas et al., 2001). Removal of the lipids reduces the helical content of the HBsAg proteins and recognition by monoclonal antibodies (mAbs) (Gavilanes et al., 1990). Reconstitution of these particles stripped of their lipids with both neutral and negatively charged phospholipids restores the original morphology of the particles and topology of the proteins. However, the helical content and antigenic activity is restored only with acidic phospholipids (Gomez-Gutierrez et al., 1994, 1995). Several observations suggest that the lipid content affects the immunogenicity of HBsAg. Triton X-100-extracted particles were reported to be more immunogenic than the native particles (Skelly et al., 1981), while incorporation of HBsAg into liposomes composed of PC and cholesterol induced higher levels of antibodies (Manesis et al., 1979). Furthermore, treatment of HBsAg with phospholipase C, which removes the phosphoalcohol head groups of the phospholipids, enhances its immunogenicity (Baijot, 1991; Diminsky, 1992; Treichel et al., 1994; Ryu et al., 2000; De Falco et al., 2001). Attachment of the S protein to annexin V and apolipoprotein H has been reported (Hertogs et al., 1993; Mehdi et al., 1994).

Methods

- **rHBsAg.** Purified rHBsAg (subtype ayw) produced in _Saccharomyces cerevisiae_ (lots DVP23 (752 µg/ml in PBS) and DVP93/1 (1 mg/ml) was kindly provided by GlaxoSmithKline. The purity of these rHBsAg preparations is judged by HPLC analysis as well as SDS–PAGE with Coomassie staining and is > 98%. rHBsAg is composed of well-defined sub-virus particles, which contain the non-glycosylated S protein only. Similar preparations are used worldwide as human HBV vaccines after adsorption to aluminium hydroxide.

- **pHBsAg.** pHBsAg (subtype ayw or ayw) was purified by precipitation with PEG-8000, potassium bromide floatation, caesium chloride-gradient ultracentrifugation, agarose 4B gel filtration and matrix cellulose-sulfate-affinity chromatography. Three different pHBsAg preparations were used. The first contained 280 µg/ml of protein and was stored at 4 °C for several months after purification. The second was stored in lyophilized form and contained 500 µg/ml of protein upon reconstitution. The third was stored at −70 °C immediately after purification from plasma and contained 500 µg/ml of protein.

- **pHBsAg** was stripped of its phospholipids and reconstituted with different phospholipids, as described previously (Gavilanes et al., 1990; Gomez-Gutierrez et al., 1994, 1995). The phospholipids used were PC (from egg), PS (from bovine brain) and 1,2-dioleoyl-phosphatidylglycerol (DOPG). pHBsAg, rHBsAg stripped of its lipids (Del-pHBsAg) and pHBsAg reconstituted with PC (PC-pHBsAg), PS (PS-pHBsAg) and DOPG (DOPG-pHBsAg) were in 10 mM Tris–HCl pH 7.0, 50 mM NaCl buffer containing 280, 300, 160, 300 and 290 µg/ml of protein, respectively.

- **Soluble CD14 (sCD14) and LBP.** sCD14 and LBP were expressed in CHO cells and purified, as described elsewhere (Stelter et al., 1999), or were purchased from Biometec.

- **Antibodies.** Mouse anti-human CD14 and anti-CD14–FITC (clone P9) and streptavidine–phycoerythrin (Strep–PE) were from Becton Dickinson. Mouse anti-human CD14 and anti-CD14–FITC (clone My4) were obtained from Immunotech. Mouse anti-human CD18–FITC (clone 6.7) was purchased from Pharmingen. Ascaline fluid of mouse anti-human CD14 clones biG3 (IgG3) and biG11 (IgG1), purified mouse anti-human CD14 clone biG2 (IgG2a) and rabbit anti-human CD14 antisera were from Biometec. Mouse anti-α and anti-γ were a kind gift from DiaSorin. Human anti-HBsAg clones F47B and F949 were a kind gift from L. Sillekens (Centraal Laboratorium van de Bloedbank, Amsterdam). Human anti-α was developed in the laboratory. The following isotypic controls were used: mouse IgG1 (Becton Dickinson) or ascaline fluid of LMBH6 (Vanlandschoot et al., 1998) and IgG1–FITC (Becton Dickinson), mouse IgG2a (Pharmitgen), mouse IgG2a–FITC (Caltag), mouse IgG2b and IgG2b–FITC (Coulter) and rabbit IgG (Sigma). For FACS analysis, non-labelled antibodies were detected with goat anti-rabbit IgG–FITC (Pharmitgen) and rabbit anti-mouse F(ab’)2–FITC (DAKO). Human antibodies were detected with rabbit anti-human F(ab’)2–FITC. Goat anti-human–HRP (Sigma) serum was used for ELISA.
**Results**

Recent observations we made suggested that CD14 was involved in the binding of b-rHBsAg to monocytes: (1) b-rHBsAg particles bind almost exclusively to the CD14+ cell population of PBMCs; (2) attachment of b-rHBsAg to the THP-1 pre-monocytic cell line occurred only upon 1,25-dihydroxyvitamin D3-induced differentiation; (3) upon phorbol myristate acetate (PMA) treatment of PBMCs, a 2- to 3-fold reduction in the attachment of b-rHBsAg was observed (Vanlandschoot et al., 2002). As one of its numerous biological effects, 1,25-dihydroxyvitamin D3 causes the expression of CD14, whereas PMA treatment is known to cause the rapid shedding of CD14 (Pedron et al., 1995; Bazil & Strominger, 1991). These different observations suggested that CD14 might be a receptor for rHBsAg.

**CD14-specific antibodies can inhibit attachment of b-rHBsAg to monocytes**

PBMCs were incubated with increasing amounts of anti-CD14-FITC clone My4 or P9. After washing, the cells were incubated with b-rHBsAg. As shown in Fig. 1, a partial inhibition of attachment of b-rHBsAg was obtained when cells were pre-incubated with increasing concentrations of antibody P9. My4 almost completely inhibited the binding of b-rHBsAg to the monocytes, even at the lowest concentration used. (Fig. 1b). A mAb directed against CD18, another LPS-binding molecule, had no effect on b-rHBsAg attachment (data not shown). These results strongly suggest that CD14 is indeed involved in the attachment of rHBsAg to monocytes.

**Expression of human CD14 in a non-monocytic cell line results in attachment of b-rHBsAg and non-biotinylated rHBsAg**

Next, we examined the ability of CD14 to induce binding of b-rHBsAg when expressed in a non-monocytic cell line (CHO). As shown in Fig. 2(a), both CD14-specific mAbs (P9 and My4) recognized the CD14-transfected CHO cells (CHO-CD14) and not the control cells (CHO-DHFR). Attachment of b-rHBsAg was observed only in the CHO-CD14 cell line. Despite the very high purity of the (b-)rHBsAg preparations, the possibility remained that some unknown (biotinylated) yeast contaminant was binding to the cells. To exclude this possibility and to demonstrate attachment of rHBsAg, CHO-CD14 cells were incubated with 5 or 25 µg/ml non-biotinylated rHBsAg.
lated rHBsAg in 2% HS–HBSS, followed by different S protein-specific mAbs. Cells were analysed by FACS analysis. As expected, rHBsAg (serotype ad) was detected only by the mAb specific for the d serotype and not by the mAb specific for the y serotype (Fig. 2b). No signal was obtained with the isotype control antibody. When CHO-DHFR cells were tested, no signal was obtained with the mAb specific for the d serotype (Fig. 2c), again demonstrating that the attachment of rHBsAg depends on the presence of CD14.

**Attachment of b-rHBsAg is inhibited by sCD14**

A soluble form of CD14 (sCD14) exists in serum (3–6 µg/ml) and might be capable of binding to rHBsAg. b-rHBsAg was incubated on ice with increasing amounts of purified sCD14 in 2% HS–HBSS. PBMCs were added after 1 h and further treated and analysed as described. As shown in Fig. 3, sCD14 reduced the binding of b-rHBsAg to monocytes, although complete inhibition was not obtained even at the highest concentration of sCD14 used. This result suggests that b-rHBsAg interacts with sCD14, providing further evidence for an interaction with membrane-bound CD14 as well.

**Residues on CD14, involved in the binding of LPS, are important for attachment of b-rHBsAg to the cells**

To identify the region on CD14 involved in the binding of rHBsAg to the cells, inhibition experiments with several mAbs

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**Fig. 2.** (a) b-rHBsAg binds to CHO cells transfected with human CD14. CHO cells transfected with the empty plasmid (CHO-DHFR) or cells expressing human CD14 (CHO-CD14) were incubated with anti-CD14–FITC clone P9, clone My4 or b-rHBsAg in 2% HS–HBSS. After two washes with the same buffer, b-rHBsAg was detected with Strep–PE. Cells were washed twice and analysed. The grey areas represent cells stained with isotypic controls or Strep–PE only. (b) The S protein of rHBsAg particles is detected on CHO-CD14 cells by a serotype d-specific mAb. CHO-CD14 cells were incubated for 90 min on ice with 5 or 25 µg/ml rHBsAg in 1% HS–HBSS. After two washes, cells were incubated for 1 h with 5 µg/ml of different mouse mAbs as indicated. After two washes, mAbs were detected with rabbit anti-mouse–FITC. (c) CHO-CD14 and -DHFR cells were incubated with 5 or 25 µg/ml rHBsAg in 1% HS–HBSS. After two washes, cells were incubated for 1 h with 5 µg/ml of mouse anti-d. After two washes, the mAb was detected with rabbit anti-mouse–FITC.
Phospholipids determine binding of HBsAg

**Fig. 3.** sCD14 inhibits binding of b-rHBsAg to monocytes. b-rHBsAg was incubated on ice with increasing amounts of sCD14 in 2% HS–HBSS. After 1 h, PBMCs were added. Data shown represent the average of three separate experiments. Error bars represent SD.

**Fig. 4.** Binding of b-rHBsAg to monocytes is blocked by mAbs My4, biG4 and biG11, which bind to the LPS-binding site of CD14. PBMCs were incubated with 0–25 µg/ml (grey bars), 1 µg/ml (white bars) and 2–5 µg/ml (black bars) of different CD14-specific antibodies and 2–5 µg/ml of isotype controls in 2% HS–HBSS. After 1 h of incubation on ice, b-rHBsAg was added and the cells were incubated for another 80 min. After two washes, b-rHBsAg was detected with Strep–PE. The data shown represent the average ± SD of two separate experiments.

with known epitope specificity were performed. Amino acids 39–44 are important for the binding of mAbs My4 and biG11, which are known to block the binding of LPS, the best studied ligand for CD14. Amino acids 33–39 are important for binding of mAb biG4, which, in contrast to My4 and biG11, does not block LPS-binding. mAbs P9 and biG2, which cannot block the binding of LPS, interact with epitopes defined by amino acids 135–146 and 147–152, respectively (Stelter et al., 1997). As shown in Fig. 4, mAb My4 blocked binding of b-rHBsAg very efficiently. Good inhibition was obtained with 2–5 µg/ml of mAbs biG4 and biG11. Very weak inhibition was observed with mAb P9, while biG2 did not inhibit binding of b-rHBsAg. From this experiment, it is concluded that amino acids 33–44, which are important for binding LPS, are also involved in the attachment of b-rHBsAg to the cell. To confirm these results, the binding of b-rHBsAg to CD14 mutated at positions 39, 40, 41, 43 and 44 was investigated using mutant CD14(A39–41,43,44) (Stelter et al., 1997); a rabbit anti-CD14 polyclonal serum efficiently recognized this mutant (Table 1). The mutant was recognized also by several mAbs, such as P9 and biG2, although binding was reduced compared to wt CD14. As expected, mAb biG4 did not bind to CD14(A39–41,43,44) and neither did b-rHBsAg. This observation confirms the results obtained with the antibody-inhibition experiments.

### LBP enhances attachment of b-rHBsAg to monocytes

Attachment of b-rHBsAg to monocytes was enhanced at low serum concentrations (1–3%). At higher concentrations (7–10%), binding was slightly inhibited compared to the serum-free control. These effects were not observed when BSA was used at similar protein concentrations. Incubation of serum at 56 °C for 30 min destroyed the factor, enhancing the attachment of b-rHBsAg to PBMCs, and thus demonstrating the thermolability of this factor (Vanlandschoot et al., 2002). LBP is a 60 kDa glycoprotein found in the plasma of all species studied so far. LBP is best known for its ability to catalyse the transfer of LPS to the major LPS receptor, CD14. Because human LBP is known to be inactivated rapidly at 56 °C (Meszaros et al., 1995) and because of the remarkable similarity between binding of LPS and rHBsAg to CD14+ cells, LBP was considered a likely candidate for the factor that enhances attachment of rHBsAg. Indeed, when purified LBP was used instead of serum, binding of b-rHBsAg was strongly enhanced.

### Table 1. Substitution of amino acids 39, 40, 41, 43 and 44 of human CD14 with alanine abrogates attachment of b-rHBsAg to CHO cells

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<td>b-rHBsAg</td>
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<td>CHO-CD14</td>
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<td>CHO-CD14(A39–41,43,44)</td>
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Around a rabbit anti-CD14 polyclonal serum.
in a dose-dependent manner (Fig. 5a). Further evidence for the role of LBP was obtained using two different LBP-specific mAbs. When these were included during incubation of PBMCs with b-rHBsAg in 2% HS–HBSS, attachment of b-rHBsAg was completely abrogated (Fig. 5b). Taken together, these experiments show that LBP is indeed the serum factor which enhances binding of rHBsAg to CD14+ cells.

Study of the role of phospholipids in the binding of HBsAg to CD14+ cells

Based on observations made with yeast-expressed HBsAg, natural pHbsAg was examined for its potential to bind to CD14+ cells. For this, the capacity of pHbsAg to reduce the binding of b-rHBsAg was examined. Surprisingly, even high doses (up to 50 µg/ml) of pHbsAg, in the presence of 2% HS, did not inhibit the attachment of b-rHBsAg to PBMCs. Additionally, unlike rHBsAg, pHbsAg was not detected by S-specific mAbs on the surface of CD14-expressing CHO cells, although binding of these mAbs to pHbsAg was demonstrated clearly (data not shown here but the results of similar experiments are shown below). Despite the differences in protein composition between natural HBsAg (S, M and L) and yeast-derived HBsAg (only S), it was considered more likely that differences in lipid content were responsible for the observed discrepancy. pHbsAg consists of 25–30% lipids (by weight) and differences in lipid content between plasma- and yeast-derived HBsAg have been reported (Kim & Bisell, 1971; Van der Meeren et al., 1994). Additionally, both CD14 and LBP have been shown to bind several phospholipids (Schromm et al., 1996; Wurfel & Wright, 1997; Yu et al., 1997).

Three phospholipids were examined first for their potential to block binding of b-rHBsAg to PBMCs. Subsequently, lipids of pHbsAg were extracted and the particles were reconstituted with these three phospholipids. The different pHbsAg preparations were examined for their antigenic structure and capacity to bind to CD14+ cells. The lipids used were chosen because of possible differences in binding to LBP or CD14 and differences in restoring the antigenic structure of pHbsAg (Gomez-Gutierrez et al., 1994, 1995; Schromm et al., 1996; Yu et al., 1997): PC does not bind to CD14 or LBP and leads to partial restoration of HBsAg antigenicity only; PS has been shown to bind to CD14 and LBP and to restore normal antigenicity of pHbsAg; DOPG has been reported to bind to LBP but it is not known if it binds to CD14 and reconstitution of HBsAg with this lipid results in enhanced antigenicity.

Effect of phospholipids on the attachment of b-rHBsAg to PBMCs

Cells were incubated with PC, PS and DOPG in 2% HS–HBSS. After washing, PBMCs were incubated with b-rHBsAg. Of the three lipids examined, PC did not inhibit attachment of b-rHBsAg. Both PS and DOPG reduced strongly the binding of b-rHBsAg to monocytes (Fig. 6). Because the lipids were removed before b-rHBsAg (in fresh 2% HS–HBSS) was added to the PBMCs, the lipids attached to the cells caused the inhibition.

The lipid content of HBsAg can influence the efficiency of binding to CD14-expressing cells

pHBsAg was stripped of its lipids and reconstituted with PC, PS or DOPG. rHBsAg and pHbsAg were antigenically
indistinguishable. Extraction of the lipids reduced recognition by mAbs F47B, F9H9 and anti-a. Reconstitution with PC did not restore this binding, while increased binding of mAbs was observed to PS- and DOPG-pHBsAg (data not shown). These results are in agreement with previous reports (Gomez-Gutierrez et al., 1994, 1995).

The LBP- and CD14-dependent attachment of rHBsAg, pHBsAg, Del-pHBsAg, PC-pHBsAg, PS-pHBsAg and DOPG-pHBsAg to the membrane of CHO cells was examined. As shown in Fig. 7, LBP already induced attachment of rHBsAg to CHO-DHFR control cells. Of the different pHBsAg preparations, only DOPG-pHBsAg bound to CHO-DHFR cells. Stronger binding of rHBsAg, PS-pHBsAg and DOPG-pHBsAg to CHO-CD14 was demonstrated, while even in the presence of LBP, no attachment was seen for pHBsAg, Del-pHBsAg and PC-pHBsAg to these cells. However, Del-pHBsAg and PC-pHBsAg are hardly recognized by mAb F47B, which was used to detect the particles on the cell surface. Therefore, the capacity of the different HBsAg preparations to inhibit binding of b-rHBsAg to PBMCs was examined (Fig. 8). Of the different preparation, 10 and 50 µg/ml, together with 0.5 µg/ml LBP in 1% HS–HBSS, were used to saturate all possible binding sites on the monocytes. After washing, PBMCs were incubated with b-rHBsAg. The use of 10 µg/ml of rHBsAg clearly inhibited (80%) the attachment of b-rHBsAg. Hardly any inhibition, even with 50 µg/ml, was obtained with pHBsAg, Del-pHBsAg and PC-pHBsAg. Both PS- and DOPG-pHBsAg inhibited binding of b-rHBsAg very efficiently. Taken together, these experiments show that plasma-purified HBsAg does not gain this characteristic when certain phospholipids are incorporated into the particles.

An antibody specific for the C-terminal end of the S protein reduces binding of rHBsAg to CHO-CD14

It is obvious from the results described above that the phospholipid moiety determines the capacity to bind to monocytes. However, it is not clear if the interaction occurs between the cellular receptor and the lipids of rHBsAg or between the S protein and the receptor. Previously, a dose-dependent reduction in binding of b-rHBsAg to monocytes was observed, with mAb F47B only (Vanlandschoot et al., 2002). This was not unexpected because biotinylation interferes strongly with the binding of mAbs F9H9 and anti-a to rHBsAg. Although recognition of b-rHBsAg by mAb anti-a was reduced only slightly, doses of up to 20 µg/ml did not inhibit attachment of b-rHBsAg to PBMCs. This observation suggested that some specific region on the S protein might be involved in the interaction.

Using CHO-CD14 cells, it is possible to detect non-biotinylated rHBsAg on the cell membrane and, therefore, to
study the effect of the other mAbs (anti-\(d\), anti-\(a\), F9H9 and F47B) on the attachment of rHBsAg to the cell surface. All these mAbs detected rHBsAg bound to the cell surface of CHO-CD14 cells (data not shown). Binding of rHBsAg pre-incubated with the different mAbs was compared to the binding of rHBsAg only. Particles were detected with the mouse mAb anti-\(d\). As a control, the effect of the mAbs on the recognition of rHBsAg already bound to the cell surface by mAb anti-\(d\) was determined. As shown in Fig. 9, identical signals were observed if rHBsAg was pre-incubated or not with mAb anti-\(d\), which suggests that this mAb does not inhibit binding. Human mAbs anti-\(a\) and F9H9 did not interfere with recognition by anti-\(d\) of rHBsAg already bound to the cells. Pre-incubation of rHBsAg with these mAbs did not result in reduced detection by mAb anti-\(d\), which suggests that these two human mAbs do not inhibit binding. Surprisingly, mAb F47B interfered with the detection by mAb anti-\(d\) of rHBsAg on the cell surface. Nevertheless, after pre-incubation of rHBsAg with F47B, a further reduced detection by mAb anti-\(d\) was obtained; this suggests that mAb F47B can (partially) inhibit the attachment of rHBsAg to the cell surface, an observation made before. Taken together, these results indicate that the C-terminal region of the S protein might also play a role in the attachment of rHBsAg to CD14-expressing cells.

**Discussion**

CD14 is present as a cell surface molecule, attached to the membrane through a glycosylphosphatidylinositol linkage, and in a soluble form in the circulation (Haziot et al., 1988). Numerous data show its possible involvement in many immune processes. Most importantly, CD14 is the major LPS receptor (Wright et al., 1990). The binding of LPS to CD14 is facilitated by LBP. Several of our observations demonstrate a remarkable similarity between attachment of LPS and rHBsAg to the cell membrane and suggest that rHBsAg binds to monocytes through interaction with LBP and CD14. (1) Attachment of rHBsAg is restricted to CD14\(^+\) cells (monocytes) of PBMCs. (2) THP-1 and CHO cells, which do not express CD14, do not bind rHBsAg efficiently. Highly enhanced attachment of rHBsAg to these cells can be obtained after induction of CD14 by 1,25-dihydroxyvitamin D\(3\) treatment or by transfection of CD14 cDNA. (3) PMA-induced shedding of CD14 results in reduced binding of rHBsAg (Vanlandschoot et al., 2002). (4) Binding of rHBsAg is blocked efficiently by several CD14-specific mAbs that bind to a region that is considered important for binding of LPS (Stelter et al., 1997). Because two other CD14-specific mAbs that do not block LPS binding to CD14 do not also block attachment of rHBsAg, we conclude that attachment of rHBsAg occurs through a domain on CD14 that is identical to or largely overlaps with the LPS-binding pocket. The lack of rHBsAg binding to CHO-CD14\((29^{-1}1,43,44)A\) demonstrates further the importance of the LPS-binding pocket for attachment of rHBsAg. (5) sCD14 can block binding of rHBsAg to membrane-bound CD14. (6) Purified recombinant LBP induces strong binding of rHBsAg to monocytes or CHO-CD14, while LBP-specific mAbs block the binding. Nevertheless, three observations suggest that additional membrane proteins are involved in the attachment of rHBsAg to the cell membrane. Firstly, CD14 is a glycosylphosphatidylinositol anchored membrane protein and therefore an additional rHBsAg-interacting protein is required to signal the suppressive action of rHBsAg. Secondly, saturating amounts of rHBsAg do not block the attachment of CD14-specific antibodies (data not shown). Finally, LBP can already induce a moderate but significant attachment of rHBsAg to CHO-DHFR control cells.

In contrast to rHBsAg, which binds to the membrane of CD14\(^+\) cells through interaction with LBP, pHBsAg does not. The results from the reconstitution experiments demonstrate clearly that this capacity to bind to the cell surface is determined by the phospholipid content of the particles. Attachment to CD14 cells does not occur upon reconstitution of pHBsAg with PC. Upon reconstitution of Del-pHBsAg with PS or DOPG, highly efficient attachment is observed. The lipid content of plasma-purified and S. cerevisiae-derived HBsAg particles has been determined (Gavilanes et al., 1982; Van der Meer et al., 1994). PC and neutral lipids are the major components, phosphatidylethanolamine being a minor one. The most striking difference is the abundance (27–32%) of PI in the yeast-derived particles. This phospholipid is not present in natural HBsAg. Like PS, PI has been shown to bind to LBP and CD14. Based on these observations, it is proposed here that PI and PS are the phospholipids that determine the LBP-
dependent attachment of rHBsAg to the membrane of CD14-expressing cells. The question remains whether attachment of rHBsAg to the cells results solely from a phospholipid–receptor interaction. PC does not bind to CD14 or LBP, while PS is known to bind to LBP and CD14. DOPG is known to bind LBP (Schromm et al., 1996; Wurzel & Wright, 1997; Yu et al., 1997). Because DOPG blocks binding of rHBsAg, DOPG most probably binds to the cell surface through interaction with LBP and CD14. Therefore, it is attractive to conclude that binding of rHBsAg to the cells results solely from a phospholipid–receptor interaction. However, a precise conformational structure of the S protein, which depends on the presence of PS and PI, might be required for HBsAg to interact with CD14 and LBP. Indeed, the possible involvement of the C-terminal end of the S protein cannot be excluded, as demonstrated by the antibody-inhibition experiments. This region, amino acids 160–207, has been predicted to form two membrane-spanning domains (Stirk et al., 1992). However, several mAbs have been reported to bind to this region, which was suggested recently to lie on the surface of the membrane with the hydrophilic face in contact with an aqueous environment (Paulij et al., 1999; Jolivet-Reynaud et al., 2001). Mutations in the region of amino acids 112–145 can affect the recognition of the 160–207 amino acid region by mAbs. It is thought that the C-terminal region is close to a region around residue 120 of the same monomer or the adjacent monomer (Chen et al., 1996). The observation that mAb F47B interferes with binding of mAb anti-d supports further this new structural concept.

The total lack of PI and the presence of only trace amounts of PS in pHBsAg is remarkable, if one considers that HBsAg, when expressed in mammalian cells (like CHO, human hepatoma cells and mouse fibroblast cells) or yeast cells (S. cerevisiae and Hansenula polymorpha) contains always 4–7% of PS and/or phosphatidylinositol at least (Gavilanes et al., 1982; Van der Meeren et al., 1994; Satoh et al., 1990, 2000; Diminsky et al., 1997). Furthermore, reconstitution experiments have demonstrated that good recovery of antigenicity is obtained only when negatively charged phospholipids, like PI and PS, are used (Gomez-Gutierrez et al., 1994, 1995). Although the results of such reconstitution experiments do not prove that these lipids are required for correct folding in vivo, the fact that HBsAg particles assemble in the membrane of the endoplasmic reticulum, which contains 10–20% molar PI, makes the lack of PI in pHBsAg even more puzzling. However, LBP and sCD14 are known to catalyse the transfer of charged phospholipids (Schromm et al., 1996; Wurzel & Wright, 1997; Yu et al., 1997). This raises the possibility that during HBV infection, PI and PS are initially present in particles produced by infected hepatocytes and that these two lipids are removed by LBP, CD14 or other lipid transfer molecules.

Whatever the in vivo relevance of our observations, the discovery of the rHBsAg–CD14 interaction is probably very important for future HBV vaccine development. Recombinant vaccines replaced the plasma-derived vaccines in the course of the 1990s. These former vaccines have proven to induce a similar rate of seroconversion and protection against HBV as the latter (Leroux-Roels et al., 2001). However, considering the effects of the phospholipid composition on the antigenic and immunogenic structure (Kekley et al., 1981; Manesis et al., 1979; Baijot, 1991; Gomez-Gutierrez et al., 1994, 1995; Diminsky et al., 2000), it seems likely that qualitative and quantitative differences can exist in the antibodies obtained upon immunization with different HBsAg preparations. Indeed, reduced anti-HBsAg levels after immunization with the yeast-derived vaccine compared to the plasma-derived vaccine has long been recognized (Heijtink et al., 1985; Jilg et al., 1984). It was proposed recently that this is due to differences in the antigenic structure and incompatibility of HBsAg used in the quantification assays (Heijtink et al., 2000). Our observations indicate that the anti-inflammatory and immunosuppressive potential of yeast-expressed HBsAg is another factor that might affect the induction of HBsAg-specific antibodies.

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References


Phospholipids determine binding of HBsAg


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