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Branched oligosaccharide structures on HBV prevent interaction with both DC-SIGN and L-SIGN

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SUMMARY. Hepatitis B virus (HBV) is a DNA virus that infects the liver as primary target. Currently, a high affinity receptor for HBV is still unknown. The dendritic cell specific C-type lectin DC-SIGN is involved in pathogen recognition through mannose and fucose containing carbohydrates leading to the induction of an anti-viral immune response. Many glycosylated viruses subvert this immune surveillance function and exploit DC-SIGN as a port of entry and for *trans*-infection of target cells. The glycosylation pattern on HBV surface antigens (HBsAg) together with the tissue distribution of HBV would allow interaction between HBV and DC-SIGN and its liver-expressed homologue L-SIGN. Therefore, a detailed study to investigate the binding of HBV to DC-SIGN and L-SIGN was performed. For HCV, both DC-SIGN and L-SIGN are known to bind envelope glycoproteins E1 and E2. Soluble DC-SIGN and L-SIGN specifically bound HCV virus-like particles, but no interaction with either HBsAg or HepG2.2.15-derived HBV was detected. Also, neither DC-SIGN nor L-SIGN transfected Raji cells bound HBsAg. In contrast, highly mannosylated HBV, obtained by treating HBV producing HepG2.2.15 cells with the α-mannosidase I inhibitor kifunensine, is recognized by DC-SIGN. The α-mannosidase I trimming of N-linked oligosaccharide structures thus prevents recognition by DC-SIGN. On the basis of these findings, it is tempting to speculate that HBV exploits mannose trimming as a way to escape recognition by DC-SIGN and thereby subvert a possible immune activation response.

Keywords: DC-SIGN, hepatitis B virus, L-SIGN, viral recognition.

INTRODUCTION

Hepatitis B virus (HBV) infects the liver as primary target, resulting in the majority of cases in self-limiting acute hepatitis. Nevertheless, more than 350 million people are chronically infected with HBV worldwide [1]. Despite the high incidence of infection, a cellular receptor for HBV entry is still unknown. Several putative binding factors have been described for the HBV surface antigens (HBsAg), such as human serum albumin [2], asialoglycoprotein receptor [3], heparin [4] and mannose binding lectin [5], but their exact role in HBV attachment and uptake remains unclear [6].

Hepatitis B virus is a DNA virus, consisting of a core particle enveloped by small (S), middle (M) and large (L) surface antigens, generally referred to as HBsAg. All three surface antigens contain a common S domain, both M and L proteins contain a preS2 domain and L exclusively contains a preS1 domain [7]. The liver and peripheral blood of HBV infected individuals can reach levels of 10^9–10^{10} infectious particles per mL. In addition, HBsAg is secreted from infected hepatocytes as spherical subviral particles and filaments, which can accumulate up to 100 μg/mL in peripheral blood [7,8]. S is the main component of both HBV virions and HBsAg subviral particles, while M and L are highly enriched on HBV virions. Post-translational modifications of the surface antigens are crucial for HBV life cycle; myristoylation of the preS1 domain is essential for infectivity [6,9], while inhibition of N-glycosylation of the preS2 domain prevents secretion of viral particles

Abbreviations: CHO, Chinese Hamster Ovary; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; HBsAg, HBV surface antigens; HCV, hepatitis C virus; FITC, fluorescein isothiocyanate; PRR, pattern recognition receptors; VLP, virus-like particles.

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[10,11]. Recently, post-translational N-glycosylation of the preS1 domain has been reported as well and although it seems dispensable for HBV morphogenesis it might be involved in viral attachment [12].

The first step in clearance of a viral infection involves recognition of the virus by the innate immune system, mediated through host pattern recognition receptors (PRR) such as Toll-like receptors and C-type lectins [13,14]. C-type lectins recognize highly conserved pathogen-derived carbohydrate structures, leading to internalization, antigen processing and presentation to T cells [14]. A prototypical member of the C-type lectin family is the dendritic cell-specific lectin DC-SIGN [15], which recognizes a broad range of glycosylated pathogens through mannose or fucose containing carbohydrates, including HIV-1, hepatitis C virus (HCV), Ebola virus, Dengue virus, measles virus, human herpesvirus 8, SARS coronavirus, cytomegalovirus, *Mycobacterium tubercolosis*, *Helicobacter pylori*, *Streptococcus pneumonia* and *Neisseria meningitidis* [16–18]. The outcome of this interaction is pathogen-dependent. Many of these DC-SIGN-binding pathogens have evolved to subvert the immune surveillance function of DC-SIGN for their own benefit and re-direct the internalization route to nonlysosomal compartments for protection and transmission to target cells [16]. Some pathogens can also modulate adaptive immune responses through interaction with DC-SIGN; e.g. *M. tuberculosis* exploits DC-SIGN to escape immune surveillance by inhibition of the immunostimulatory function of dendritic cells [16,19].

It is well established that envelope glycoproteins E1 and E2 of HCV interact with both DC-SIGN [15] and its homologue L-SIGN [20] expressed on sinusoidal endothelial cells in liver and lymph nodes. For HCV virus-like particles, DC-SIGN interaction leads to efficient capture, internalization and transport to nonlysosomal compartments within immature dendritic cells, thereby protecting the virus from degradation [21,22]. Similarly, HCV virus-like particles interact with L-SIGN expressed on liver sinusoidal endothelial cells (LSEC) *in situ*, and are targeted to nonlysosomal early endosomes in L-SIGN transfected Raji cells [22]. DC-SIGN and L-SIGN are also thought to play an important role in HCV viral dissemination by transferring HCV from the circulation to hepatocytes, the main HCV target cells [23].

Several reports have shown the presence of HBV on or within dendritic cells [24–27], suggesting the involvement of a dendritic cell-specific receptor such as DC-SIGN. Patient-derived HBsAg is preferentially internalised by human LSEC in a mixed culture with human hepatocytes (K. Esser and U. Protzer, unpublished data) – as it has also been reported for duck HBV [28] – implying a potential interaction between HBV and L-SIGN. The HBV glycosylation pattern together with the cellular localization prompted us to investigate the possible role of both DC-SIGN and L-SIGN in binding of HBV.

METHODS

Antibodies and viral glycoproteins

The following antibodies were used: DC-SIGN and L-SIGN specific antibody AZN-D2 [29]; mouse anti-DC-SIGN conjugated with fluorescein isothiocyanate (FITC) (clone DCN46; BD Biosciences, San Jose, CA, USA); mouse anti-HCV E2 (4H6B2; Innogenetics, Ghent, Belgium); sheep anti-HBsAg preS1, mouse anti-HBsAg preS2 and biotinylated human anti-HBsAg (F-9H9-E, all kind gifts of R. Heijtink, Erasmus MC, Rotterdam, The Netherlands); rabbit anti-HBsAg-FITC (Acris Antibodies GmbH, Hiddenhausen, Germany); peroxidase-conjugated goat anti-human immunoglobulin G1 (Jackson Immunoresearch, West Grove, PA, USA); peroxidase-conjugated rabbit anti-FITC (Dako, Glostrup, Denmark); peroxidase-conjugated goat anti-mouse (Caltag, Carlsbad, CA, USA); peroxidase-conjugated streptavidin (Dako).

Recombinant HBV surface antigen HBsAg (containing both S and preS2 domains) purified from transfected Chinese Hamster Ovary (CHO) cells was kindly provided by M. van Roosmalen (bioMérieux, Boxtel, The Netherlands). Yeast cell-derived HCV glycoproteins E1 and E2 reconstituted as virus-like particles (VLP) were kindly provided by S. Depraetere (Innogenetics).

HepG2.2.15-derived HBV

HepG2.2.15 cells [30] were grown until confluence in Williams' E medium (Gibco, Paisley, UK) supplemented with 5% foetal calf serum (FCS, Hyclone, Logan, UT, USA). Secreted HBV and subviral particles were concentrated from the medium as described before [27], to a final concentration of $0.96 \times 10E9$ HBV particles/mL. To enrich for HBV over subviral particles, HepG2.2.15 culture supernatants were loaded on a PBS equilibrated 1 mL bed volume Hi Trap™ Heparin HP column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). After fifteen column volumes PBS wash, HBV particles were eluted in 20 mL elution buffer (350 mM NaCl, 20 mM Tris–HCl, pH 7.4) and collected in elution fractions of 2 mL at a flow rate of 1 mL/min. Elution fractions were dialyzed against PBS. As a negative control, the same procedure was followed with supernatant from untransfected HepG2 cells. The elution fractions were assessed for the presence of HBV particles with L, M and S-specific enzyme-linked immunosorbent assay (ELISA), by capturing HBV with anti-preS1, anti-preS2 and anti-S specific antibodies respectively. Bound HBV was detected with a biotinylated human anti-HBsAg (F-9H9-E). Quantification of HBV particles was done by COBAS® TaqMan HBV Test (Roche Diagnostics GmbH, Mannheim, Germany) after viral DNA isolation using the High Pure System Viral Nucleic Acid Kit (Roche Diagnostics, Penzberg, Germany). To generate highly mannosylated HBV, HepG2.2.15 cells were treated with the α-mannosidase I inhibitor kifunensine (5 days, 20 μg/mL; Calbiochem, Darmstadt, Germany).

Untreated HepG2.2.15 cells and kifunsine treated HepG2 cells served as negative controls. The final concentrations of highly mannosylated and native HBV were $1.4 \times 10E8$ and $2.2 \times 10E8$ HBV particles/mL, respectively.

Cells

Stable Raji transfectants expressing wild-type DC-SIGN or L-SIGN were generated as previously described [31,32]. Monocyte-derived dendritic cells (moDC) were cultured from monocytes in the presence of IL-4 and GM-CSF (500 and 800 U/mL, respectively; Schering-Plough, Brussels, Belgium). At day 6, the phenotype of the cultured moDC was confirmed by flow cytometric analysis [22]. Expression levels of DC-SIGN and L-SIGN on transfected Raji cells and moDC were determined by flow cytometry with the DC-SIGN and L-SIGN specific AZN-D2 antibody.

Plant lectin ELISA

Heparin purified, glycan modified or native HepG2.2.15-derived HBV were captured on ELISA plates (Maxisorb, Nunc, Roskilde, Denmark) with mouse anti-preS2. Recombinant HBsAg (0.5 μg/well), HCV VLP (0.25 μg/well) and mannan (0.25 μg/well; Sigma-Aldrich, St Louis, MO, USA) were coated directly onto the plate for 18 h at 4 °C. After blocking with 1% bovine serum albumin for 1 h at 37 °C, the following biotinylated plant lectins were added for 2 h at room temperature at a concentration of 5 μg/mL: Con A (*Concavalin A*; recognizes α-glucose and α-mannose), GNA (*Galanthus nivalis* agglutinin; α-mannose), WGA (wheat germ agglutinin; *N*-acetylglucosamine), PNA (*Arachis hypogaea* agglutinin; β-galactose, N-acetylgalactosamine), RCAII (*Ricinus communis* agglutinin; β-galactose, *N*-acetylgalactosamine) or LTA (*Lotus tetragonolobus* agglutinin; fucose; all from Sigma Aldrich [33]). Lectin binding was detected using peroxidase-conjugated streptavidin and absorbance was measured at 450 nm.

Recombinant DC-SIGN-Fc binding ELISA

The DC-SIGN-Fc binding assay was performed as previously described [34]. In short, recombinant HBsAg (0.5 μg/well), HepG2.2.15-derived HBV and HepG2 controls were captured on Maxisorb ELISA plates (Nunc) with mouse anti-preS2. HCV VLP (0.25 μg/well) were coated directly on the plates. After blocking with 1% bovine serum albumin for 30 min at 37 °C, soluble DC-SIGN-Fc was added and bound DC-SIGN was detected after incubation with peroxidase-labelled anti-human immunoglobulin G1 antibody. Specificity of DC-SIGN-Fc binding was determined by blocking with either mannan (100 μg/mL; Sigma-Aldrich) or EGTA (10 mM; Sigma-Aldrich). To assess the coating efficiency, HBsAg and HBV were detected with biotinylated human anti-HBsAg (F-9H9-E) and HCV VLP were detected with mouse anti-HCV E2 (4H6B2). HBV concentrated by centrifugation or purified by heparin column gave similar results in DC-SIGN-Fc binding assays.

Soluble DC-SIGN/L-SIGN lysate ELISA

Raji DC-SIGN/L-SIGN transfectants were lysed for 4 h at 4 °C in lysis buffer (1% NP40, 150 mM NaCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ in PBS) supplemented with ethylenediamine tetraacetic acid (EDTA)-free protease inhibitors (Roche Diagnostics). HBsAg, HBV and medium controls were captured on ELISA plates with a mouse anti-preS2 antibody. HCV VLP were coated directly on the plates. After blocking with 5% bovine serum albumin for 30 min at 37 °C, Raji DC-SIGN/L-SIGN lysates were added for 2 h at RT. Bound DC-SIGN/L-SIGN was detected with a FITC-conjugated DC-SIGN/L-SIGN specific antibody (clone DCN46) followed by a peroxidase-conjugated rabbit anti-FITC antibody. Specificity of binding was determined in the presence of mannan (100 μg/mL; Sigma-Aldrich). To assess the coating efficiency, HBsAg, HBV and HCV VLP were detected with specific antibodies. HBV concentrated by centrifugation or purified by heparin column gave similar results in soluble DC-SIGN/L-SIGN lysate ELISA.

Cellular DC-SIGN/L-SIGN bindings assay

Untransfected Raji cells, DC-SIGN or L-SIGN-transfected Raji cells and moDC were incubated with or without HBsAg (5 μg/mL) for 2 or 18 h at 37 °C and binding was measured by flow cytometry after intracellular staining with an anti-HBsAg-FITC antibody (Acris Antibodies GmbH). Uptake was compared to lectin-mediated binding of dextran-FITC (100 μg/mL, 40 000 MW; Molecular Probes, Invitrogen, Carlsbad, CA, USA). Specificity of binding was determined by 30 min preincubation with mannan (100 μg/mL).

RESULTS

Characterisation of purified HBV particles

Secreted HBsAg subviral particles outnumber the HBV virions at least 100-fold in both patient serum and culture supernatant of HepG2.2.15 cells [7]. To enrich for HBV virions, culture supernantant of HepG2.2.15 cells was fractionated over a heparin column and different elution fractions were assessed by HBV L, M and S-specific ELISA. The input fraction contained mainly the S protein, representing the relative high level of secreted spherical subviral particles (Fig. 1a). Elution fraction 1 and 2 however, were highly enriched for both M and L

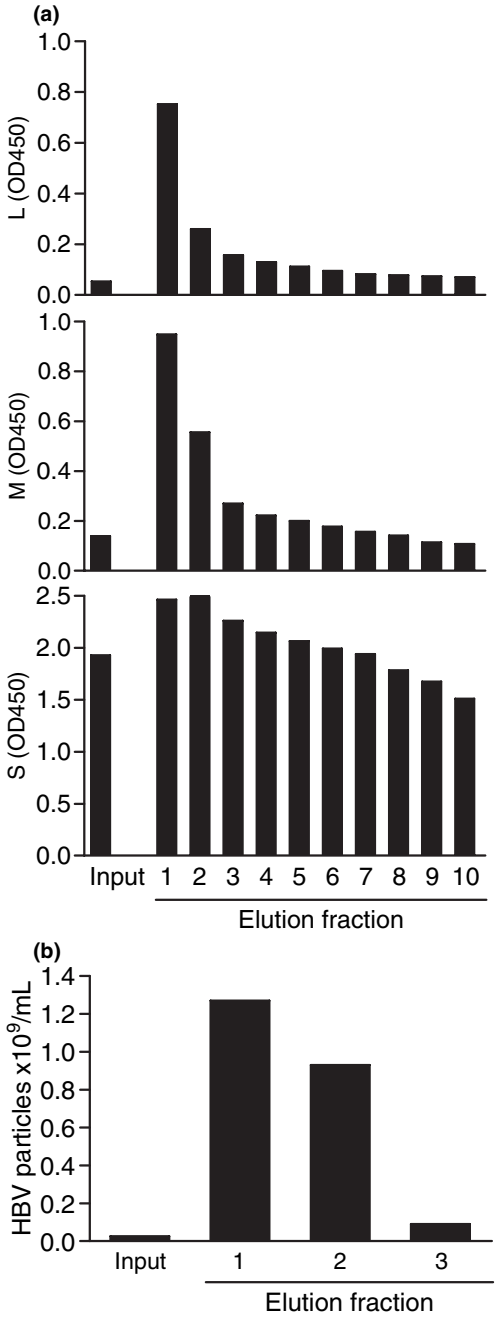

Fig. 1 Characterization of purified HBV particles. (a) HBV particles were purified from HepG2.2.15 culture supernatant by heparin column and both input and different elution fractions were assessed by L, M and S-specific capture ELISA. (b) Quantification of HBV particles in both input and elution fraction 1, 2 and 3 by COBAS® TaqMan HBV Test.

proteins confirming the increased level of HBV virions in these fractions. Quantification of HBV-DNA of the input fraction and elution fraction 1–3 confirmed an almost 50-fold enrichment of HBV virions in fraction 1 (0.026 to 1.27×10^9 HBV particles per mL, Fig. 1b).

HBsAg and HBV do not interact with recombinant DC-SIGN-Fc

The interaction between DC-SIGN and recombinant HBsAg or HepG2.2.15-derived HBV was determined in a binding ELISA where coated HBsAg, HBV, HCV VLP or HepG2 medium controls were incubated with soluble recombinant DC-SIGN-Fc. The detected signal of the HepG2 medium control was never above background. Strikingly, DC-SIGN did not interact with HBsAg nor with whole virus particles, whereas DC-SIGN did interact with HCV virus-like particles, consisting of purified yeast recombinant E1 and E2 HCV envelope proteins (Fig. 2a and [22]). Specificity of binding

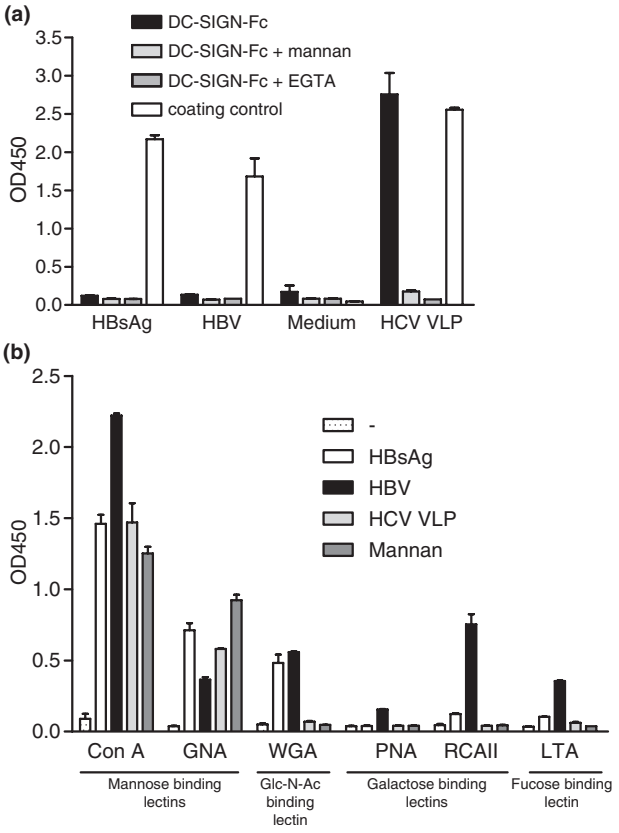

Fig. 2 HBV and its surface antigen HBsAg do not interact with recombinant DC-SIGN. (a) DC-SIGN interaction with CHO-derived recombinant HBV surface antigen HBsAg and HepG2.2.15-derived HBV was determined in an Fc-based ELISA, as described in Methods. Supernatant of the nonvirus producing cell line HepG2 was used as negative control and HCV VLP were used as a positive control. Specificity of binding was determined in the presence of mannan or the calcium chelator EGTA. To assess the coating efficiency, HBsAg, HBV and HCV VLP were detected with specific antibodies. Data are shown as mean ± SD of duplicate measurements; one representative experiment out of four is shown. (b) Glycan analysis of HBsAg and HBV by plant lectin ELISA. For abbreviations and detailed oligosaccharide specificity of the indicated lectins see Methods.

was determined by blocking with either mannan, a yeast cell derived polycarbohydrate that competitively binds the carbohydrate binding site of mannose-specific lectins, or the calcium chelator EGTA, and both agents indeed reduced DC-SIGN binding to HCV to background level. Coating controls demonstrated that approximately equal amounts of HBsAg, HBV and HCV VLP were coated.

Based on their oligosaccharide specificity, several plant lectins were used in a glycan analysis of HBsAg and heparin purified HBV (Fig. 2b). Strong binding to Con A, GNA, WGA, PNA, RCAII and LTA demonstrated the presence of high and/or complex mannose, *N*-acetylglucosamine, galactose and fucose containing carbohydrate structures on both HBsAg and HBV. For DC-SIGN-glycoprotein interaction the high mannose and fucose containing oligosaccharide structures are most important [35]. Of note, carbohydrate structures on both HCV VLP and mannan mainly consist of mannose, as indicated by the exclusive binding to Con A and GNA.

HBsAg and HBV do not interact with soluble DC-SIGN from cell lysates

To exclude the possibility that the lack of interaction between HBV and recombinant DC-SIGN was because of improper multimerization of DC-SIGN-Fc, binding of HBsAg and HBV to native DC-SIGN was studied with DC-SIGN derived from transfected Raji cells [31,32]. As described before, flow cytometric analysis of Raji transfectants showed high expression levels of DC-SIGN (Fig. 3a, [22]). In a bindings ELISA, coated HBsAg or HCV VLP were incubated with lysates of mock or DC-SIGN transfected Raji cells and bound DC-SIGN was detected with an anti-DC-SIGN antibody.

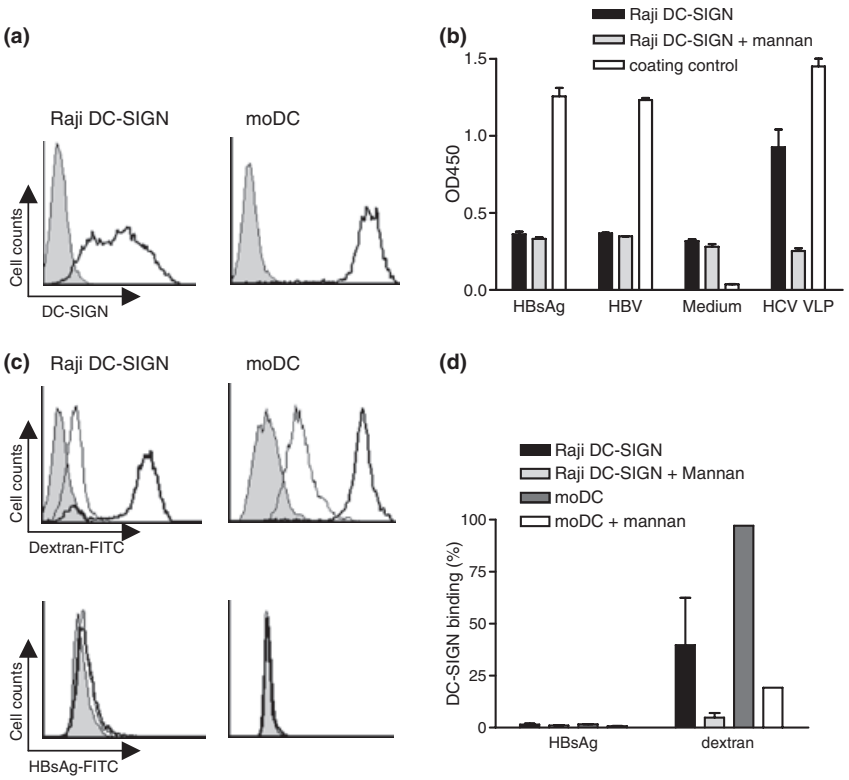

Fig. 3 HBsAg and HBV do not interact with cellular DC-SIGN. (a) Expression levels of DC-SIGN on DC-SIGN transfected Raji cells and monocyte-derived dendritic cells (moDC) were determined by flow cytometry with AZN-D2 (bold black line). Filled graphs represent isotype controls. (b) HBsAg and HBV interaction with cellular DC-SIGN was determined by the soluble DC-SIGN lysate ELISA, as described in Methods. Interaction with HCV VLP was determined as a positive control. Specificity of binding was determined in the presence of mannan. To assess the coating efficiency, HBsAg, HBV and HCV VLP were detected with specific antibodies. Data are shown as mean ± SD of duplicate measurements; one representative experiment out of three is shown. (c, d) Interaction of HBsAg with DC-SIGN transfected Raji cells (n = 2) or DC-SIGN expressing moDC (n = 1) in culture was determined by flow cytometry, after 2 h HBsAg incubation at 37 °C. Specificity of binding was determined by 30 min preincubation with mannan. Lectin-mediated binding of dextran-FITC to DC-SIGN was determined as a positive control. (c) Histogram plots of dextran-FITC (upper panel) and HBsAg (lower panel) binding to Raji DC-SIGN and moDC. Filled graphs represent background without substrate, bold black lines represent substrate binding at 37 °C and black lines show the specific inhibition with mannan. (d) Summary of DC-SIGN binding experiments, data are shown as mean ± SD.

DC-SIGN derived from Raji transfectants did not bind HBsAg nor whole HBV particles, whereas HCV VLP showed mannan-sensitive binding to DC-SIGN (Fig. 3b). Control incubation with anti-HCV and anti-HBsAg specific antibodies indicated equal amounts were coated. No background binding was observed for either HBsAg or HCV VLP using the lysate of untransfected Raji cells (data not shown).

HBsAg does not interact with cellular DC-SIGN

In addition to ELISA, HBsAg interaction with cellular DC-SIGN was also determined. DC-SIGN is highly expressed on moDC (Fig. 3a). Therefore, both Raji-DC-SIGN and moDC were incubated with HBsAg and binding was measured by flow cytometry. Neither DC-SIGN-positive Raji cells nor moDC interacted with HBsAg after 2 and 18 h incubation (Fig. 3c and data not shown). In contrast, both DC-SIGN-transfected Raji cells and moDC efficiently internalised the DC-SIGN ligand dextran-FITC in a mannan-sensitive manner (Fig. 3c). Mock transfected Raji cells remained dextran-FITC negative (data not shown). Figure 3d summarizes the results of two independent binding assays.

HBsAg and HBV do not interact with soluble L-SIGN

In a similar manner, the interaction between cellular L-SIGN and both HBsAg and HBV was determined using L-SIGN-transfected Raji cells. Flow cytometric analysis showed L-SIGN expression levels equal to DC-SIGN expression (Fig. 4a [22]). Soluble L-SIGN interacted neither with HBV nor with HBsAg in ELISA, whereas L-SIGN did interact with HCV VLP (Fig. 4b). Medium controls were always at background level. Moreover, cellular L-SIGN expressed by Raji transfectants did not bind to HBsAg, while it bound dextran-FITC in a mannan-sensitive manner (Fig. 4c,d).

DC-SIGN binds highly mannosylated HBV

Thus, neither HBsAg nor HBV interacts with DC-SIGN or L-SIGN, whereas both C-type lectins interact with HCV and other viruses such as HIV-1. These data suggest that the N-linked glycosylation of HBV is distinct from that of other viruses. To evaluate whether indeed native HBV glycosylation does not meet the requirements for DC-SIGN interaction, glycan modified HBV was generated by treating

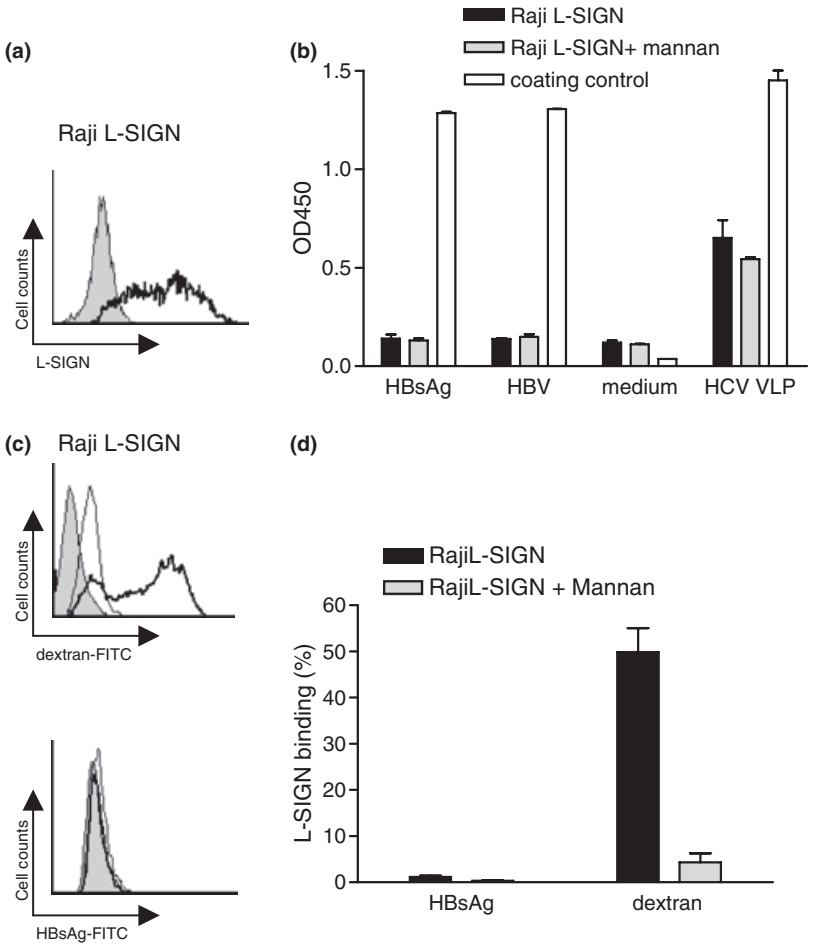

Fig. 4 HBsAg and HBV do not interact with cellular L-SIGN. (a) Expression level of L-SIGN on L-SIGN transfected Raji cells was determined by flow cytometry with AZN-D2 (bold black line). Filled graph represents isotype control. (b) HBsAg and HBV interaction with cellular L-SIGN was determined by soluble L-SIGN lysate ELISA. Data are shown as mean ± SD of duplicate measurements; one representative experiment out of three is shown. (c, d) Interaction of HBsAg with L-SIGN transfected Raji cells in culture was determined by flow cytometry, as described in Fig. 3. L-SIGN binding of dextran-FITC was determined as a positive control. (c) Histogram plots of dextran-FITC (upper panel) and HBsAg (lower panel) binding to Raji L-SIGN. Filled graphs represent background without substrate, bold black lines represent substrate binding at 37 °C and black lines show the specific inhibition with mannan. (c) Results of two independent L-SIGN binding experiments, data are shown as mean ± SD.

HBV-producing HepG2.2.15 cells with the α-mannosidase I inhibitor kifunensine. Kifunensine causes the accumulation of $Man_{7-9}GlcNAc_2$ oligosaccharides on glycoproteins by inhibiting mannose trimming in the endoplasmic reticulum [36]. Carbohydrate analysis of glycan modified HBV indeed demonstrated an increased amount of mannose structures compared to native HBV, shown by a two-fold increase in binding to the mannose-specific plant lectins Con A and GNA while binding to the N-acetylglucosamine-specific lectin WGA was unchanged (Fig. 5b). Control incubations with an anti-HBsAg specific antibody showed that similar amounts of native and highly glycosylated HBV were coated (Fig. 5a). The interaction between highly mannosylated HBV and DC-SIGN was studied by DC-SIGN-Fc-based ELISA (Fig. 5c). Strikingly, HBV binding to DC-SIGN was observed after kifunensine treatment of the virus producing cells, and the binding could be inhibited by both mannan and EGTA. The protein structure of HBV is therefore compatible with DC-SIGN binding, but the native glycosylation, that exclusively determines DC-SIGN interaction, is not. This observation also excludes the possibility that lack of DC-SIGN-HBV interaction is observed due to too low sensitivity of the DC-SIGN assays used in this study. Supernatant of untreated HepG2.2.15 and HepG2 cells showed no binding to DC-SIGN. Minor background binding was observed with supernatant of kifunensine-treated HepG2 cells, due to the accumulation of large amounts of highly mannosylated proteins in the medium during treatment. These data indicate that mannose trimming of the oligosaccharide structures present on HBV prevents interaction with DC-SIGN.

DISCUSSION

One of the key questions in HBV biology focuses on the attachment and entry mechanisms used by HBV to infect hepatocytes, the main target cells. Several putative binding receptors are proposed, but none of them are confirmed as high affinity receptors for HBV. Related to the issue of HBV entry is the way HBV reaches the hepatocyte: Is there direct contact between HBV in the circulation and hepatocytes, possibly through fenestrations in the LSEC [37]? Or are other cell types involved in HBV dissemination, similar to HIV transport by dendritic cells for *trans*-infection of T cells [31] and HCV capture by LSEC promoting infection of hepatocytes [38]?

Since HBsAg are glycoproteins, the involvement of a C-type lectin receptor in viral recognition seems plausible. The best-characterised C-type lectins are the dendritic cell-expressed DC-SIGN and its LSEC-expressed homologue L-SIGN, both capable of recognizing a broad range of glycosylated pathogens, including viruses, bacteria and protozoa [16]. The fact that HBV has been found attached to both

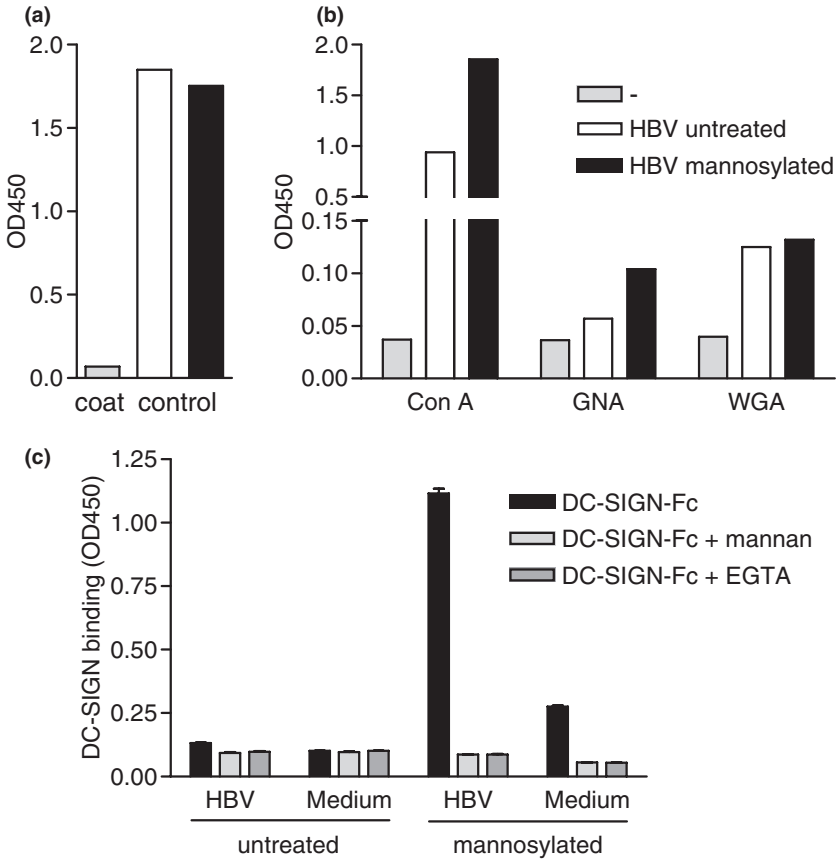

Fig. 5 DC-SIGN specifically binds highly mannosylated HBV. Highly mannosylated HBV was generated by treating HBV producing HepG2.2.15 cells with the α-mannosidase I inhibitor kifunensine (20 μg/mL) for 5 days. Untreated HepG2.2.15 cells and kifunensine treated HepG2 cells served as negative controls. (a, b) Glycan analysis of untreated and mannosylated HBV by the mannose-specific lectins Con A and GNA and the N-acetylglucosamine-specific lectin WGA. In the coating control, the captured amount of untreated and mannosylated HBV is detected with an anti-HBS specific antibody. (c) The interaction of highly mannosylated HBV with DC-SIGN was studied by DC-SIGN-Fc ELISA, as described in Fig. 2. Specificity of binding was determined in the presence of mannan or EGTA. Supernatant of untransfected HepG2 cells is shown as medium control. Data are shown as mean ± SD of duplicate measurements; one representative experiment out of three is shown.

dendritic cells [24–27], and LSEC [28] leads to DC-SIGN and L-SIGN as likely candidate receptors.

The present study conclusively shows the lack of interaction between DC-SIGN and purified HepG2.2.15-derived HBV with its native glycosylation. Moreover, recombinant HBsAg were recognized by neither DC-SIGN nor L-SIGN. Glycan analysis of both HBV and HBsAg demonstrated that lack of DC-SIGN binding was not due to aberrant glycosylation of virions and glycoproteins used in this study, since the observed glycosylation pattern was consistent with previously reported complex-type carbohydrate structures on HBV [39]. Notably, while mannose and N-acetylglucosamine seem to be present in similar amounts on both HBV and HBsAg, glycan structures on virions are enriched for galactose, N-acetylgalactosamine and fucose. This difference in observed glycosylation pattern might be the result of O-glycans present on the preS1 domain [39].

DC-SIGN has been shown to interact with high mannose-containing glycans on various viruses, such as HCV, HIV and Ebola [16]. Even though data demonstrate that these viruses can exploit DC-SIGN for their own benefit, the majority of the captured virus is degraded and routed into the antigen presentation pathway [40–42], thereby allowing the induction of an anti-viral immune response. Although HBV glycoproteins are involved in viral recognition, as they are known to bind mannose binding lectin and the asialoglycoprotein receptor, here it is shown that α-mannosidase I trimming of N-linked oligosaccharide structures prevents recognition by DC-SIGN. HBV is thus recognized by DC-SIGN as soon as one of the enzymes in the formation of complex glycans, α-mannosidase I, is inhibited and highly mannosylated virions are generated. Previous reports have shown the importance of HBV glycosylation for viral secretion [11,43] and indeed we observe a decrease in secretion of highly mannosylated *vs* native HBV, under similar culture conditions.

We cannot rule out that patient sera contain a subpopulation of these highly glycosylated viremia that would allow interaction with DC-SIGN. Preliminary data on HBV derived from several patient sera with a high viral load did not show binding to DC-SIGN-Fc (data not shown), thereby indicating that DC-SIGN may not be involved in recognition of HBV *in vivo* either. On the basis of our findings, it is tempting to speculate that HBV exploits mannose trimming as a way to escape recognition by DC-SIGN and thereby subvert a possible immune activation response. Further studies are needed to address this issue.

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CONFLICTS OF INTEREST

All authors have nothing to disclose.

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