Search for Hepatitis B Virus Cell Receptors Reveals Binding Sites for Interleukin 6 on the Virus Envelope Protein

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Summary

The major target organ for hepatitis B virus (HBV) is the liver. However, cells other than hepatocytes, including peripheral blood lymphocytes and monocytes, may become infected with HBV. The cell receptor binding site was assigned to the preS(21-47) segment of the HBV envelope protein. HBV receptors were detected on human liver and hepatoma cells, on B lymphocytes, and, as shown here, on monocytes, and T cell lines, activated by *Escherichia coli* lipopolysaccharide and concanavalin A, respectively. The cell receptors for HBV have not been characterized until now. The detection of HBV receptors and their "activation antigen" characteristic on distinct cells suggested paths for identification of the receptors with already defined cell surface proteins. This search revealed that interleukin 6 contains recognition sites for the preS(21-47) sequence and mediates HBV-cell interactions. Thus, HBV belongs to a group of viruses utilizing cytokines or cytokine receptors for replication and interference with the host immune system.

Hepatitis B virus (HBV),[1] a member of the family of hepadnaviridae (1), is a major human pathogen implicated in primary hepatocarcinoma. Its major target organ is the liver but cells other than hepatocytes, including PBL, may become infected (2). The HBV envelope (env) consists of three distinct related proteins, designated S, M, and L protein, which are products of a single *env* gene. These proteins share a common 226 COOH-terminal amino acid sequence (S protein). M protein has an additional 55 amino acid sequence located at the NH_2 terminus (preS2 sequence). L protein differs from M protein by an additional NH_2-terminal 108 or 119 amino acid sequence designated as preS1. The site mediating the attachment of HBV to human hepatoma HepG2 cells (3-5) and to human liver plasma membranes (6, 7) encompasses amino acid residues (21-47) of the preS1 sequence.

The presence of receptors for the preS (21-47) region of the HBV env protein was demonstrated also on cells of nonhepatic origin, including PBL, hematopoietic cell lines of the B cell lineage, and some carcinoma and simian virus 40 (SV-40)-transformed cell lines (2). HBV DNA or RNA was detected not only in B lymphocytes but also in T lymphocytes (8) and in PBMC (9) of HBV-infected humans and chimpanzees. However, preS1-specific cell receptors for HBV were not detected on T cells, PBMC, or promonocytic cell lines

(2; see below). To resolve this apparent discrepancy, we searched for the expression of HBV receptors in cell lines of the latter two lineages after activation with lectins and LPS, respectively. The emergence of HBV receptors could indeed be demonstrated after activation of the cells. The constitutive expression of HBV receptors in several cell lines and their induced expression in the additional lines facilitated the design of strategies to define the receptors. The quest to characterize preS1-specific receptors for HBV led to the identification of IL-6 as a cell attachment site for HBV. Thus, HBV appears to belong to a group of viruses utilizing interactions between virally encoded proteins and cytokines or cytokine receptors as steps in replication (10-16).

Materials and Methods

Attachment of Cells to Immobilized Ligands. The following human cells and cell lines were used: PBL isolated as described (2); purified PBMC (Advanced Biotechnologies, Inc., Columbia, MD); HepG2 hepatoma cells; Cates 1B (embryonic carcinoma); Wish (amnion); SK-N-SH (neuroblastoma); U937; RPMI 8226 and U266 (myeloma); Namalwa and Cess (B lymphoblast); MOLT-3, MOLT-4, CEM, Jurkat, HUT 78, H9, MT-4 (all seven T cell leukemia lines); FS-4 (foreskin); and COS-1 (SV-40-transformed African Green monkey kidney cells). The cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD) or from donors listed in Acknowledgments. The cells were propagated as described before (2, 3) or as recommended by ATCC. For ligand binding studies, the cells were used either untreated, treated for 5 min at

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[1] *Abbreviations used in this paper:* HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; PI-PLC, phosphatidylinositol-specific phospholipase C; SV-40, simian virus 40.

pH 4 as described (17), or treated with phosphatidylinositol-specific phospholipase C (PI-PLC; 1 U/ml for 1 h at 37°C in 0.14 M NaCl, 0.01 M Tris, 0.02% NaN$_3$, pH 7.2 [TS]). PI-PLC was a gift from Dr. M. G. Low (Columbia University, New York, NY) or was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). These treatments did not affect cell viability as determined by trypan blue exclusion tests.

Selected T cell lines, PBMC, and the promonocytic cell line U937 were stimulated by adding to the appropriate tissue culture media Con A (50 μg/ml; Sigma Chemical Co., St. Louis, MO) and *Escherichia coli* LPS (5 μg/ml; catalog no. 055:B5; Sigma Chemical Co.), respectively, for time periods indicated in Results.

PreS(12-47)- and preS(12-32)-cellulose and cellulose with covalently linked serum-derived hepatitis B surface antigen (HBsAg) containing the preS1 sequence were all prepared as described before (2, 3). Magnetic beads with attached mAb to CD antigens and cytokine receptors, respectively, were prepared as follows. Magnetic beads with covalently attached goat anti–mouse IgG and IgM and anti–rat IgG, respectively (depending on the mAb used) (300 μl; Advanced Magnetics, Inc., Cambridge, MA), were mixed with 50 μg of the respective mAb overnight at 4°C. Unadsorbed antibodies were removed, the beads were washed four times with 0.14 M NaCl, 0.01 M phosphate, 0.02% NaN$_3$, pH 7 (PBS), and resuspended in 300 μl of TS. mAb to CD antigens were obtained from Amac, Inc. (Westbrook, ME) or from donors listed in Acknowledgments. Antibodies to IL-2R β chain were obtained from Endogen (Boston, MA). Monoclonal and polyclonal rabbit antibodies to IL-6 were obtained from Genzyme (Cambridge, MA). The rabbit anti-IL-6 corresponded to an IgG prepared from serum by ammonium sulfate precipitation. 1 mg of the partially purified IgG was reported by the manufacturer to neutralize 100 ng of IL-6 after prolonged incubation.

The attachment of cells to the cellulose derivative was quantitated as described before (2, 3). The SD of relative cell counts, each based on 14 OD determinations, was ≤ ± 8% (2). To detect the attachment of cells to mAb-coated magnetic beads, 50 μl of beads (∼5 × 10^8 particles/ml) was mixed with 5 × 10^5 cells in 0.5 ml of TS containing 1 mM CaCl$_2$ and 10 mg/ml of BSA (TS-Ca-BSA). After mixing for 30 min at 20°C, attached and unattached cells were separated magnetically and quantitated by measuring lactate dehydrogenase activity as described before (2, 3).

The inhibitory effect of mAbs, of polyclonal rabbit antiserum to partially purified HBV "receptor" (final dilution, 1:5; references 2 and 3), and of distinct cytokines on the interaction between cells and the respective immobilized ligands was followed by adding the respective substances to the cell-ligand mixtures otherwise following conditions to study the attachment of cells to the respective immobilized ligands. The final concentrations of the respective inhibitors are presented in Results. In addition to antisera and mAbs, the following cytokines were studied for inhibitory activity: IL-3 and IL-6 produced in CHO cells (both from Genetics Institute, Cambridge, MA); IL-7 (Sterling Drug, Inc., Malvern, PA); IL-5 (from *E. coli*), leukemic inhibitory factor, and TGF-α and -β (all four from Amgen, Thousand Oaks, California). CHO cell- and yeast-derived IL-6 was also obtained from Genzyme, and *E. coli*-derived IL-6 from R & D Systems (Minneapolis, MN).

Measurement of Association between IL-6 and the preS1 Region of the HBV env Protein. The association between IL-6 and the peptide preS(21-47) was measured by affinity chromatography. PreS(21-47)-cellulose (1.6 mg of the cellulose derivative in a final volume of 100 μl TS-Ca-BSA) was mixed with 500 ng of IL-6. After incubation for 1 h at 20°C, the cellulose derivative was pelleted by centrifugation, transferred to a minicolumn, and eluted with 4 M

MgCl$_2$ at 20°C. IL-6 was determined in the original preparation and the wash and eluate by ELISA after diluting the specimens 1,000-fold. The ELISA kit to quantify IL-6 (Intertest 6X) was obtained from Genzyme. The kit uses yeast IL-6 as a calibration standard and detects IL-6 from mammalian cells with a lower sensitivity (∼1/15) as compared with *E. coli* or yeast IL-6 (own data). Control experiments were carried out with preS(12-32)-cellulose. The respective cellulose derivatives contained 5-10 mg of peptides per gram of cellulose.

The formation of complexes between HBsAg containing the preS1 sequence and IL-6 was detected by a sandwich ELISA utilizing wells coated with anti-preS1 mAb F35.25 (1 μg per well; reference 5) or with goat antibodies to S protein (obtained as part of an ELISA kit from Nuclear Medical Laboratories, Irving, TX), and biotinyated polyclonal rabbit anti-IL-6. Graded quantities of distinct HBsAg preparations were incubated with IL-6 in the diluent used for the Intertest 6X kit for 30 min at 25°C, followed by 90 min at 4°C. Subsequently, the mixtures were transferred to wells coated with the respective (anti-S or anti-preS1) antibodies. After overnight incubation, the quantity of attached IL-6 was determined using reagents from the Intertest 6X kit. The following preparations of HBsAg were utilized in these tests: L protein produced in yeast (18); recombinant HBsAg (produced in CHO cells) either lacking (19) or expressing the preS1 sequence (∼10 ng of the preS1 sequence per μg of HBsAg; Bio-Technology General, Rehovot, Israel); serum-derived HBsAg (containing 50 ng of the preS1 sequence per μg of HBsAg; reference 3), and recombinant HBsAg consisting of S protein produced in yeast (20).

Ultrafiltration was used to demonstrate directly the association between HBsAg and IL-6 and to determine the equilibrium between bound and unbound IL-6. Distinct quantities of HBsAg preparations and of recombinant L protein listed above (10 ng to 1 μg) were mixed with IL-6 (5-89 ng in 1 ml of TS-Ca containing 1 mg/ml of BSA and 0.25 mg/ml of gelatine prefiltered through a 0.22-μ filter). After incubation for 2 h at 20°C, the mixtures were transferred into Ultrafree-CL filters (Millipore, Bedford, MA) and centrifuged at 2,000 revolutions/min. The filtrates were assayed for IL-6 by ELISA after appropriate dilution. Based on preliminary experiments, filters with a cut-off of 3 × 10^2 kD were selected, since unbound IL-6 was not retained by these filters, while both HBsAg and L protein, existing predominantly in the form of aggregates (18), were retained by the ultrafiltration membranes of this porosity. The experiments were carried out with rIL-6 produced in CHO cells (Genetics Institute). Similar results were obtained with IL-6 produced in yeast and *E. coli*, respectively.

Results

Induction of HBV Receptors in Cells That Do Not Constitutively Express the Receptors. HBV DNA and RNA were detected in peripheral B cells, T cells, and monocytes (8, 9). On the other hand, HBV receptors were detected only on cells belonging to the B cell lineage (2). To resolve this apparent discrepancy, we wished to determine whether or not the expression of HBV receptors can be induced in T cell lines. HUT-78 and MOLT-3 cells stimulated with Con A attached to a peptide from the preS1 sequence of the HBV env protein (preS[21-47]) covalently attached to cellulose, indicating the presence of HBV receptors on these cells (Fig. 1). Expression of the receptors appeared to be maximal after activation for 24 h and gradually declined after prolonged in-
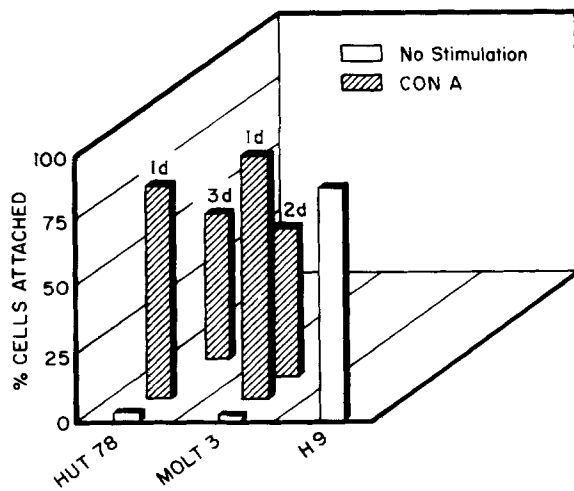
Figure 1. Effect of preactivation by Con A (50 μg/ml) of distinct T cell lines on their attachment to preS(21-47)-cellulose. The time interval for stimulation in days is indicated on top of the bars. Results obtained with unstimulated cell lines are shown in the first (front) row.

cubation with Con A. HUT-78 and MOLT-3 cells not exposed to Con A failed to attach to the cellulose derivative, indicating the absence of HBV receptors on nonstimulated cells. On the other hand, H9 cells, related to the prototype HUT-78 cell line (21), expressed HBV receptors constitutively. The expression of HBV receptors was also induced by Con A in Jurkat cells (66% attachment to preS[21-47]-cellulose) but not in the T cell lines CEM, MOLT-4, and MT-4 (<10% attachment; data not shown). Cells that attached to preS(21-47)-cellulose also attached to HBsAg containing the preS1 sequence covalently linked to cellulose (HBs-cellulose). However, they did not attach to another peptide from the preS1 sequence, preS(12-32) linked to cellulose. Attempts to induce the expression of HBV receptors on the majority of T cells from PBL by Con A were unsuccessful (data not shown).

Results of experiments to be described later indicated that

recognition sites for the HBV env protein were also inducible by LPS in PBMC and a promonocytic cell line, U937 (see Fig. 8).

Treatment of Cells with PI-PLC Diminishes the Surface Exposure of Recognition Sites for the HBV env preS(21-47) Sequence. A subset of cell surface proteins are anchored to the membrane by covalent linkage to an oligosaccharide which is glycosidically linked to PI. They can be released from cells by PI-PLC (22, 23). Several leucocyte cell differentiation (CD) antigens, including activation antigens, are PI anchored (24). Since HBV receptors are expressed or inducible on several lineages of hemopoietic cells in addition to hepatoma and liver cells, it was of interest to determine whether or not treatment with PI-PLC renders these cells nonreactive with the preS(21-47) ligand. Susceptibility to cleavage by PI-PLC, if demonstrable, was expected to facilitate the characterization of HBV receptors and their possible identification with already characterized cell surface proteins. The percentage of HepG2 cells attaching to (21-47)- or HBs-cellulose decreased to background levels after treatment with PI-PLC (Fig. 2). Receptors for HBV became gradually reexpressed on the treated cells after subsequent cultivation in tissue culture media. Treatment with PI-PLC resulted in decreased attachment to preS1 ligands of most other cell lines tested. However, RPMI 8226 myeloma cells, H9, and Con A-activated MOLT-3 and HUT-78 T cell lines treated with PI-PLC continued to adsorb to the cellulose derivatives. A cell-specific heterogeneity in sensitivity of PI-anchored membrane antigens to release by PI-PLC was noted before (23) and may explain the observed exceptions. A decrease in the ability of PI-PLC to remove PI-anchored surface proteins from activated T cells was also reported (25). These results suggest that similar binding sites for the preS1 region of the HBV env protein are attached to the cell membranes of most, if not all cells tested, through a PI anchor.

Expression of Leukocyte Differentiation CD Antigens on HepG2 Cells and Their Distinctiveness from Binding Sites for HBV. Since preS1-specific receptors for HBV, first detected on human hepa-
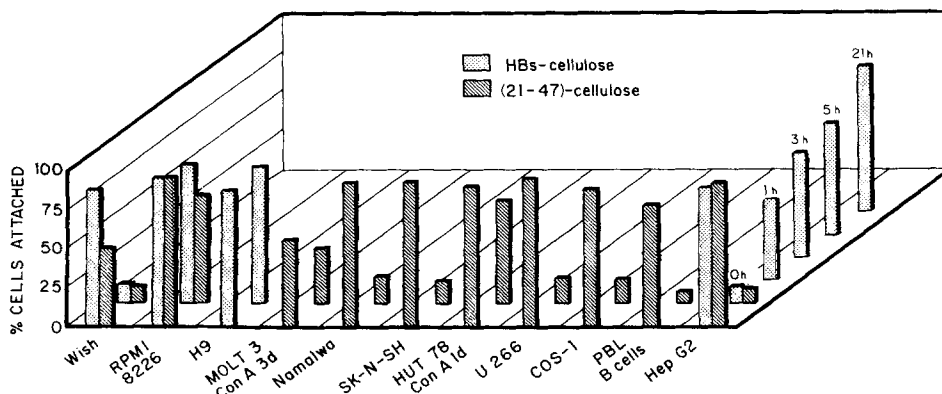

Figure 2. Effect of treatment with PI-PLC (1 U/ml, 1 h at 37°C) of distinct cell lines on their attachment to HBsAg-cellulose and preS(21-47)-cellulose, respectively. The attachment of untreated cells to the cellulose derivatives is shown in the first (front) row; the attachment of cells treated with PI-PLC is shown in the second row. Treated HepG2 cells were washed to remove PI-PLC and subsequently grown in tissue culture media (see Materials and Methods) for time intervals indicated on top of the bars. The attachment of these cells to HBsAg-cellulose was then determined (rows 3-6). HUT 78 Con A 1d and MOLT 3 Con A 3d are designations for the respective cell lines treated 1 and 3 d, respectively, with Con A.

toma cells and liver cell membranes (3–7), were also detected on PBL B cells and on activated T cell lines and PBMC, it seemed possible that a CD antigen might also function as an HBV receptor. This possibility appeared attractive, since some CD antigens were identified as receptors for other viruses (CD4, CD21, and CD54; 26–29). Therefore, the expression of CD antigens on HepG2 cells was studied. Because of the expression of HBV receptors on several hemopoietic cell lineages, the studies were limited to those CD antigens that are not considered specific markers to distinguish one cell lineage from another. Platelets and RBCs did not react with preS1 ligands. Therefore, CD antigens expressed on the latter two cell types were not searched for their expression on HepG2 cells. Among the 33 CD antigens searched for, only five appeared to be expressed on the surface of HepG2 cells abundantly enough to mediate the attachment of these cells to magnetic beads coated with the respective anti-CD mAbs (Fig. 3). These antigens were: CD58 (LFA-3), CD59, CD69, CD71 (transferrin receptor), and CD73 (ecto-5'-nucleotidase). Three of these antigens (CD58, CD59, and CD73) are linked to the cell membrane by a PI anchor and two antigens (CD69 and CD71) are defined as activation markers (24). Another activation antigen, 4F2 (30), was also present on the surface of HepG2 cells.

After establishing the surface expression of several CD antigens on HepG2 cells, experiments were carried out to determine whether or not any of these antigens recognized the preS(21-47) site on the HBV env protein. First, it was determined whether or not any of the mAbs directed against each of the six antigens referred to above would inhibit the attachment of HepG2 cells to preS(21–47)- and HBs-cellulose, respectively. Second, the effect of polyclonal rabbit antibodies to the HBV receptor (3) on the attachment of HepG2 cells to magnetic beads coated with mAbs against the respective CD antigens and 4F2 was investigated. In no case were there any inhibitory effects observed, suggesting that none of these CD/activation antigens functioned as receptors for the preS1 region of the HBV env protein.

Inhibitory Effect of IL-6 on the Attachment of Cells Expressing the HBV Receptor to (21-47)- and HBs-Cellulose. After excluding all CD antigens tested as candidate HBV receptors, the possibility was considered that cytokine receptors might be involved in the interaction between the preS1 region of the HBV env protein and cells. To address this possibility, the inhibitory effect of several cytokines on the reaction between HepG2 cells and preS1-specific ligands was examined. In complementary experiments, the expression of some cytokine receptors on HepG2 cells was assayed using experimental procedures analogous to those for detection of the expression of CD antigens (Fig. 3).

IL-3, IL-5, IL-7, TGF-α and -β, and leukemic inhibitory factor, all at concentrations of 5–20 μg/ml, did not inhibit the attachment of HepG2 cells to preS(21-47) cellulose. The IL-2R α chain (CD 25; Fig. 3) and IL-2R β chain (data not shown) were not detectable on HepG2 cells. On the other hand, IL-6Rs were expressed on HepG2 cells, as detected by both cytofluorometry and by attachment of these cells to anti-IL-6R-coated beads (data not shown), in agreement with already published data (31). The inhibitory effect of IL-6 on the interaction between HepG2 cells and preS(21-47)-cellulose was investigated originally to determine whether or not IL-6R blockade would interfere with cell attachment. Results shown in Fig. 4 indicate that IL-6 indeed inhibited the attachment of HepG2 cells to preS(21-47)-cellulose in a dose-dependent fashion. This inhibitory effect was abrogated by anti-IL-6 mAbs. The attachment of HepG2 cells to HBsAg-cellulose was also inhibited by IL-6 at similar concentrations (data not shown). The interaction of other selected cell lines with the preS1-specific ligand was also inhibited (Fig. 4).

IL-6 and Not IL-6R Carries the Binding Site for the preS(21-47) Segment of the HBV env Protein. The inhibition of attachment of HepG2 cells to preS(21-47)- and HBs-cellulose by IL-6 suggested the possibility the IL-6R carried sites directly involved in attachment of HBV. However, this notion is incompatible with the following findings. (*a*) The inhibitory effect of IL-6 occurred at concentrations that are by several orders of magnitude higher than physiological concentrations. (*b*) Since the number of IL-6R sites per cell for different cell types is <2 × 10^4 (31, 32), and the K_d for the IL-6/IL-6R reaction is between 100 and 700 pM, the concentration of IL-6 required to prevent attachment of HepG2 and of other cells to preS(21-47)- or HBs-cellulose (>20 nM) by far exceeded the concentration of IL-6 required for occupancy of cellular IL-6R sites. (*c*) Cells reported to have IL-6R, for ex-
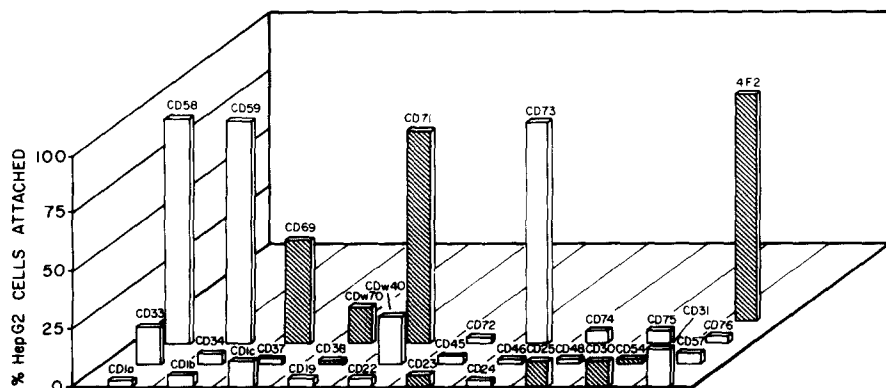

Figure 3. Occurrence of leucocyte CD antigens on HepG2 cells as determined from their attachment to magnetic beads with covalently linked anti–mouse IgG, subsequently coated with mAb to distinct CD antigens. Hatched columns correspond to activation antigens. HepG2 cells also did not attach to magnetic beads coated with mAb to CD14, CD28, and CD39, respectively.
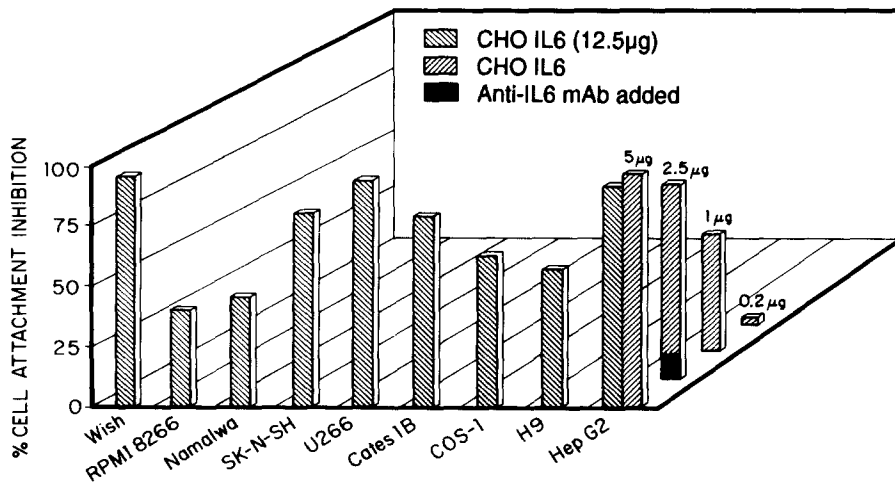
Figure 4. Inhibition by IL-6 of attachment of distinct cell lines to preS(21-47)-cellulose. IL-6 produced in CHO cells (CHO IL6; 12.5 μg; from Genetics Institute) was added to suspensions of different cell lines before mixing them with preS(21-47) cellulose. The inhibitory effect of CHO IL-6 (from Genzyme), at distinct levels, indicated on top of the bars was studied using HepG2 cells. The inhibitory effect of CHO IL-6 (2.5 μg) was abrogated by anti-IL-6 mAb (500 μg) but not by normal isotype-matched IgG.

ample, CESS, HL-60, unstimulated U937 promonocytic cells, and peripheral T cells (32, 33), do not express detectable binding sites for the preS1 region of the HBV env protein (3; data not shown). (d) The attachment of HepG2 cells to magnetic beads coated with anti-IL-6R mAbs was not inhibited by antibodies to the HBV receptor generated as described before (3), indicating the distinctiveness of IL-6R and receptors for HBV. Therefore, explanations other than the direct involvement of IL-6R in the binding of HBV had to

be established to understand the inhibitory effect of IL-6 on the interaction between cells and preS1-specific ligands.

Further experiments were designed to determine whether or not IL-6 reacted with the preS1 ligand and thereby inhibited the interaction between cells and preS(21-47)- or HBs-cellulose. First, IL-6 was chromatographed on a minicolumn of preS(21-47)-cellulose. Most (92.9%) of the IL-6 adsorbed to the cellulose derivative and was subsequently eluted (49%) by 4 M MgCl2 (Fig. 5). When similar experiments were carried out with preS(12-32)-cellulose, previously shown not to attach any of the cell lines reacting with the preS1 ligand (2, 3), most of the IL-6 remained unabsorbed. To confirm that attachment of IL-6 to preS1-specific cellulose derivatives indeed prevented their subsequent interaction with cells expressing
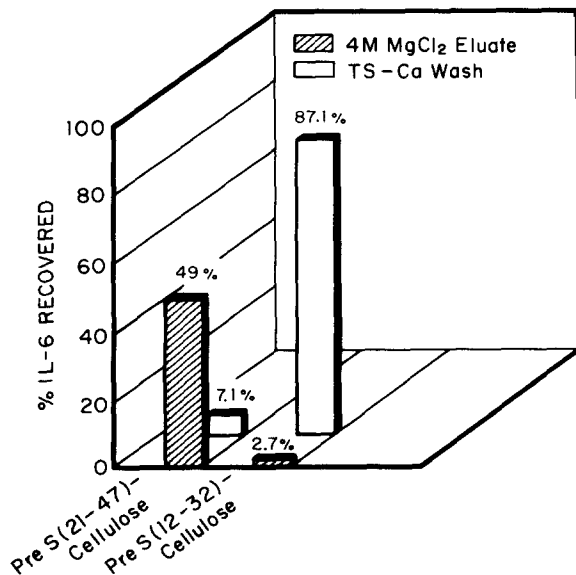

Figure 5. Attachment of IL-6 to preS(21-47)-cellulose. IL-6 (500 ng) was added to preS(21-47) cellulose and control, preS(12-32)-cellulose (1.6 mg of each cellulose derivative in a final volume of 100 μl), each suspended in 0.14 M NaCl, 0.01 M Tris, 0.02% NaN3, 1 mM CaCl2, pH 7.2 (TS-CA), containing 10 mg/ml BSA. After incubation for 1 h at 4°C and centrifugation, the supernatants were removed, the cellulose derivatives were washed with TS-CA, and subsequently eluted at 20°C with 4 M MgCl2. IL-6 was determined by ELISA in the supernatants and eluates, and the percentages of recovered IL-6 in these fractions were determined (numbers on top of the bars).
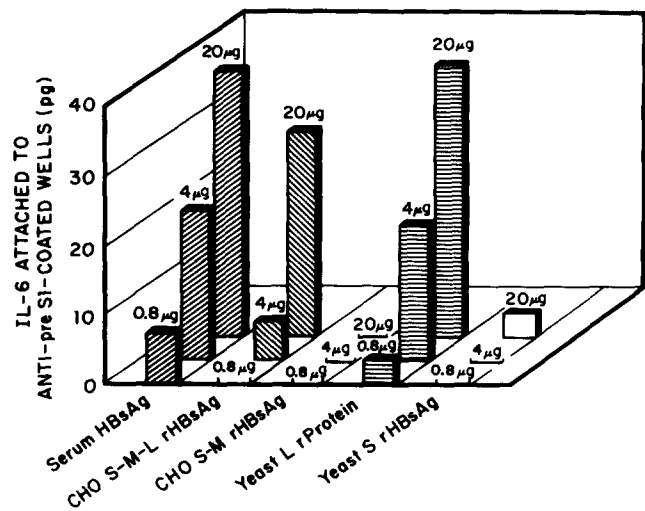

Figure 6. HBsAg-mediated attachment of IL-6 to wells of polystyrene plates coated with anti-preS1 F35.25. The following preparation of HBsAg were used: serum-derived HBsAg containing S, M, and L protein; rHBsAg produced in CHO cells and either consisting of S, M, and L protein (CHO S-M-L rHBsAg) or of S and M protein only (CHO S-M HBsAg; 19); rHBsAg produced in yeast consisting of S protein (S rHBsAg; 20); and L protein produced in yeast (18).

the HBV receptor, preS(21-47)- and HBs-cellulose (40 mg each) were premixed with IL-6 (10 μg), washed with 0.14 M NaCl, 1 mM CaCl₂ containing 10 mg/ml of BSA (TS-Ca-BSA), and their ability to attach HepG2 cells was tested. The cellulose derivatives adsorbed 7 μg of IL-6 as determined by ELISA and attached only background numbers of HepG2 cells (11%) in comparison with the respective cellulose derivatives not pretreated with IL-6, which attached 93-97% of the cells. These results provide evidence that the inhibitory effect of IL-6 on the reaction between preS(21-47)-cellulose and cells expressing HBV receptors can be ascribed to a specific interaction between the preS(21-47) region of the HBV env protein and IL-6.

To show that IL-6 reacted not only with preS1-specific ligands covalently linked to cellulose but also with HBV env proteins containing the preS1 sequence, a test for detecting HBsAg-IL-6 complexes was designed. Such complexes were detected in an ELISA utilizing polystyrene plates coated with antibodies to HBV env proteins (anti- preS1 or anti-S) and biotinylated antibodies to IL-6 as a probe. The association between IL-6 and HBV env proteins could be demonstrated only for HBsAg containing the preS1 sequence but not for HBsAg containing S protein or S protein and the preS2 sequence only (Fig. 6). These results confirmed that the preS1 region of the HBV env protein contained sites recognizing IL-6. Similar results were obtained by an ELISA in which plates coated with antibodies to S protein, present in each of the envelope protein constituents of HBV, were used (Fig. 7).

The association between IL-6 and the preS1 region of the HBV env protein was confirmed by microfiltration using 3 × 10² kD cut-off ultrafiltration membranes. IL-6 in the absence of HBV env proteins containing the preS1 sequence

Figure 8. Effect of preactivation with LPS of human PBMC and promonocytic U937 cells on their attachment to preS(21-47) cellulose. The attachment of activated U937 cells was abrogated in the presence of polyclonal rabbit anti-IL-6 (200 μg) (third row). The effect of anti-IL-6 on attachment of LPS-activated PBMC was not tested.

or in the presence of HBsAg containing S and M protein, or S protein only, was quantitatively recovered in the ultrafiltrate. However, IL-6 was partially recovered in the retentate (33-87%, depending on the initial concentrations of the
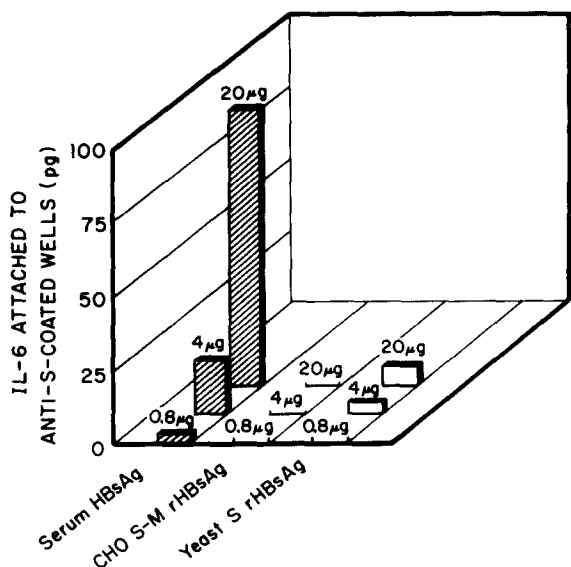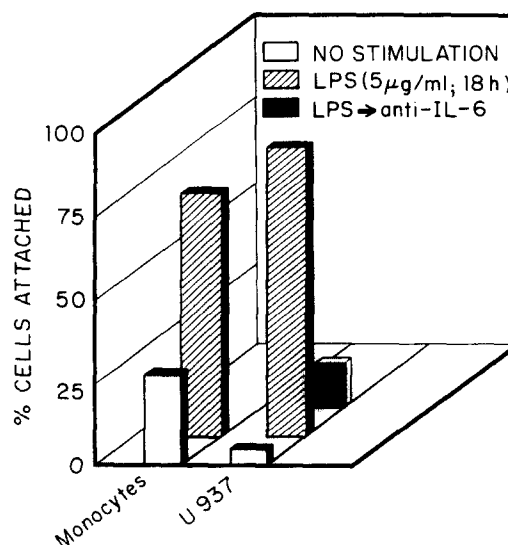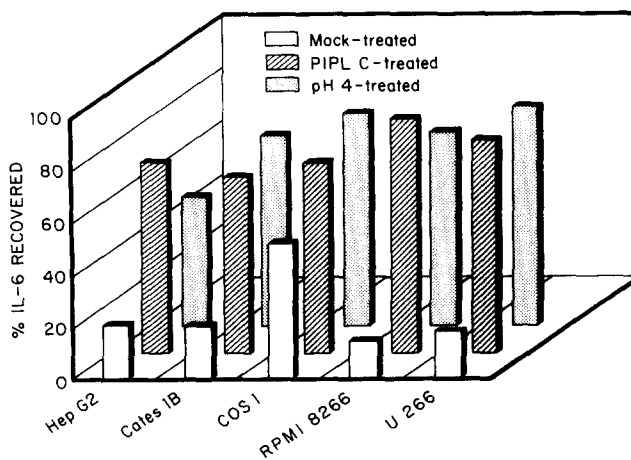

Figure 7. HBsAg-mediated attachment of IL-6 to wells of polystyrene plates coated with polyclonal guinea pig anti-HBs (anti-S protein). For further explanations, see legend to Fig. 6.

Figure 9. Release of IL-6 from distinct cell lines by PI-PLC or by exposure to pH 4. Cells (10⁶) were treated for 1 h at 37°C with PI-PLC (100 μl; 1 U/ml in TS; second row), mock treated with TS (first [front] row), or incubated in 10 mM citrate, 0.14 M NaCl, pH 4 (third row). IL-6 was determined in supernatant fluids after centrifugation of the treated cells, and in lysed pelleted cells. The percentage of IL-6 recovered in the supernatant fluids was calculated and plotted. The concentration of IL-6 in lysates of untreated cells was in the range of 2.5-5.1 ng/ml, as calculated from a calibration curve relating ELISA readings to concentration of an IL-6 standard. Similar results were obtained with Wish, SK-N-SH, and Con A-activated HUT 78 cells.

reactants) when mixtures of IL-6 and serum-derived HBsAg and recombinant L protein, respectively, were submitted to ultrafiltration. The retention of IL-6 under the latter circumstances was nearly completely suppressed (94%) when the peptide preS(12-47) was added at 50-M excess. The K_a for the reaction between IL-6 and the preS1 segment of the HBV env protein was calculated from the results of these experiments (K_a = 4.0 ± 0.94 × 10^9).

Cell-associated IL-6 Is Involved in Recognition of the preS(21-47) Segment of the HBV L Protein. Purified IL-6 reacts with the preS1 region of the HBV env proteins, and the preS(21-47) segment of the sequence is involved in this interaction. Blocking of this region of the HBV env protein by IL-6 prevented the interaction of HBV env proteins and of the peptide preS(21-47) with cell surface recognition sites. The following results provide evidence that these recognition sites are located on cell surface–associated IL-6.

Human PBMC do not produce IL-6. LPS induces the synthesis of IL-6 in PBMC, which are the major source of IL-6 produced by LPS-treated whole blood cultures (34, 35). The majority of human PBMC and promonocytic U937 cells did not attach to preS(21-47)-cellulose. However, after stimulation with LPS, these cells attached to this cellulose derivative (Fig. 8) and to HBs-cellulose (data not shown) but not to preS(12-32)-cellulose. The attachment of LPS-activated U937 cells to preS(21-47)-cellulose was abrogated by polyclonal antibodies to IL-6. Polyclonal anti-IL-6 also inhibited the attachment to preS(21-47)-cellulose of other cell lines (U266, H9, FS-4, HepG2, COS-1, Namalwa, SK-N-SH, Cates 1B, and Con A–activated MOLT-3) by 45–78%.

Partially purified preparations of the HBV receptor were prepared by affinity chromatography on preS(21-47)-cellulose (3). A lysate from 10^7 HepG2 cells was chromatographed on a minicolumn of preS(21-47)-cellulose (8 mg) under conditions described before for the receptor purification. A major portion (78% = 570 pg) of IL-6 originally present in the cell lysate was attached to the cellulose derivative and subsequently eluted by 4 M $MgCl_2$. This indicates that IL-6 is selectively removed from crude cell lysates in agreement with the observed binding between IL-6 and the preS(21-47) segment of the HBV L protein. IL-6 was also removed from crude cell lysates by affinity chromatography on columns of immobilized anti-"receptor antibodies" prepared as described before (3; data not shown).

Unlike mature forms of some other growth factors (36), IL-6 is not cleaved from integral membrane precursors. Therefore, it seemed possible that IL-6 involved in the interaction with the preS(21-47) segment of the preS1 sequence was peripherally associated with cell membranes. To confirm that this indeed was the case, cells were pretreated at pH 4, conditions known to release cell-associated lymphokines while preserving cell viability (17), and the attachment of the treated cells to preS(21-47)-cellulose as well as the release from the cells of IL-6 were measured. Treatment at pH 4 abrogated the attachment to preS(21-47)-cellulose of the following cell lines tested: HepG2, RPMI 8226, H9, FS-4, Wish, SK-N-SH, Namalwa, Cates 1B, U266, COS-1, Con A–activated

HUT 78, and LPS-activated U937 cells. The release of IL-6 at pH 4 from cell lines expressing the highest levels of IL-6 was assayed by ELISA. Results shown in Fig. 9 indicate that most of the IL-6 was released from the cells at pH 4. These results indicate that cell-associated IL-6 is indeed involved in the interaction between cells and cellulose derivatives with immobilized preS1 ligands. Not surprisingly, partial release of IL-6 was also observed with mock-treated cells, in agreement with the observed shedding of HBV "receptors" into the extracellular environment (2).

Initial attempts to characterize the receptor for HBV indicated that treatment of distinct cell lines with PI-PLC resulted in decreased reactivity with the preS(21-47) binding site (Fig. 2). Subsequent results indicated that the component recognizing the latter site was IL-6. However, IL-6 is not attached to cell membranes by a PI anchor. To resolve this apparent discrepancy, IL-6 was measured in supernatant fluids after treating cells with PI-PLC. Most of the cell-associated IL-6 was released after such treatment (Fig. 9). These results agree with the proposed role of IL-6 as a mediator of HBV attachment to cells, and suggest that IL-6 is functioning as an HBV receptor anchored to the cell surface through another PI-PLC-releasable component(s). Preliminary results suggest that this component(s) is a proteoglycan.

Discussion

Although the liver is the major target organ for HBV, cells other than hepatocytes, including PBL, may become infected with the virus. In accordance with this, receptors for HBV were detected on cells of both hepatic and extrahepatic origin (2). It was anticipated that attempts to detect and characterize HBV receptors on several cell lines, rather than only on cells of hepatic origin, would provide clues concerning the nature of HBV receptors and an explanation for multiple target cells (organs) for HBV. These expectations lead to an experimental path finally revealing that IL-6 carries a binding site for HBV. The production of IL-6 by a variety of cells (37) may explain the miscellany of target cells for HBV. Some of the cells (PBMC, promonocytic, and T cell lines) did not express IL-6 constitutively and did not react with the cell receptor binding site on HBV, but expressed both IL-6 and the binding site for HBV after activation. This might explain the reported replication of HBV in PBMC (9) and peripheral T lymphocytes (8), and possibly contribute to explaining the reactivation of latent hepatitis virus infection of circulating lymphoid cells after activation with LPS (38).

Aplastic anemia has occasionally been diagnosed in patients infected with HBV, and it was observed that exposure of human bone marrow mononuclear cells to HBV resulted in suppression of in vitro growth of several hematopoietic progenitor cells (39). The interaction between IL-6 and HBV, documented herein, might offer an explanation for the observed inhibition of proliferation and differentiation of hematopoietic colony-forming cells by HBV.

The interaction between growth factors or growth factor receptors with viral envelope proteins (10, 11, 14, 15), and

mimicking of cytokines or their receptors by virally encoded proteins (12, 16), provide new insights into the replication of the corresponding viruses and explanations for their effects on the host immune system. The demonstration that HBV utilizes IL-6 as a target for attachment to cells suggests that this virus should be included in an apparently expanding family of viral pathogens capable of modulating cell growth and differentiation.

IL-6 involved in the interaction between HBV and cells appeared to be associated with the cell periphery through another component, attached to the cell membrane through a PI anchor. Preliminary results suggest that this component(s) is a proteoglycan (own unpublished data). This observation appears to agree with the proposed role of proteoglycans as modulators of growth factor activities (40), although no experimental evidence for the association of IL-6 with proteoglycans has been reported so far. Some of the proteoglycans

are linked to the cell membrane by a PI anchor (41), possibly explaining the release of IL-6- and HBV-binding activity from cells by PI-PLC. IL-6 and proteoglycans may possibly play a dual role in attachment of HBV to cells.

The interaction between IL-6 and the preS1 region of the HBV envelope appears to be responsible for the primary interaction of HBV with cells. The precise details of cell-HBV interactions, probably involving other cell membrane components (proteoglycans?), and possibly also regions on the HBV env protein other than the preS(21-47) segment, leading to productive infection of cells, remain to be established. This task is complicated by the unavailability of a system for quantitating infectious HBV. The location of the preS(21-47) binding site on IL-6 and its similarity to or distinctiveness from the domain involved in interaction with IL-6R also remain to be explored.

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