Cells Transfected with Human Interleukin 6 cDNA Acquire Binding Sites for the Hepatitis B Virus Envelope Protein

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Summary

Earlier studies revealed that human interleukin 6 (IL-6) contains recognition sites for the hepatitis B virus (HBV) envelope (env) protein, and that IL-6 and anti-IL-6 antibodies, respectively, inhibited the interaction of cells expressing a receptor for HBV with the preS(21–47) segment of the HBV env protein, encompassing the complementary attachment site for IL-6. This suggested that IL-6 mediates HBV-cell interactions. We report that: (a) Chinese hamster ovary cells transfected with human IL-6 cDNA and *Spodoptera frugiperda* ovarian insect cells infected with recombinant baculovirus carrying human IL-6 cDNA expressed receptors for the preS(21–47) region of the HBV env protein, indicating that expression of IL-6 on the surface of cells is sufficient to endow them with receptors for HBV. (b) Among peptides covering the entire sequence of human IL-6 and the corresponding antipeptide antibodies, the peptide IL-6[35–66] and anti-IL-6[35–66] most effectively inhibited the interaction between human hepatoma HepG2 cells and the preS(21–47) ligand, suggesting that this region of the human IL-6 sequence encompasses a binding site for the HBV env protein. (c) Studies with replacement set peptides from the preS(21–47) sequence indicated that residues 21–25, 28, 31, 33–35, 39, and 43–45 can be replaced by alanine (serine) residues, while all the other residues are essential for maintaining the cell receptor/IL-6 binding activity. Further delineation of complementary sites on IL-6 and on the HBV env protein may contribute to the design of compounds inhibiting HBV replication.

Hepatitis B virus (HBV)[1] is a major human pathogen implicated in primary hepatocarcinoma. The envelope (env) of the virus consists of three related proteins (S, M, and L) that are all products of a single *env* gene. These proteins share a common 226-residue 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. A segment of the preS1 sequence encompassing amino acid residues 21–47 is involved in the attachment of HBV to human hepatoma HepG2 cells (1) and to isolated human liver plasma membranes (2–4). 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 (5, 6), representing, in addition to the liver, target cells for HBV (7–9).

The preS(21–47) region of the HBV env protein encom-

passes a binding site for IL-6 (6). The following findings indicated that cell-associated IL-6 mediates the interaction between the preS1 region of the HBV env proteins and cells of hepatic or extrahepatic origin: (a) this interaction was inhibited by IL-6 and by anti-IL-6 antibodies; (b) stimulation of T cells and PBMC with Con A and LPS, respectively, induced the production of both IL-6 and of recognition sites for the preS1 region of the HBV env protein; and (c) exposure of IL-6-producing cells to phosphatidylinositol-specific phospholipase C (PI-PLC) or to low pH resulted in the release of cell-associated IL-6 and in reduced cell binding to the preS1(21–47) ligand (6). These results suggested that cell-associated IL-6 either represented a cell surface receptor for HBV or a constituent of a multicomponent receptor complex. This report provides direct evidence that cell surface–associated IL-6 is sufficient to endow cells with binding activity for the preS1 region of the HBV env protein by demonstrating that nonhuman cells transfected with human IL-6 cDNA acquired receptors for HBV. Furthermore, regions on the IL-6 protein essential for recognition of the preS(21–47) ligand were delineated, and amino acid residues within the preS(21–47) region essential for cell receptor binding were identified.

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

Materials and Methods

Cells Expressing Human IL-6. The following cell lines were used: human HepG2 hepatoma cells; Chinese hamster ovary (CHO) cells transfected with IL-6 cDNA (CHO-IL-6; obtained from Sandoz Technology Ltd., Basel, Switzerland through the courtesy of the Genetics Institute, Cambridge, MA); and *Spodoptera frugiperda* Sf9 ovarian cells infected with recombinant baculovirus carrying IL-6 cDNA (B-IL-6 cells). The recombinant baculovirus was prepared by Invitrogen Corporation (San Diego, CA) as follows. *Escherichia coli* MC1061 containing plasmid pCSF309 carrying IL-6 cDNA (67153; American Type Culture Collection, Rockville, MD) was grown for large scale preparation of the plasmid DNA (10). The plasmid was purified by equilibrium centrifugation in CsCl-ethidium bromide gradients. The isolated plasmid DNA as well as the pVL1392 transfer vector were digested with the restriction enzyme EcoRI. The pVL1392 DNA was subsequently treated with alkaline phosphatase to prevent self-ligation of the compatible EcoRI ends. The EcoRI-cut plasmid pCSF309 and the EcoRI-cut and phosphatase-treated transfer vector pVL1392 were subsequently subjected to electrophoresis in a low-melting point agarose gel to purify the 1.16-kb fragment containing IL-6 cDNA and the vector DNA, respectively. The respective DNA fragments were recovered from the gel and ligated overnight. Chemically competent DH1αF' *E. coli* cells were transformed with the ligated DNA. Colonies containing the desired clone were identified by performing small-scale plasmid preparations and by EcoRI and AccI restriction digests to check for appropriate inserts and their orientation, respectively. Positive colonies were selected and grown in 20-ml cultures from which plasmid DNA was prepared by purification on columns (Qiagen, Inc., Chatsworth, CA). The purified plasmid DNA was used to transfect Sf9 insect cells along with linearized wild-type baculovirus DNA following the Invitrogen MaxBac manual (Invitrogen Corporation). Recombinant baculovirus produced by the transfected cells was purified and checked for purity by the PCR. High titer recombinant baculovirus stocks were prepared and used to infect Sf9 cells. For studies on the surface expression of IL-6, Sf9 cells were infected at a multiplicity of infection corresponding to 5. The infected cells (B-IL-6 cells) were analyzed for expression of IL-6 48–72 h after infection. Uninfected Sf9 cells, Sf9 cells infected with wild-type baculovirus, and with recombinant baculovirus containing cDNA for the glucocorticoid binding protein, respectively, were used in control experiments.

Peptide Synthesis. Peptides from the preS region of the HBV env protein, preS(12–32), preS(12–47), and preS(21–47), were synthesized and linked to cellulose as described before (1, 5, 6). Replacement set peptides from the preS(21–47) sequence were synthesized on a multiple-peptide synthesizer (350 MPS; Advanced ChemTech, Louisville, KY) using 1,3-diisopropylcarbodiimide and 1-hydroxybenzotriazole activation in a 9-fluorenyl-methoxycarbonyl (Fmoc)-mediated synthesis. The peptides were cleaved off the resin by TFA/thioanisole/ethanedithiol/anisole (90:5:3:2) for 4 h. The peptide-resin mixtures were freeze dried and the peptides were extracted from the resin with 20% acetic acid. Each of the peptides was purified on a C18 reverse-phase column with a linear 0.1% TFA/H$_2$O-100% acetonitrile gradient. Fractions corresponding to the major peak of absorbance at 214 nm were lyophilized and used for the experiments described below. To confirm the correctness of the sequence, the peptide with no substitutions and the peptide with proline at position 47 replaced by alanine were sequenced on a sequencer (477A/120A; Applied Biosystems, Inc., Foster City, CA). The sequences were proven to be correct. All peptides were synthesized in the form of amides and had a glycine-glycine-cysteine spacer at the COOH terminus. The NH$_2$ terminus of each peptide was acetylated.

Peptides from the sequence of IL-6 were synthesized in the form of amides on a peptide synthesizer (9600; MilliGen/Biosearch, Milford, MA) using benzotriazolyloxytris (dimethylamino) phosphonium hexafluorophosphate and 1-hydroxybenzotriazole activation in a Fmoc-mediated synthesis following Technical Bulletin no. 900-03 from Biosearch. Peptide cleavage was accomplished as described above. The peptides were precipitated with diethyl ether and lyophilized. When submitted to reverse-phase chromatography on C18 columns using a linear gradient as described above, a single major absorbance peak at 214 nm was discernible. The sequence of each peptide was confirmed by direct sequencing.

Immunofluorescence. Cell-bound human IL-6 was determined by indirect immunofluorescence. 10^6 CHO or CHO-IL-6 cells pelleted and prewashed with PBS containing 10 mg/ml BSA (PBS-BSA) were mixed with 100 μl PBS containing 5% normal goat serum (PBS-NGS) and with either control normal mouse IgG or with anti–human IL-6 mAb (10 μg in 100 μl; Genzyme, Cambridge, MA). After 45 min at 4°C, the cells were washed three times with 1 ml PBS-BSA. Subsequently, 50 μl of FITC-labeled goat F(ab')$_2$ anti–mouse IgG (Tago, Inc., Burlingame, CA), diluted to 3.5 μg/ml in PBS-NGS, was added in the dark for 30 min at 4°C. The cells were washed three times with cold PBS and then resuspended in 400 μl of 1% formaldehyde in PBS. Flow cytometry analysis was then performed in FACS IV® or FACScan® flow cytometers (Becton Dickinson Immunocytometry Systems, San Jose, CA). Surface expression of IL-6 on B-IL-6 cells was measured under similar conditions, except that the cells were preincubated for 30 min at 25°C with brefeldin A (4 μg/ml; Boehringer Mannheim Biochemicals, Indianapolis, IN) to inhibit secretion of IL-6 (11, 12), and that Graces' medium was used instead of PBS-BSA.

Preparation and Testing of Antipeptide Antisera. Two NZW rabbits were immunized with 200 μg of the respective peptides from IL-6 (see Fig. 4) in unconjugated form in combination with CFA. The rabbits were boosted with 200-μg doses of peptides in combination with IFA in biweekly intervals. 2 wk after each immunization, blood samples were taken and analyzed for antibodies by ELISA. 8–10 wk after the initial immunization, the rabbits were killed after collecting blood by cardiac puncture.

Wells of 96-well polystyrene plates (Immulon II; Dynatech Laboratories, Inc., Chantilly, VA) were coated either with purified IL-6 (500 ng/well) produced by CHO-IL-6 cells or with the respective synthetic peptides from IL-6 (4 μg/well; 200 μl in 0.1 M Tris, pH 8.8) overnight at 20°C. The wells were postcoated with BSA and gelatin (10 and 2.5 mg/ml, respectively). Control as well as antipeptide antisera were serially diluted (starting dilution, 1:≥20) in a mixture of FCS and goat serum (9:1), 0.1% Tween 20, adjusted to pH 8.0 (BG). The diluted sera (200 μl) were added to IL-6- or peptide-coated wells and incubated overnight at 25°C. The wells were washed with 0.14 M NaCl, 0.01 M Tris, 0.01% sodium merthiolate, pH 7.2 (TS). Subsequently, the wells were incubated with goat anti–rabbit IgG linked to horseradish peroxidase (Boehringer Mannheim Biochemicals) (diluted 1:1,000 to 1:2,000 in TS containing 5% normal goat serum and 0.05% Tween 20). After incubation for 2 h at 37°C, the wells were washed with TS, and peroxidase activity bound to the wells was determined using a kit from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). Absorbance at 450 nm was determined in a microplate reader (MR600; Dynatech Laboratories, Inc.). Dilution endpoints corresponding to the highest dilution at which the ratio of absorbance corresponding to positive samples divided by absorbance corresponding

to equally diluted normal sera was ≥2.1 were calculated as described by Ritchie et al. (15). ELISAs with mouse anti-IL-6 mAbs (Genzyme, Cambridge, MA) were carried out under similar conditions, except that horseradish peroxidase-labeled anti–mouse instead of anti–rabbit, IgG was used.

Binding of Cells to Anti-IL-6 Magnetic Beads and to preS(21–47) Cellulose. PreS(21–47) cellulose was prepared as described before (1, 6). Magnetic beads with bound anti-IL-6 mAb were prepared as follows: magnetic beads with covalently attached goat anti–mouse IgG (300 μl; Advanced Magnetics, Inc., Cambridge, MA) were mixed with 50 μg of mouse anti-IL-6 mAb overnight at 4°C. Unadsorbed antibodies were removed, the beads were washed four times with PBS, and resuspended in 300 μl of TS. The attachment of HepG2, CHO, CHO-IL-6, and of Sf9 insect cells, either uninfected or infected with wild-type or recombinant baculoviruses, to preS(21–47) cellulose and anti-IL-6 magnetic beads, respectively, was quantitated as described before (6). In some cases, the cells were pretreated with brefeldin A (4 μg/ml) for 30 min either at 37°C (CHO-IL-6 cells) or at 25°C (Sf9 cells) to inhibit secretion of IL-6. Conditions for treating cells with PI-PLC or at pH4 were the same as already described (6).

The inhibitory effect of antisera to peptides from IL-6 (final dilution, 1:10) and of peptides from IL-6 and from the preS(21–47) sequence (final concentration, 2 mg/ml), respectively, was followed by adding the respective substances to cell–preS(21–47) cellulose mixtures, otherwise following conditions described above.

Results

Expression of IL-6 cDNA in Cells Endows Them with Receptors for the preS1 Region of the HBV env Protein. To determine whether or not the biosynthesis of human IL-6, but of no other human gene products, is sufficient for expression of cell receptors for the preS(21–47) region of the HBV env protein, cells transfected with IL-6 cDNA or infected with a recombinant baculovirus with inserted IL-6 cDNA were assayed for the presence of HBV receptors. CHO-IL-6 cells secreted relatively large quantities of IL-6 ($\sim$250 μg/10^7 cells/72 h). Sf9 cells, derived from *S. frugiperda* ovarian cells, infected with recombinant baculovirus carrying IL-6 cDNA, produced $\sim$1% of that amount of IL-6. IL-6 was detectable on the surface of each of these cell lines by cytofluorometry (Fig. 1). IL-6 was not detectable on the surface of CHO cells and of uninfected Sf9 cells.

The surface expression of IL-6 on CHO-IL-6 cells was confirmed by the observation that these cells adsorbed to magnetic beads with attached anti-IL-6 mAbs (Fig. 2 *A*). CHO-IL-6 cells did not bind to control magnetic beads carrying normal mouse IgG. Control CHO cells bound to the anti-IL-6 beads nonspecifically to a much lesser extent that CHO-IL-6 cells. Pretreatment of CHO-IL-6 cells with PI-PLC resulted in their decreased binding to anti-IL-6 beads. Pretreatment at pH 4 had a minimal effect on the binding of these cells to anti-IL-6 beads. CHO-IL-6 cells, unlike CHO cells, also bound to preS(21–47) cellulose (Fig. 2 *B*). Treatment of the cells with either PI-PLC or at pH 4 resulted in decreased binding to the cellulose derivative. These results demonstrate that surface expression of human IL-6 on cells of nonhuman origin resulted in appearance of binding sites for the preS(21–47) segment of the HBV env protein. CHO-IL-6 cells
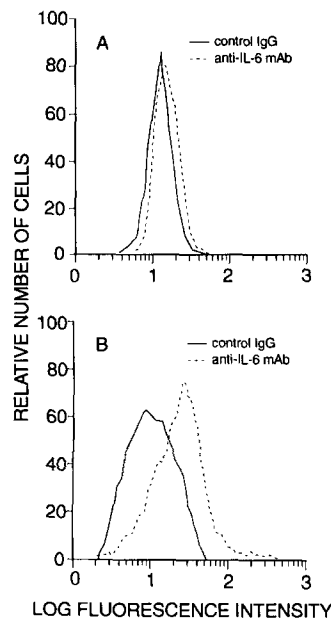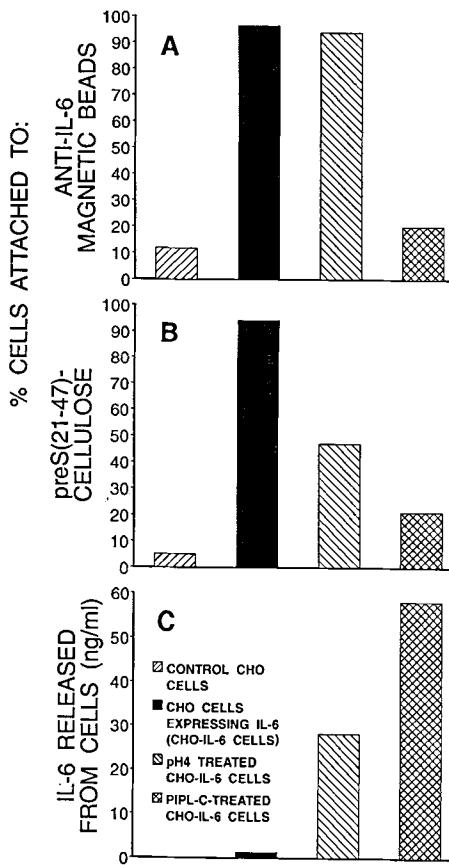

Figure 1. Expression of IL-6 on the surface of CHO cells transfected with IL-6 cDNA and secreting the IL-6 protein (*A*), and of Sf9 cells infected with recombinant baculovirus carrying IL-6 cDNA (*B*). Cells pretreated with brefeldin A (4 μg/ml) for 30 min to block secretion of IL-6 (11, 12) were reacted with mouse anti-IL-6 mAb or with control normal mouse IgG. Subsequently, the cells were washed and reacted with FITC-labeled anti–mouse IgG. The FITC-labeled cells were measured by flow cytometry.

bound also to cellulose with covalently linked hepatitis B surface antigen (HBsAg) containing the preS1 sequence but not to control cellulose with covalently linked BSA or the peptide preS(12–32) (data not shown).

To confirm that decreased binding to preS(21–47) cellulose of CHO-IL-6 cells pretreated with PI-PLC or at pH 4 can indeed be attributed to release of IL-6 from these cells, IL-6 was measured in the supernatant fluids after treating the cells. Similar levels of IL-6 were found in cells treated by either method and in mock-treated cells. This was due to continued secretion of IL-6 from the cells during the 30-min treatment period. To block the secretion of IL-6, the cells were pretreated with brefeldin A and subsequently exposed to either PI-PLC or to pH 4. Under these conditions, significant differences in the release of IL-6 between PI-PLC-, pH 4–, and mock-treated cells were observed (Fig. 2 *C*). Thus, decreased binding to preS(21–47) cellulose of PI-PLC- or pH 4–treated CHO-IL-6 cells can indeed be explained by the release of surface-bound IL-6 from the cells. Treatment of CHO-IL-6 cells with brefeldin A did not have any effect on their binding to either anti-IL-6 magnetic beads or to preS(21–47) cellulose (data not shown).

Since transfection of mammalian CHO cells with IL-6 cDNA led to the expression of both IL-6 and of binding sites for the HBV env protein, it was of interest to determine whether or not expression of IL-6 in cells phylogenetically more distant from human cells than are CHO cells would also lead to appearance of binding sites for the preS region of the HBV env protein. Sf9 insect cells infected with recombinant baculovirus carrying IL-6 cDNA (B-IL-6 cells) expressed on their surface both IL-6 (Fig. 3 *A*) and HBV receptors (Fig. 3 *B*), unlike uninfected cells and cells infected with wild-type baculovirus or with recombinant baculovirus carrying cDNA for the glucocorticoid binding protein. Exposure of B-IL-6 cells to pH 4 or to PI-PLC resulted in the release of IL-6 into
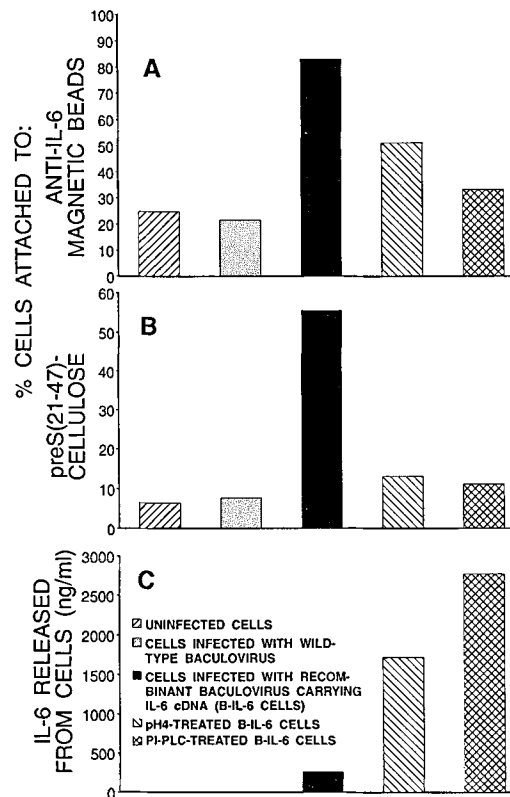
Figure 2. Surface expression of IL-6 on CHO cells transfected with IL-6 cDNA and secreting the IL-6 protein (CHO-IL-6), and their binding to preS(21–47) cellulose. (*A*) Attachment of control CHO cells and of CHO-IL-6 cells that were either mock treated, exposed to 10 mM citrate, 0.14 M NaCl, pH 4, for 5 min at 25°C, or treated with PI-PLC (1 U/ml, 1 h at 37°C) to anti-IL-6 magnetic beads. (*B*) Attachment of CHO and CHO-IL-6 cells (either untreated or treated with PI-PLC or exposed to pH 4) to preS(21–47) cellulose. (*C*) Release of IL-6 from CHO-IL-6 cells by PI-PLC or by exposure to pH 4. Cells (10⁶) pretreated with brefeldin A (4 µg/ml) for 30 min at 37°C were treated for 1 h at 37°C with PI-PLC (100 µl; 1 U/ml in TS), mock treated with TS for 1 h at 37°C, or incubated at pH 4 for 5 min at 25°C. IL-6 was detected in the supernatant fluids after centrifugation of the treated cells. IL-6 was not detectable in supernatants after treating control CHO cells.

Figure 3. Surface expression of IL-6 on Sf9 cells (derived from *S. frugiperda* ovarian cells) infected with recombinant baculovirus carrying IL-6 cDNA (B-IL-6 cells), and their binding to preS(21–47) cellulose. (*A*) Attachment of control uninfected or wild-type baculovirus-infected Sf9 cells and of B-IL-6 cells that were either mock treated, exposed to 10 mM citrate, 0.14 M NaCl, pH 4, for 5 min at 25°C, or treated with PI-PLC (1 U/ml, 1 h at 37°C) to magnetic anti-IL-6 beads. (*B*) Attachment of control Sf9 and of B-IL-6 cells (either untreated or treated with PI-PLC, or exposed to pH 4) to preS(21–47) cellulose. (*C*) Release of IL-6 from B-IL-6 cells 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), mock treated with TS for 1 h at 37°C, or incubated at pH 4 for 5 min at 25°C. IL-6 was determined in the supernatant fluids after centrifugation of the treated cells. IL-6 was not detectable in supernatants after treating control Sf9 cells. For further experimental details, see legend to Fig. 2.

the medium (Fig. 3 *C*) and in diminution of surface-exposed IL-6 and of binding sites for the preS(21–47) ligand.

In summary, these results indicate that expression of human IL-6 in cells, independent of their origin, also leads to the expression of receptors for the preS1 region of the HBV env protein.

Delineation of Binding Sites on IL-6 for the preS(21–47) Region of the HBV env Protein. Since the preS(21–47) region of the HBV sequence was recognized by IL-6 both in solution and in cell-associated form (6), it was of interest to define the preS1 binding domain on the IL-6 protein. To accomplish this, the inhibitory effect of synthetic peptides from the IL-6 sequence (Fig. 4), and of the corresponding antipeptide antisera on the binding of HepG2 cells to preS(21–47) cellulose, was investigated. Synthetic peptides covering the entire

sequence of IL-6, except the peptide corresponding to the signal sequence, reacted with a polyclonal rabbit anti-IL-6 antiserum (Fig. 5 *A*). The antibody dilution endpoint of this antiserum, as measured by ELISA on IL-6-coated wells, was 1:10⁵, i.e., ~30-fold higher than that obtained with the synthetic peptides [1–35] and [120–143], respectively, optimally recognized by this antiserum. This suggested that the synthetic IL-6 peptides together did not represent all epitopes exposed on the intact IL-6 protein. All peptides from IL-6 were highly immunogenic, and except for the signal peptide [(-28)–(-1)], elicited antibodies recognizing IL-6 (Fig. 5 *B*). However, the titer of antibodies recognizing IL-6 was generally lower than that of antibodies reacting with the respective homologous peptides, in agreement with the conclusion that the immunological crossreactivity between IL-6 and synthetic peptides from IL-6 was partial.

A

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-28          -20          -10           -1
 MNSFSTSAFGPVAFSLGLLLVLPAAFPA
 1           10           20           30
 PVPPGEDSKDVAAPHRQPLISSERIDKQIR
 31          40           50           60
 YILDGISALRKETCNKSNMCESSKEALAEN
 61          70           80           90
 NLNLPKMAEKDGCFQSGFNEETCLVKIITG
 91          100          110          120
 LLEFEVYLEYLQNRFESSEEQARAVQMSTK
 121         130          140          150
 VLIQFLQKKAKNLDAITTPDPTTNASLLTK
 151         160          170          180
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 181
 LRQM
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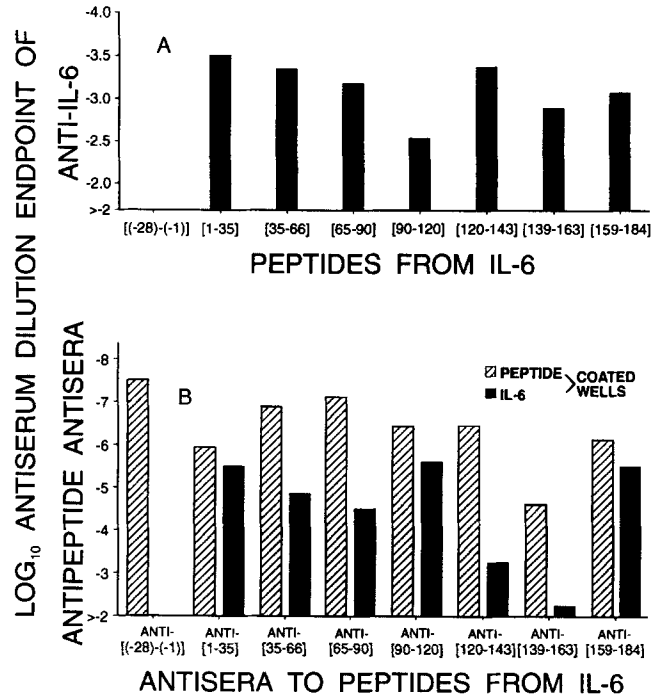
B

1. [(-28)-(-1)]*KKC*   5. *CG*[90-120]
2. [1-35]*GC*   6. *CGG*[120-143]
3. [35-66]   7. [139-163]*GGC*
4. [65-90]   8. *CGG*[159-184]

Figure 4. Amino acid sequence of IL-6 (*A*) (13, 14) and of synthetic peptides from IL-6 (*B*). The peptides are consecutively numbered from 1 to 8. Amino acids added to either the NH_2 or COOH termini for ease of synthesis or for providing spacers for coupling of the peptides to carriers are shown in italics. The sequence [(-28)-(-1)] corresponds to the IL-6 signal sequence.

Figure 5. Antigenicity and immunogenicity of synthetic peptides from the sequence of IL-6. (*A*) Serial dilutions of rabbit antiserum against IL-6 (having an antibody dilution endpoint of 1:102,400 as measured by ELISA on IL-6-coated plates) and a control normal rabbit serum, respectively, were serially diluted in BG and added to wells of polystyrene plates coated with peptides covering the entire sequence of IL-6 (see Fig. 4). After overnight incubation at 20°C, the wells were washed, and rabbit IgG bound to the wells was determined by ELISA using horseradish peroxidase–labeled goat anti–rabbit IgG. The dilution endpoints of anti–IL-6, corresponding to the highest dilution at which the OD_{450} readings corresponding to bound IgG from the anti–IL-6 serum were 2.1 times higher than that corresponding to bound IgG from the control normal rabbit serum, were calculated and plotted. (*B*) Serial dilutions in BG of rabbit antisera against distinct peptides from the sequence of IL-6 were added to wells coated either with the homologous peptides (4 μg/well) or with rIL-6 (500 ng/well). Control experiments were carried out with dilutions of normal rabbit serum. Antibody dilution endpoints were determined as described for *A*. For further details, see Materials and Methods.
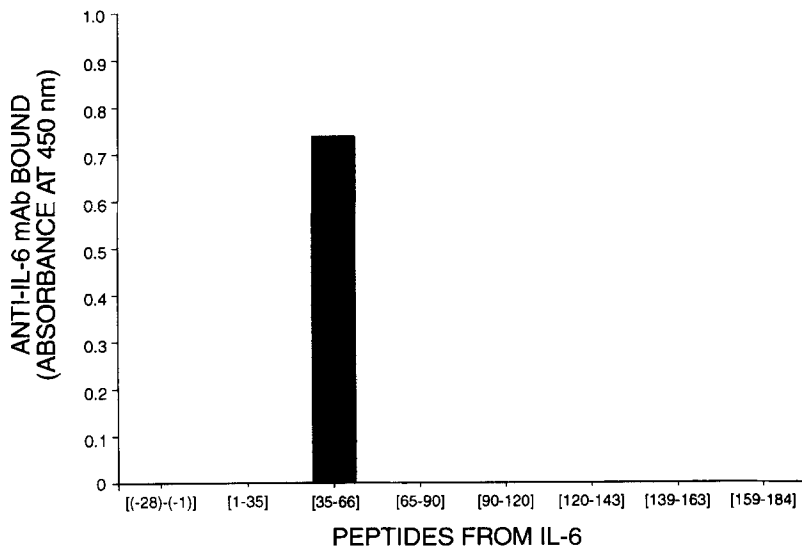
Since the synthetic peptides from IL-6 at least partially mimicked the corresponding region on the IL-6 protein at the level of immunological recognition, it was expected that they might also be suitable for delineating the preS(21–47) binding site within the IL-6 amino acid sequence. Among the IL-6 peptides tested for inhibitory activity on the interaction between HepG2 cells and preS(21–47) cellulose, peptide [35–66] appeared to be the most effective (Fig. 6 *A*). Peptides [1–35] and [90–120] were less effective in this respect. However, on molar basis, peptide [35–66] had only ∼1/5,000 of the inhibitory activity of IL-6 (6). All other peptides lacked inhibitory activity. Reduced and alkylated peptide [35–66] had no inhibitory activity, indicating that cysteine residues 44 and 50 (Fig. 4 *A*), and possibly a disulfide bond between them, were essential for inhibitory activity. Among the antipeptide antisera tested, only anti-[35–66] significantly inhibited the binding of HepG2 cells to preS(21–47) cellulose (Fig. 6 *B*). In summary, assaying of peptides from IL-6 and of the corresponding antipeptide antisera for inhibition of binding of HepG2 cells to preS(21–47) cellulose suggested that the [35–66] region of the IL-6 sequence overlaps a complementary site for the preS(21–47) segment on the HBV env preS1 sequence. This conclusion was further supported by results of epitope mapping of anti-IL-6 mAbs (Fig. 7), shown before (6) to inhibit the interaction between the HBV env protein and HepG2 cells.

Identification of Amino Acid Residues within the preS(21–47) Sequence Essential for Recognition by the Cell Receptor for HBV. Results of earlier studies identified the preS(21–47) segment of the HBV L protein as a binding site for the cell receptor for HBV (1). Results shown above indicated that the [35–66] segment of IL-6 overlaps a complementary site for the preS(21–47) sequence. For a better understanding of

molecular details of the interaction between the HBV env protein and IL-6, the cell receptor binding site for HBV, it was important to determine which amino acid residues on the complementary sequences are essential for the binding reaction to occur. Since the mimicry of the preS1 region, involved in this interaction, by the synthetic peptide preS(21–47) was more complete (16) than that of the complementary IL-6 sequence by the IL-6 peptide [35–66] (Figs. 5 and 6), we decided to initiate such studies by first measuring the activity of replacement set peptides from the preS(21–47) sequence. Alanine replacement set peptides corresponding to this sequence were synthesized, and their inhibitory effect on the attachment of HepG2 cells to preS(21–47) cellulose was studied. The results revealed that residues 21–25, 28, 31, 34, 35, 39, and 43–45 could all be replaced by alanine (A), and that the A residue at position 33 could be replaced by serine (S) without impairing the inhibitory activity of the peptides (Fig. 8). All
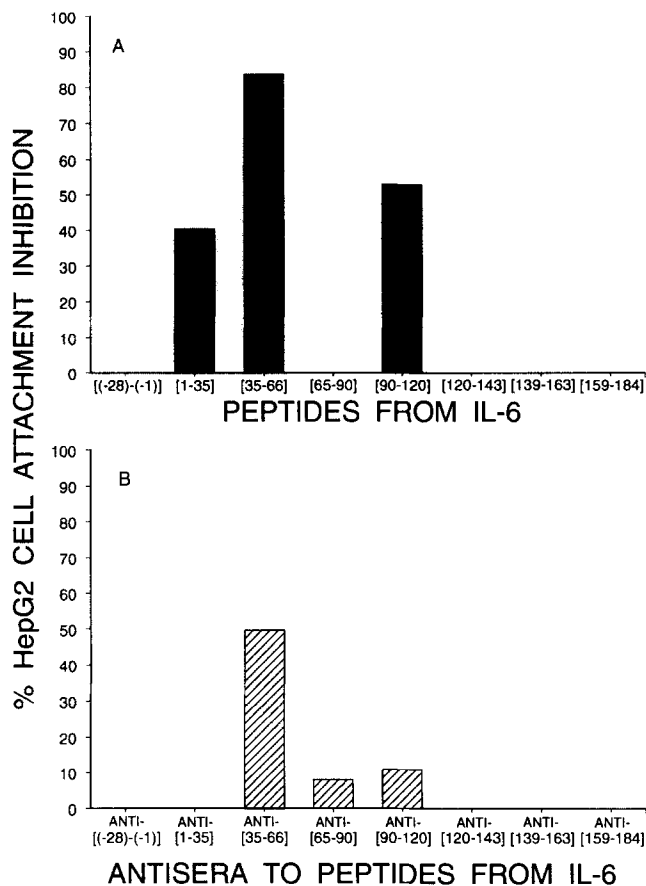
Figure 6. Inhibition by synthetic peptides from IL-6 and by the corresponding antipeptide antisera of attachment of HepG2 cells to preS(21–47) cellulose. (A) The final concentration of each peptide was 2 mg/ml. (B) Each antiserum was diluted 10-fold in 0.14 M NaCl, 0.01 M Tris, 0.02% NaN₃, 1 mM CaCl₂, pH 7.2 (TS-CA), containing 10 mg/ml BSA. The same diluent was also used for the peptides. The proportion of cells bound to preS(21–47) cellulose in the absence of peptides or antipeptide antisera ranged between 91 and 97%.
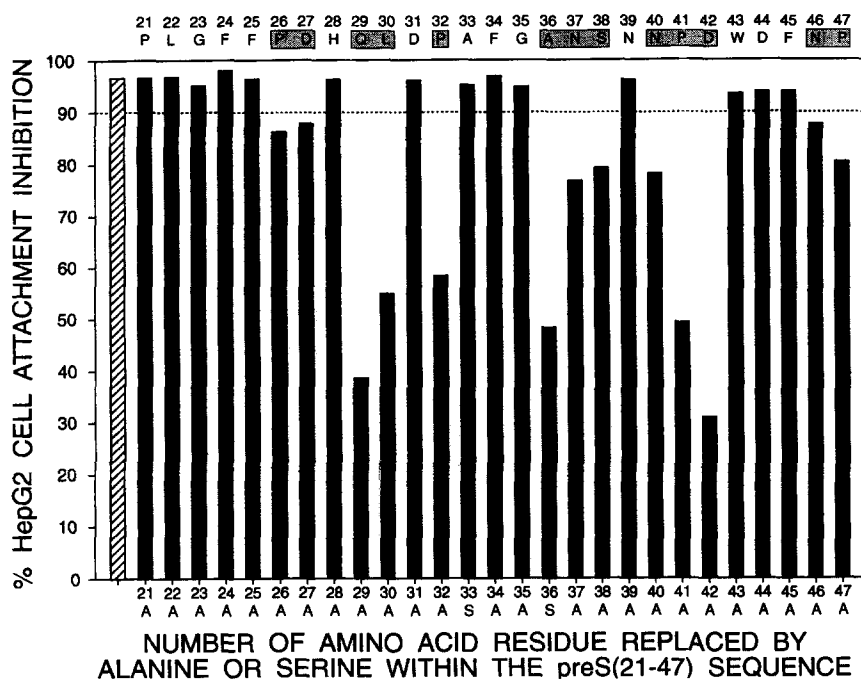
other replacements, including substitutions of four proline residues at positions 26, 32, 41, and 47, had a deleterious effect.

Identification of essential amino acid residues within the IL-6 sequence, possibly corresponding to contact residues involved in the IL-6-HBV env protein interaction, will require the design of synthetic molecules mimicking the binding site for the preS(21–47) region with greater fidelity than the [35–66] peptide from IL-6. The design of such mimetics may contribute to the development of antiviral agents against HBV.

Discussion

Results of earlier studies (6) indicated that: (a) IL-6 in solution reacted with the L protein constituent of the HBV envelope and with synthetic peptides preS(12–47) and preS(21–47) from the preS1 segment of L protein; (b) the attachment of HepG2 cells and of other cells expressing a receptor for the preS1 region of the HBV env protein with immobilized L protein or with preS(21–47) cellulose was inhibited by IL-6 and by anti-IL-6 antibodies; (c) activation of T cells and PBMC resulting in biosynthesis of IL-6 also led to the expression of receptors for HBV; and (d) treatment of cells expressing receptors for HBV with PI-PLC or their brief exposure to pH 4 resulted in release of IL-6 and in decreased binding of the cells to the preS1-specific ligands. These results indicated that IL-6 contains recognition sites for the preS(21–47) sequence and mediates HBV-cell interactions, but did not establish whether or not other cell surface components were necessary for the association between cells and the preS1 region of the HBV env proteins to occur. Data presented here indicate that: (a) the expression of IL-6 in cells of nonhuman origin transfected by human IL-6 cDNA is sufficient to endow these cells with receptors recognizing the preS(21–47) segment of the HBV L protein; and (b) other cell membrane components are probably not required for this recognition. Treatment of the transfected cells with PI-PLC

Figure 7. Epitope mapping of anti-IL-6 mAbs. Anti-IL-6 mAbs (100 ng/well), shown to inhibit the binding of IL-6 to the preS1 region of the HBV env protein (6), were added to wells coated with synthetic peptides from IL-6 (500 ng/well). The bound mAbs were determined using horseradish peroxidase–labeled anti–mouse IgG. The OD₄₅₀ values corresponding to control mouse IgG bound to the peptide-coated wells were all 0.

Figure 8. Inhibitory effect on the attachment of HepG2 cells to preS(21–47) cellulose of replacement set peptides corresponding to the preS(21–47) sequence. The final concentration of each peptide was 2 mg/ml. The replacement set consisted of peptides in which amino acid residues in the preS(21–47) sequence were replaced by alanine (*A*). Alanines at positions 33 and 36, respectively, were replaced by serines (S). The preS(21–47) sequence is presented on top and the replacements are indicated on the abscissa. The inhibitory effect of the parent peptide is presented by the hatched bar. The inhibitory effect of the replacement set peptides is presented by black bars. The horizontal dashed line corresponds to 90% inhibition of HepG2 cell attachment. Amino acid residues, the replacement of which led to a decrease of cell attachment inhibition to <90%, are boxed.

or their exposure to pH 4 resulted in release of IL-6 and in decreased binding activity for the preS(21–47) ligand, as was observed earlier with human cells expressing IL-6 either constitutively or after activation (6). IL-6 released by PI-PLC from HepG2 cells or from IL-6-c-DNA-transfected cells had a molecular mass similar to that of IL-6 secreted into the medium (23–30 kD), as determined by immunoblotting after electrophoresis in polyacrylamide gels under reducing and denaturing conditions (our unpublished data), in agreement with other reports (17, 18). In addition to ~27-kD species, less abundant larger molecular mass forms of IL-6 (~47–89 kD) were discerned under nondenaturing conditions by molecular exclusion chromatography on Sephadex G-150 for PI-PLC-released IL-6, as well as for IL-6 secreted either from HepG2 cells or from transfected cells (our unpublished data), in agreement with results reported by May et al. (17, 19). These results suggest that PI-PLC-released IL-6 is predominantly not associated with another carrier protein(s) or that possible complexes of IL-6 with other proteins are dissociated during molecular exclusion chromatography, suggesting a weak binding between IL-6 and a putative carrier molecule. A possible association between IL-6 and mannose binding proteins has been recently suggested (20). The mechanism whereby IL-6 becomes released by PI-PLC from cells remains to be elucidated, since the COOH terminus of IL-6 does not fulfill the sequence requirements needed for attachment of a phosphatidylinositol glycan (13, 14, 21).

rIL-6 produced in *E. coli* lacking saccharide residues, unlike IL-6 from eukaryotic cells (17, 18, 22), also had binding sites for the preS(21–47) region of the HBV L protein and inhibited the attachment of HepG2 cells to preS(21–47) cellulose (our unpublished data). This indicated that the binding site for the preS(21–47) ligand is located on the protein moiety

of IL-6, offering the opportunity to locate this site within the IL-6 sequence using synthetic peptides from IL-6. Among peptides covering the entire sequence of IL-6 and the corresponding antipeptide antibodies, peptide [35–66] and the homologous anti-[35–66] antipeptide antiserum were the most effective in inhibiting the attachment of HepG2 cells to preS(21–47) cellulose. These results suggest that the [35–66] segment of the IL-6 sequence is part of a binding site for the preS1 region of the HBV env protein, although other regions of the IL-6 sequence appear to contribute directly or indirectly (through conformational effects) to this binding site.

Studies with point and deletion mutants of IL-6 and with mAbs of defined specificity suggested that the COOH-terminal portion of IL-6 encompassing residues [151–183] is essential for biological activity and contains the IL-6R binding site (23–26). On the other hand, studies with NH2-terminally deleted mutants suggested that residues [29–34] are essential for biological activity of IL-6, while residues [1–28] could be deleted without affecting activity (27). Cumulatively, these results suggest that both the COOH- and NH2-terminal regions are essential for biological activity of this cytokine (28). Any internal deletions within the sequence of IL-6, reported to result in loss of biological activity (28), probably prevent the proper folding of IL-6. This agrees with the experimentally determined or proposed structures for four helix bundle cytokines, including IL-6 (29–33). The binding site for the preS(21–47) ligand, tentatively assigned to a region of IL-6 encompassing residues [35–66], is likely to be distinct from the IL-6R binding site assigned to residues [178–183] (25). The distinctiveness of preS(21–47) and IL-6R binding sites is also suggested by the observation that the inhibitory activity of the IL-6 [35–66] peptide on attachment of HepG2 cells to the preS(21–47) ligand is abolished after

reduction and alkylation of cysteine residues 35 and 50, while a mutant IL-6 protein in which all four cysteine residues are replaced by serines was reported to retain biological activity (34). This raises the possibility of developing antiviral compounds mimicking the receptor binding site for HBV on IL-6 but not displaying undesirable biological effects of the intact IL-6 molecule. In this respect, it also appears important to note that: (a) there is no significant sequence homology between the preS(21–47) ligand and IL-6R (35), and the IL-6 signal transducer gp130 (36), respectively; (b) the interaction of HepG2 cells and of other cells expressing the receptor for the preS1 region of the HBV env protein with the preS(21–47) ligand (5) and binding of rIL-6 to the HBV L protein or to the synthetic peptides preS(12–47) and preS(21–47), respectively, required the presence of Ca^{2+} ions (6, our unpublished data), while there is so far no evidence that the biological activity of IL-6 is manifested only at the presence of Ca^{2+}. Notwithstanding the Ca^{2+} dependence of the IL-6-preS(21–47) association, consensus sequences characteristic for calcium binding proteins could be recognized on neither the IL-6 or the preS1 sequences.

Association between IL-6 exposed on the surface of cells and the preS1 region of the HBV env L protein is probably an important, but possibly not sufficient, step for entry of HBV into cells, resulting in initiation of the virus replication cycle. Additional cell receptor binding sites (37, 38) and dividing target cells (39) may be required for virus replication. Transfection of nonhuman cells with cDNAs coding for human cellular proteins may help identify steps required for the life cycle of HBV. Cells transfected with human IL-6 cDNA and expressing receptors for the preS1 region of the HBV env protein represent a step towards accomplishing this goal.

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